AIX-MARSEILLE UNIVERSITÉ
FACULTÉ DES SCIENCES MEDICALES ET PARAMEDICALES
ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE
INSTITUT HOSPITALO-UNIVERSITAIRE MEDITERRANEE INFECTION

THESE DE DOCTORAT

Présentée par

Younes LAIDOUDI

Né le 13 Mars 1992, à Sétif, Algérie

Pour obtenir le grade de DOCTEUR d'Aix-Marseille Université

Discipline : Sciences de la Vie et de la Santé

Spécialité : Maladies Infectieuses

Helminthoses vectorielles : Caractérisation, diagnostic et moyens de contrôle

Vector-borne helminths: Characterization, diagnosis and control tools

---

Soutenue publiquement le 26/11/2020

Composition du jury :

Pr Anthony LEVASSEUR	Aix-Marseille Université	**Président du jury**
Pr Bruno CHOMEL	Université de Californie-Davis (USA)	**Rapporteur**
Pr Florence FENOLLAR	Aix-Marseille Université	**Examinatrice**
Dr Coralie MARTIN	Muséum Nationale d'Histoire Naturelle	**Rapporteure**
Dr Vét Emilie BOUHSIRA	Ecole Nationale Vétérinaire de Toulouse	**Examinatrice**
Dr Oleg MEDIANNIKOV	IHU Méditerranée Infection	**Directeur de la thèse**

Codirection de la thèse :

Dr Vét Bernard DAVOUST	IHU Méditerranée Infection	**Codirecteur**

Aix-Marseille Université - Faculté des Sciences Médicales et Paramédicales
Institut Hospitalo-Universitaire (IHU) Méditerranée Infection
Microbes, Evolution, Phylogénie et Infection (MEΦI)
Institut de Recherche pour le Développement (IRD 198)
Assistance Publique - Hôpitaux de Marseille (AP-HM)

Table des matières

Avant-propos

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille. Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse. Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis, associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Pr Didier RAOULT

Dédicaces

Je dédie ce travail à la mémoire de mon ***cher papa Khaled « Merdessii ».***

J'aurais tant aimé que tu sois présent papa.

Aucune dédicace ne saurait exprimer mon respect, mon amour éternel et ma considération pour les sacrifices que tu as consenti pour mon instruction et mon bien-être. Je te remercie pour tout le soutien et l'amour que tu m'as apportés depuis mon enfance et j'espère que ta bénédiction m'accompagnera toujours. J'espère que, du monde qui est le tien maintenant, tu apprécies cet humble geste comme preuve de reconnaissance de la part d'un fils qui a toujours prié pour le salut de ton âme. Puisse Dieu, le Très Haut, te garder dans son vaste paradis.

A ma chère maman*, mon adorable maman, celle qui est toujours présente et continue de l'être pour faire mon bonheur. Merci pour t'être sacrifiée pour que tes enfants grandissent et prospèrent. Merci de trimer sans relâche, malgré les péripéties de l'âge, de la santé, de la vie, pour le bien-être de tes enfants. Enfin ! Merci tout simplement d'être… maman. Aucune dédicace ne saurait exprimer tout l'amour que j'ai pour toi maman, ta joie et ta gaieté me comblent de bonheur. Que ce modeste travail exauce tes vœux tant formulés, et soit le fruit de tes innombrables sacrifices, que je n'acquitterai jamais assez.*

A mes chers et adorables frères et sœurs, *en témoignage de mon affection fraternelle, de ma profonde tendresse et reconnaissance, je vous souhaite une vie pleine de bonheur et de succès et que Dieu, le tout puissant, vous protège et vous garde.*

Remerciements

Qu'il me soit permis de présenter ici mes remerciements au Professeur **Didier RAOULT** de m'avoir donné son accord pour la réalisation de mes travaux de thèse au sein de l'unité de recherche **MEPHI**, à l'Institut Hospitalo-Universitaire (IHU) Méditerranée Infection à Marseille, France. J'ai eu le grand plaisir de bénéficier de son riche savoir. Merci aussi pour l'attention particulière que vous nous avez portée durant cette pandémie du Covid.

Je tiens à dire ma reconnaissance envers le Docteur **Oleg MEDIANNIKOV**, mon directeur de thèse, qui, malgré les charges qui sont les siennes, a accepté sans réserve, de diriger cette thèse. Il s'y est grandement impliqué par ses directives, ses remarques et suggestions, mais aussi par ses encouragements dans les moments clés de son élaboration. Je tiens à le remercier aussi pour cette liberté qu'il m'a permise, sans laquelle le chercheur ne saurait affirmer sa manière de penser et de procéder, sa manière d'être, bref toute sa personnalité.

Je remercie le Docteur vétérinaire **Bernard DAVOUST**, mon co-directeur de thèse. Merci cher Docteur pour votre gentillesse et votre spontanéité avec lesquelles vous avez bien voulu diriger ce travail. J'ai eu le grand plaisir de travailler sous votre codirection, et j'ai trouvé auprès de vous le conseiller et le guide qui m'a reçu en toute circonstance avec sympathie, sourire et bienveillance. Vos conseils incontestables ainsi que vos qualités humaines vous valent l'admiration et le respect de tous. Vous êtes et vous serez pour moi l'exemple de rigueur et de droiture dans l'exercice de la profession. Veuillez, cher Docteur, trouver dans ce modeste travail l'expression de ma haute considération, de ma sincère reconnaissance et de mon profond respect.

Mes remerciements vont également aux Professeurs **Domenico OTRANTO, Maria-Stefania LATROFA et Raquel Adriana HERNANDEZ-AGUILAR** qui ont eu l'amabilité de discuter avec moi sur certains points clés de mon analyse. Leurs remarques pertinentes m'ont amené à reconsidérer ma position et à réviser bien des points. Je les remercie aussi pour leur disponibilité chaque fois qu'ils ont été sollicités.

Je saisi aussi cette occasion pour prononcer un mot de gratitude à l'égard du Professeur **Idir BITAM**, pour ses qualités scientifiques, pédagogiques, mais surtout humaines. Merci d'avoir guidé mes premiers pas sur le chemin de la recherche scientifique, et de m'avoir un jour conseillé de travailler sous la direction des Docteurs Bernard DAVOUST et Oleg MEDIANNIKOV.

Mes remerciements touchent également le Professeur **Florence FENNOLAR** pour l'intérêt qu'elle a porté à mon travail. Je vous remercie également pour votre aide scientifique et vos conseils incontournables durant ce travail.

Je tiens à remercier le Professeur **Anthony LEVASSEUR** pour avoir accepté de présider le jury de cette thèse, mais aussi pour son aide précieuse et ses orientations relatives à certaines de mes analyses. Ses remarques pertinentes m'ont amené à reconsidérer ma position et à bien mener à terme mes travaux.

Un grand merci aux membres du jury, le Professeur **Bruno CHOMEL** et les Docteurs **Coralie MARTIN et Emilie BOUSHIRA,** qui ont accepté, sans réserve aucune, d'évaluer cette thèse à sa juste valeur, et de me faire part de leurs remarques sûrement pertinentes qui, avec un peu de recul, contribueront, sans nul doute, au perfectionnement du présent travail. Veuillez croire, chers rapporteurs, à l'assurance de mon respect et de ma reconnaissance.

Je ne manque pas non plus de dire un grand merci aux membres de **CEVA Santé Animale**. Ainsi, je remercie, énormément, Monsieur le Dr vétérinaire **Pascal RAOUL**, directeur du développement de nouveaux produits, Madame le Dr (PhD) **Marie VARLOUD**, responsable technique Parasitologie pour les animaux de compagnie, Monsieur **Gilles NESPOULOUS**, directeur du groupe de commercialisation et du diagnostic, Monsieur le Dr vétérinaire **Gautier BARTHELEMY,** directeur de l'évaluation des projets pour les animaux de compagnie et Monsieur le Dr vétérinaire **Hamadi KAREMBE**, directeur de l'innovation au département R&D Pharmacologie. Je n'aurais jamais pu réaliser ce travail sans votre appui scientifique, votre soutien moral et l'aide financière de CEVA. Veuillez tous accepter, ainsi que la Direction de CEVA, l'expression de mes sincères et profonds remerciements.

Je remercie **Muriel MILITELLO, Jean-Pierre, Nathalie et Nathalie** ainsi que tout le personnel du laboratoire NSB3. Merci pour votre aide et votre disponibilité. Je ne manque pas non plus de remercier le Professeur **Philippe PAROLA,** ainsi que tous les personnels de l'Insectarium de l'IHU.

Mes remerciements vont aussi à Mesdames **Micheline PITACCOLO** et **Alexia BATTISTINI**. Merci beaucoup de m'avoir facilité toutes les démarches administratives tout en me laissant me concentrer sur mon travail.

Je ne remercierai jamais assez, pour leur soutien, leurs conseils judicieux et leur aide précieuse, mes collègues et amis **Handy, Hacène, Wafa, ElHadj, Maps, Nadji, Madjid, Ghiles, Souad, Linda, Djamel, Saïd, Amar, Audrey, Clara, Mirmam, Lamine, Adama, Sergei, Alissa, Duc, Issam, Amina, Nassim, Amar, Kenza, Mossaab, Mariem** et bien d'autres encore. Ils étaient la bouffée d'oxygène qui me ressourçait durant ces trois dernières années.

Et j'en viens à ma famille. Merci aussi à ma sœur chérie, une frangine unique au monde, **Fifi**. Merci d'être toujours à mes côtés, par ta présence, par ton amour, pour donner du goût et du sens à notre vie de famille. Merci aussi à mes adorables frères et amis **Abed El-Sammed et Youcèf**. Merci à mes cousins **Sofiane et Lahcène**. Merci pour votre aide et pour avoir porté et voulu toujours le grand honneur de la famille.

Merci à ma chère **Samia**, qui, dans les longues et pénibles après-midis de rédaction, coupé du reste du monde, figé devant mon ordinateur, me tenait compagnie, me fait écouter ses belles partitions de piano, comme si elle avait pitié de moi, merci d'avoir rempli ma vie de joie et de bonheur. Je ne manquerai pas non plus de remercier sa maman **Nabila** et son papa **Foudil**, ainsi que sa chaleureuse famille.

Voici enfin le fruit du travail de trois années à l'IHU, là où j'ai eu le plaisir de côtoyer des gens venus d'horizons tellement différents. Cette aventure qu'a constitué ma thèse ne m'a pas seulement apporté un diplôme mais m'a également fait grandir à vos côtés. Certains d'entre vous ont une place particulière dans mon cœur.

Résumé

La lutte contre les maladies à transmission vectorielle (VBD) repose essentiellement sur le contrôle de trois maillons principaux : (i) l'agent pathogène lui-même, (ii) les hôtes (vecteurs et hôtes définitifs) et (iii) leurs interactions au sein de leur écosystème. La présente thèse étudie de façon inductive, d'une part, le paradigme de lutte contre les maladies vectorielles (principalement des helminthoses) des animaux et d'autre part le rôle d'animaux réservoirs dans la transmission des VBD. Nous avons étudié les bactéries du genre *Wolbachia*, endosymbiotique des arthropodes (vecteurs) et des parasites (nématodes filaires), souvent utilisées pour la lutte contre les VBD. L'approche de l'isolement et de la coculture cellulaire (cellules S2 de drosophile), nous a permis de caractériser le génome de *Wolbachia massiliensis* sp. nov. (*wChem*), souche type d'un nouveau supergroupe T, *Wolbachia* de *Cimex hemipterius* collectée au Sénégal. L'étude taxo-génomique souligne la nette distinction de cette nouvelle espèce de toutes les autres *Wolbachia*, et la nécessité de revoir la taxonomie de ce genre bactérien. L'analyse du génome de *W. massiliensis* et de ses voies métaboliques montre un profil proche de celui de la *Wolbachia* mutualistique de la filariose humaine *Brugia malayi*, faisant d'elle une nouvelle piste pour approfondir les connaissances de cette relation symbiotique. D'un autre côté, l'implication des *Wolbachia* en tant que cible moléculaire pour le diagnostic des filarioses canines a permis d'améliorer la détection de la forme occulte de ces infections. Ainsi, nous avons proposé de nouvelles approches moléculaires de diagnostic basées, essentiellement, sur la technologie TaqMan® combinant la détection multiple par qPCR, des filaires ainsi que de leurs *Wolbachia*. Les principales espèces ciblées sont celles sévissant dans le Bassin méditerranéen, à savoir, *Dirofilaria immitis, D. repens, Acanthocheilonema reconditum, Cercopithifilaria grassii, C. bainae* et *Cercopithifilaria* sp. II. Dans un second volet, on a caractérisé par biologie moléculaire et/ou morphologiquement, des nématodes de primates non-humains (PNHs) du Nouveau et de l'Ancien Monde. Nos résultats montrent qu'au moins onze espèces de nématodes gastro-intestinaux, souvent zoonotiques, ont été caractérisées à partir de fèces des PNHs d'Afrique, particulièrement *Abbreviata caucasica*, parasite pour lequel, nous avons fourni des données morphologiques et utilisé de nouveaux outils moléculaires spécifiques pour le diagnostic et la surveillance épidémiologique. Nous avons également fourni des données préliminaires sur des filaires, non identifiées auparavant, chez des singes hurleurs de l'Amazonie, infectés avec au moins trois génotypes, dont un du genre *Brugia*, potentiellement zoonotique. En outre, nous avons proposé un système de détection moléculaire (qPCR) spécifique au genre *Brugia*, afin de mieux diagnostiquer,

surveiller et comprendre le cycle de vie de ce parasite. En termes de protection contre les maladies vectorielles du chien (CVBD), nous avons prouvé d'abord, au laboratoire, l'effet protecteur d'un insecticide (Vectra® 3D) à la fois sur les hôtes traités et non traités présents à proche distance de ces derniers. Ensuite, via un essai terrain de supériorité, nous avons prouvé l'efficacité de la stratégie prophylactique mensuelle multimodale, basée sur l'utilisation de deux produits (Vectra® 3D associé au Milbactor®) contre les dirofilarioses, la leishmaniose canine (LCan) et l'ehrlichiose, en Corse. En outre, on a démontré, via un essai de non-infériorité, l'efficacité de l'artésunate dans le traitement de la LCan. Dans le cadre de la lutte biologique contre les vecteurs, on a partiellement caractérisé une amine produite par la souche bactérienne *Serratia marcescens* P400, dotée d'un effet insecticide supérieur à celui de l'ivermectine contre les larves de moustique *Aedes albopictus*. On a également détecté l'infection naturelle des tiques par des entomophages (guêpes parasitoïdes). Ces données peuvent offrir une excellente alternative de lutte biologique contre ces vecteurs. Finalement, on a démontré le rôle joué par les animaux réservoirs et sentinelles de la transmission et de la propagation des VBD. Ainsi, l'hôte canin, particulièrement le chien, en Guyane, en France métropolitaine, en Algérie et en Côte d'Ivoire, a un rôle sentinelle relatif au maintien et à la propagation des VBD (dirofilarioses, leishmanioses, et trypanosomose). En Italie, on a mis en évidence le rôle de sentinelle des rickettsioses joué par les reptiles, alors que les gorilles, en République du Congo, ont un rôle sentinelle pour des zoonoses gastro-intestinales (*Giardia lamblia, Necator americanus, Ascaris lumbricoides, Strongyloides stercoralis* et plusieurs nématodes non identifiés). Finalement, ma thèse montre l'utilité de développer et d'adapter de nouvelles stratégies de contrôle et de surveillance des maladies vectorielles. Elle vise, modestement, à suppléer au manque de mise en œuvre de techniques performantes et donc de connaissances dans la perspective d'un paradigme complet d'étude des phénomènes infectieux dans leur écosystème.

Mots-clés : *Wolbachia*, filaires, nématodes, vecteurs, sentinelle, réservoir, diagnostic, prophylaxie, traitement.

Abstract

Fighting against vector-borne diseases (VBD) relies essentially on the control of three main links: (i) the pathogen itself, (ii) the hosts (vectors and definitive hosts) and (iii) their interactions within their ecosystem. The present thesis inductively studies, on the one hand, the paradigm controls of VBD (mainly helminthiasis) of animals and on the other hand the role of sentinel animals in the transmission of VBD. We have studied the bacteria of the genus *Wolbachia*, an arthropod and filarial associated-endosymbiotic, often used for the control of VBD. The cell coculture system using *Drosophila* S2 cells allowed us to characterise the genome of *Wolbachia massiliensis* sp. nov. (wChem), a type strain of a new supergroup T. The bacteria were isolated from *Cimex hemipterius* collected in Senegal. The taxo-genomic study made it possible to distinguish clearly this new species from all other *Wolbachia*, as well as the need to review the taxonomy of this bacterial genus. Analysis of the *W. massiliensis* genome and its metabolic pathways show a profile close to that of the mutualistic *Wolbachia* of the human lymphatic filaria (*Brugia malayi*), which offers a new insight for deepening our knowledge of this symbiotic relationship. On the other hand, the involvement of *Wolbachia* as a molecular target for the diagnosis of canine filariasis has improved the detection of the occult form of these infections. However, we have proposed new molecular diagnosis approach based primarily on TaqMan® technology, combining multiple detection by qPCR, both of filaria as well as their *Wolbachia*. The main target species are those found in the Mediterranean basin, namely *Dirofilaria immitis*, *D. repens*, *Acanthocheilonema reconditum*, *Cercopithifilaria grassii*, *C. bainae* and *Cercopithifilaria* sp. II. In the second part of the present work, we characterized molecularly and/or morphologically nematodes of non-human primates (PNHs) from both the New and the Old World. Our results show that at least eleven species of gastrointestinal nematodes, often with zoonotic concern, have been characterized from faeces of African PNHs. Among them, *Abbreviata caucasica*, a parasite for which we have provided morphological data and a new specific molecular tool for its diagnosis and epidemiological surveillance. We also provided preliminary data on previously unidentified filarial parasites in neotropics monkeys (howler monkeys) of French Guiana, where at least three genotypes were identified. One of them belongs to the genus *Brugia*, a potential zoonosis. In addition, we have proposed a molecular detection system (qPCR) specific to this genus, in order to better diagnose, monitor and understand the life cycle of this parasite. In terms of protection against canine vector-borne diseases (CVBD), we evaluated in the field, the effectiveness of the multimodal monthly prophylactic strategy, based on the use of two products (Vectra® 3D

associated with Milbactor®) against dirofilarioses, leishmaniasis (LCan) and ehrlichiosis. We therefore assessed the efficacy of artesunate in the treatment of LCan using a non-inferiority trial. In the context of biological pest control, an amine produced by the bacterial strain *Serratia marcescens* P400 has been partially characterized, which has been proven an insecticidal effect higher than that of ivermectin in keeling *Aedes albopictus* larvae. A natural infection of ticks with by parasite entomophagus wasps has also been detected. These data may offer an excellent alternative for biological control of these vectors. Finally, we demonstrated the role played by sentinel animals in the transmission and spread of VBD. Thus, the canine host, particularly dogs in Guyana, in metropolitan France, in Algeria and in Côte d'Ivoire, have a sentinel role for the maintenance and spread of BVD (dirofilariosis, leishmaniosis and trypanosomiasis). We also demonstrated the sentinel role in Italy for Rickettsiales played by reptiles. Moreover gorillas, in the Republic of Congo, have a sentinel role for gastrointestinal zoonoses (*Giardia lamblia, Necator americanus, Ascaris lumbricoides, Strongyloides stercoralis* and several unidentified nematodes). Finally, my thesis shows it is useful to develop and adapt new strategies for the control and surveillance of vector-borne diseases. I modestly contribute to make up for the lack of implementation of efficient techniques and knowledge in the perspective of a complete paradigm for the study of VBD in their ecosystem.

Key words: *Wolbachia*, filaria, nematodes, vectors, sentinel, reservoir, biological control, diagnosis, prophylaxis, treatment.

Introduction

Les maladies parasitaires peuvent entraîner une morbidité et une mortalité importantes, à la fois, chez l'homme et l'animal. L'histoire des maladies parasitaires semble être riche, parfois marquée par des découvertes, des concepts nouveaux et des réponses adaptées en matière de santé publique. Ce passé est riche de la mémoire de plusieurs précurseurs dans la lutte contre les maladies infectieuses. Ainsi, Carlos Chagas, en tant qu'étudiant en médecine au Brésil, a découvert puis précisé le cycle de vie complet de la trypanosomiase américaine à *Trypanosoma cruzi* (maladie de Chagas). De son côté, Ronald Ross, un médecin militaire écossais, a découvert, en Inde, le rôle des moustiques dans la transmission du paludisme. En réalité, bon nombre des premiers progrès dans le domaine des maladies parasitaires ont été réalisés du temps de l'histoire coloniale de la fin du XIX^{e} et du début du XX^{e} siècle (Carrion et al. 2016). Les découvertes, les publications et les premiers programmes de contrôle de cette époque étaient exceptionnels et bon nombre de chercheurs coloniaux et institutions qu'ils ont fondées étaient et restent, à certains égards, des centres d'excellence scientifique (comme les instituts Pasteur). Pourtant, le domaine des maladies parasitaires souffre encore de **l'insuffisance de connaissances sur les parasites et leurs réelles distributions géographiques**. Nonobstant, des efforts de recherche et de lutte contre les maladies parasitaires sont, aujourd'hui, menés, de manière très appropriée, dans des pays à revenu faible ou intermédiaire. Ils impliquent, souvent, des chercheurs et des praticiens de pays à revenu élevé. Avec un espoir de durabilité, ces programmes se situent, désormais, pleinement, dans le cadre de véritables partenariats entre collègues internationaux. Ils mettent un fort accent sur le renforcement des capacités dans les pays où les maladies étudiées sont endémiques. L'adage « loin des yeux, loin du cœur » pourrait expliquer un certain désintérêt pour l'étude des maladies exotiques, mais, heureusement, cela est, actuellement, remis en question, sur la base, à la fois,

de **problèmes de santé publique et de développement économique**. Ainsi, il y a eu une vague récente d'intérêt et d'activités pour lutter contre le paludisme et les « **maladies tropicales négligées** » dans la perspective de promouvoir la bonne santé des personnes, essentielle à la participation au développement économique mondial et à l'édification de chaque nation. Beaucoup d'observateurs et de dirigeants plaident, actuellement, pour lutter contre les maladies tropicales en se fondant sur : le rendement économique promis par les programmes de lutte intégrée (Canning et al. 2006) ; les droits humains liés à la santé (Hunt et al. 2006) ; et le grand nombre (c'est-à-dire des milliards) de personnes infectées par des parasites.

Bien que négligées, **les filarioses humaines et animales** sont parmi les plus importantes helminthoses connues dans l'histoire. Elles continuent de causer de graves dommages et revêtent une grande importance sanitaire et socio-économique dans la plupart des zones tropicales et subtropicales du monde. Selon l'Organisation mondiale de la santé (OMS), les filarioses lymphatiques avec l'onchocercose à *Onchocerca volvulus* affectent jusqu'à 893 millions de personnes dans 49 pays dans le monde (WHO 2020). Ces affections sont connues par leur capacité à induire des invalidations graves. *Wuchereria bancrofti* est impliqué dans 95% des cas de filarioses humaines, d'autres espèces continuent à émerger et deviennent, de plus en plus, menaçantes dans le monde entier. Leur transmission vectorielle, et leur capacité à parasiter plusieurs hôtes, font d'elles des maladies rustiques, et difficiles à éradiquer. L'OMS a envisagé la complète éradication des filarioses en 2020, par l'utilisation d'une stratégie de traitement de masse préventif (praziquantel, ivermectine et albendazole) ainsi que par la lutte antivectorielle (WHO 2020).

Les récentes recherches ont également montré l'émergence en Europe, de plusieurs espèces de nématodes, filaires et autres, à transmission vectorielle (***Vector-Borne Helminthes*** : VBH) dont l'intérêt est à la fois médical et vétérinaire. Un grand nombre de VBH sont donc décrites en Europe, et certaines d'entre elles sont de plus en plus étudiées en raison du niveau

de leur gravité chez les chiens, voire chez l'homme (Otranto et al. 2011, Genchi et al. 2011, Morchón et al. 2012). Actuellement, 45% de la population humaine totale d'Europe, ainsi que leurs animaux domestiques et de compagnie, sont exposés au risque des VBH (Otranto et al. 2013). Un ensemble de facteurs biologiques intrinsèques (par exemple, la capacité vectorielle), et extrinsèques (facteurs environnementaux tels que le climat, les mouvements de population, le commerce, etc.), affectent les interactions entre les nématodes parasites, les vecteurs (arthropodes), les animaux et l'homme. Cela rend complexe le contrôle et la surveillance de ces maladies. En effet, **leur processus de propagation**, dans des zones auparavant non endémiques, a été, principalement, associé à la biologie et à l'écologie des arthropodes vecteurs et à leurs capacités à établir de nouveaux cycles de transmission, en créant des foyers d'infestation dans des populations d'hôtes sensibles.

Un autre aspect du parasitisme par les nématodes demeure également négligé. En effet, en Afrique tropicale et subtropicale, là où sévissent les filarioses humaines, il se trouve que l'endémicité de la zone est **à risque pour la population humaine comme pour les animaux domestiques et sauvages.** Par exemple, chez les **primates non-humains**, dont certains appartiennent à des espèces en voie de disparition, il a été montré qu'une cause de mortalité fréquente chez les orangs-outans juvéniles et les gorilles est liée aux infestations par des nématodes gastro-intestinaux tel que *Strongyloides* spp., *Ancyclostoma duodenale* et *Necator americanus* (Carrion et al. 2016). En effet, la **faible spécificité d'hôte** de ces nématodes et l'aspect asymptomatique qui caractérisent ces parasitoses, font d'elles une menace envahissante pour des populations vivant en communauté. Les parasitoses des singes et leurs thérapies ont fait, depuis longtemps, l'objet de publications. Pourtant, en recherchant les études réalisées dans ce domaine, il se trouve qu'elles sont, finalement, rares, et souvent basées sur les **techniques classiques dont l'apport informationnel demeure toujours incomplet**. Par conséquent, le

développement de **nouveaux outils de laboratoire** est devenu, actuellement, nécessaire, afin de mieux les étudier.

De nos jours, la lutte contre les maladies vectorielles vient se compliquer par l'émergence de phénomènes de **résistance** aux traitements, à la fois, chez les vecteurs (Dahmana et al. 2020) et les parasites (Bourguinat et al. 2015, Ponte-Sucre et al. 2017). Par conséquent, la découverte de **traitements alternatifs pour contrôler ces parasitoses** est impérative. Cependant, une meilleure compréhension de ces **maladies d'écosystème,** est prometteuse pour apporter de nouvelles solutions quant à leur contrôle. En effet, dans chaque écosystème, aucun organisme vivant n'est seul, car il est, en permanence, associé, plus ou moins étroitement, à de nombreux autres organismes, en particulier, des microorganismes. Leurs **interactions** peuvent être classées en fonction du degré d'association des organismes impliqués, de la durée de ces interactions et de leur caractère bénéfique (ou non) pour l'un et l'autre des partenaires. Toutes les situations intermédiaires existent, formant un véritable continuum. Il va des organismes libres qui ont besoin d'autres organismes pour se nourrir jusqu'aux parasites dont le cycle de vie repose, entièrement, sur des hôtes bien spécifiques. Symbiose et parasitisme permettent d'illustrer le fait, qu'au-delà de l'extrême diversité des situations, l'interaction est dans tous les cas, essentielle à la vie des partenaires, et souvent à l'origine de l'émergence de propriétés nouvelles des systèmes ainsi constitués. Des études antérieures ont révélé une relation « infection contre infection » intéressante entre divers agents pathogènes (Bustinduy et al. 2015, Griffiths et al. 2014). De plus, certaines études ont révélé que l'image classique des interactions hôtes-parasites comme étant un simple équilibre dynamique entre, seulement, deux partenaires, a, progressivement, changé, ces dernières années, avec la découverte de nombreux hôtes, naturellement, co-infectés par de multiples parasites (Shen et al. 2019). L'étude des **co-infections** s'avère, particulièrement, pertinente lorsque l'hôte est un vecteur ou la cause de la maladie elle-même (parasite), dans la mesure où

les **infections multiples** peuvent avoir d'importantes conséquences sur la transmission d'un parasite, à la fois à des échelles écologiques et évolutives. On observe un phénomène d'**antagonisme** où un agent pathogène d'une certaine espèce parvient à supprimer d'autres espèces d'agents pathogènes lors d'infections concomitantes (Shen et al. 2019). Cela se produit par l'un des deux processus suivants : le contrôle biologique des vecteurs d'agents pathogènes et l'inhibition de l'invasion, du développement et de la reproduction des agents transmis par ce vecteur. On peut prendre l'infection à ***Wolbachia*** comme l'exemple par excellence, de ces mécanismes. Le genre bactérien *Wolbachia*, à Gram négatif, représente le microbe endosymbiotique le plus commun chez les insectes et les filaires. Ce fait suscite un intérêt particulier pour la compréhension du rôle des co-infections dans le devenir des infections parasitaires. Plusieurs études ont révélé qu'en transférant *Wolbachia* aux moustiques, il est possible de **limiter la transmission de parasites**, tels que les agents du paludisme, des filarioses, ainsi que d'arboviroses émergentes (Bourtzis et al. 2014, Jeffries et Walker 2015, Le Page et Bordenstein 2013, Walker et Moreira 2011, Kambris et al. 2009, Bian et al. 2013).

De plus, les *Wolbachia* entretiennent une relation d'endosymbiose avec la majorité des filaires appartenant aux sous-familles des Onchocercinae et Dirofilariinae (principaux parasites de l'homme et des animaux vertébrés). L'endosymbiose est un type de symbiose qui représente la dépendance la plus intime entre les organismes, dans laquelle des êtres vivants (généralement, des microorganismes) vivent au sein de leurs hôtes eucaryotes (Wernegreen et al. 2012). Elle peut être caractérisée soit comme obligatoire, soit facultative, soit parasitaire, soit mutualiste ou soit commensaliste (Wernegreen et al. 2004). Chez les filaires, *Wolbachia* apparaît à l'intérieur de vacuoles des cellules hôtes, comprenant les cellules hypodermiques des cordons latéraux des mâles et des femelles, ainsi que dans la lumière des canaux excréteurs et sécréteurs, où elles peuvent être sécrétées (Makepeace et Tanya 2016), ainsi que dans le système reproducteur (les ovaires, les ovocytes et les embryons en développement), confirmant, ainsi,

la transmission verticale à travers le cytoplasme de l'œuf (Kramer et al. 2003, Landmann et al. 2010). La clairance de ces bactéries, à l'aide d'une antibiothérapie des hôtes infectés (hommes ou animaux), conduit à la mort des parasites, confirmant ainsi l'indispensabilité de cette relation endosymbiotique.

A l'occasion de mes travaux de thèse de science, menés durant les trois dernières années, j'ai, principalement, basé mes recherches sur une approche scientifique inductive ou expérimentale, c'est-à-dire à partir de faits (des données brutes, réelles, et observables) pour expliquer un phénomène. La majorité de mes travaux portent sur l'étude des **nématodes parasitaires (filaires et non filaires)** dans le but de développer des connaissances pour **mieux diagnostiquer, prévenir et contrôler les parasitoses** qu'ils causent.

Dans un premier temps, j'ai basé mes recherches sur la culture des bactéries endosymbiotiques du genre *Wolbachia*. En dépit de leur importance dans le contrôle des maladies vectorielles, ces bactéries demeurent mal étudiées depuis leur première description, il y a un siècle. Ceci est dû, d'une part, à la concentration des recherches uniquement sur la relation de ces bactéries avec leurs hôtes et non pas sur la bactérie elle-même, et d'autre part, à l'absence de techniques standardisées pour leur isolement et leur culture. Mes travaux ont donc porté sur la mise en place d'une technique permettant **l'isolement et la culture de ces bactéries exigeantes,** tout en apportant de nouvelles connaissances sur la génomique des *Wolbachia*.

Dans un deuxième temps, je me suis concentré sur l'étude de maladies vectorielles émergentes/négligées, tout particulièrement des helminthoses à transmission vectorielle. Les principales causes d'échec dans le contrôle de ces maladies transmissibles, qui conduisent à leur émergence et à leur propagation, sont l'absence de moyens efficaces de surveillance et de prévention. J'ai donc orienté mes travaux vers la **mise en place d'outils de diagnostic moléculaire et de stratégies de contrôle** pour les principales helminthoses émergentes.

La présente thèse sur articles comprend trois chapitres :

I. Le premier chapitre porte sur **la culture et la caractérisation génomique des bactéries du genre *Wolbachia***, qui ont comme but :

a. La mise en œuvre d'une technique d'isolement et de culture de ces bactéries exigeantes ;

b. La description et la caractérisation génomique des espèces isolées.

II. Le deuxième chapitre est relatif à **la détection et à la caractérisation (moléculaire et morphologique) de nématodes vectorisés**, ce qui a pour objectifs :

a. Le développement d'outils de diagnostic pour la détection des agents des filarioses en général et des filarioses canines émergentes en Europe ;

b. La caractérisation des agents de filarioses et d'autres nématodoses chez des primates non-humains en zones tropicales.

III. Le troisième chapitre concerne **l'épidémiologie et les moyens de contrôle des maladies vectorielles, principalement, les helminthoses vectorielles du chien**, ce qui nous a conduit à :

a. La mise en œuvre d'un essai clinique pour la prévention des maladies vectorielles du chien, avec l'évaluation de l'effet des antiparasitaires sur le comportement des moustiques au laboratoire ;

b. La surveillance épidémiologique des maladies vectorielles chez de potentiels réservoirs, en zone urbaines et périurbaines, avec un focus sur les helminthoses à transmission vectorielle chez l'hôte canidé.

Considérations éthiques et réglementaires

Durant mon parcours de thèse, j'ai été amené à mener des études sur l'animal, aussi bien au laboratoire que sur le terrain. Les expérimentations utilisant des animaux de laboratoire ont eu lieu au sein du laboratoire agréé NSB3 de l'IHU Méditerranée Infection, après validation du projet par le comité d'éthique d'Aix-Marseille Université et autorisation ministérielle, conformément à la réglementation mentionnée dans les articles R.214-87 à R.214-126 du code rural et de la pêche maritime. Par ailleurs, différents types de prélèvements ont été réalisés sur des animaux domestiques ou sauvages. Le respect du bien-être de l'animal a été, dans tous les cas, pris en compte, conformément aux normes dictées par l'Office International des Epizooties (OIE 2019), aux prescriptions réglementaires en vigueur en Europe (CE 1986) et à la réglementation nationale du pays d'origine. Parfois, nous étions amenés à collaborer avec des collègues vétérinaires, ou directement avec des propriétaires d'animaux en France, en Algérie ou ailleurs. En plus du respect du principe de base, concernant l'utilisation des échantillons qui doit, avant tout, bénéficier à la population d'origine et être réalisée avec des chercheurs locaux, nous avons également, dans certains cas, communiqué les résultats de nos diagnostics aux vétérinaires concernés et/ou aux propriétaires des animaux, afin que ces derniers bénéficient de la prévention ou des traitements adéquats.

Chapitre 1 :

Isolement, culture et caractérisation de *Wolbachia* à partir d'arthropodes vecteurs

Préambule

La symbiose, une relation entre les êtres vivants, fait débat depuis plus d'un siècle et demi. Elle a été décrite pour la première fois par le botaniste et mycologue allemand Heinrich Anton de Bary, en 1873, qui a inventé le terme symbiose pour décrire la vie en conjonction de deux organismes différents, généralement en étroite association, avec des effets bénéfiques pour au moins l'un d'entre eux. En 1877, Albert Bernhard Frank l'a décrite comme une relation mutualiste chez les champignons lichénisés (Moran 2006, Egerton 2015). Plus précisément, cette relation conduit à la création d'un nouveau type d'individu, elle devient une symbiogenèse, dans laquelle les organismes résultants ont de nouvelles caractéristiques (Kutschera et Niklas 2005). L'endosymbiose est, quant à elle, un type de symbiose qui représente la dépendance la plus intime entre les organismes, dans laquelle des êtres vivants (généralement microorganismes) vivent au sein de leurs hôtes eucaryotes (Wernegreen 2012). Elle peut être définie comme obligatoire, facultative, parasitaire, mutualiste ou commensaliste (Wernegreen 2004). Par ailleurs, les analyses génomiques suggèrent que ce phénomène d'endosymbiose semble être l'hypothèse la plus probable de l'origine des eucaryotes d'aujourd'hui. Selon López-García et David Moreira, la naissance de la cellule eucaryote est la résultante de **la fusion** entre une bactérie et une archée suscitée par des exigences métaboliques. Le noyau est apparu comme un moyen pour ces endosymbiontes d'empêcher l'interférence métabolique entre ces deux organismes, comme l'explique López-García "*You needed the [nuclear] membrane because you have two competing pathways*" (Pennisi 2004). Cependant, la théorie évolutive suggère que l'organisme anaérobique portant un noyau a également englouti un organisme endosymbiotique mitochondrial de nature aérobie obligatoire, probablement similaire en physiologie et mode de vie aux **alphaprotéobactéries de l'ordre des Rickettsiales** d'aujourd'hui (Martin et Mentel 2010). En effet, la transition de la bactérie endosymbiotique à une organelle permanente a entraîné un nombre massif de changements évolutifs, y compris

l'origine de centaines de nouveaux gènes et d'un système d'importation de protéines, **l'insertion de transporteurs membranaires, l'intégration du métabolisme et de la reproduction, la réduction du génome, le transfert de gènes endosymbiotiques et le transfert latéral de gènes.** Ces changements se sont produits progressivement à mesure que l'endosymbionte et l'hôte se sont intégrés (Roger et al. 2017). Bien que ces évènements se soient déroulés depuis un milliard et demi d'années, certains de ces phénomènes évolutifs sont observables avec les alphaprotéobactéries modernes. Par exemple, les *Wolbachia*, alphaprotéobactéries de la famille des Anaplasmataceae et de l'ordre Rickettsiales, présentent un génome réduit, et montrent une dépendance obligatoire entre elles et leurs hôtes, qui, quant à elle est issue de l'adaptation évolutive à la suite des phénomènes d'intégration métaboliques et de transfert latéral de gènes.

Bien que décrite au début du XX^{e} siècle, *Wolbachia*, une bactérie endosymbiotique, demeure un mystère de la microbiologie médicale. Cette α-protéobactérie de l'ordre des Rickettsiales et de la famille des Anaplasmataceae a, jusqu'à maintenant, une systématique incomplète. Malgré les différentes tentatives de classification, la bactérie appartient, actuellement, à une seule et unique espèce, à savoir : *Wolbachia pipientis* (Ferri et al. 2011). Depuis sa découverte en 1924, par Marshall Hertig et Simeon Burt Wolbach, et sa description formelle en 1936, par M. Hertig (Hertig et Wolbach 1924), la notion de supergroupe ou clade a été adoptée pour désigner les espèces de ce genre (Ramírez-Puebla et al. 2015). Ces limites de classification sont liées à l'absence de moyen de culture axénique permettant la caractérisation de la bactérie (Lindsey et al. 2016). Durant les deux dernières décennies, de nombreux progrès de recherche scientifique ont été orientés vers l'étude de la relation entre les *Wolbachia* et leurs hôtes. Il s'avère que ces bactéries entretiennent différents types de relations avec leurs hôtes, à savoir un parasitisme induisant l'incompatibilité cytoplasmique chez *Drosophila simulans*, un mutualisme nutritionnel chez les punaises de lit (*Cimex lectularius*) et

un mutualisme obligatoire chez certaines espèces de filaires (Gonza et al. 2009, Hosokawa et al. 2010). A cela, s'ajoute leur rôle dans des phénomènes physio-pathogéniques inflammatoires et des modifications de la réponse immunitaire en faveur de l'infection filarienne, faisant d'elles une efficace cible thérapeutique. Elles sont, pour cela, utilisées dans la lutte antivectorielle et/ou le contrôle des maladies vectorielles à cause du phénomène d'antagonisme (infection contre infection).

Dans mon travail, je me suis intéressé à l'isolement et à la culture des *Wolbachia*, en utilisant le système de co-culture cellulaire qui a été couplé à une étude génomique afin de décrire, caractériser et classer les bactéries isolées. J'ai eu accès à des échantillons d'une souche sauvage de punaises de lit de l'espèce *Cimex hemipterius*, collectées à Dakar (Sénégal), à partir de laquelle l'isolement et la culture de *Wolbachia* ont pu être faites. La relation entre *Wolbachia* et les punaises de lit a été bien décrite chez *Cimex lectelarius*. Ces dernières hébergent une *Wolbachia* appartenant au supergroupe F, et participe à la survie de son hôte, principalement, par la production de la biotine (vitamine B7). Ce type de voie métabolique est rare, et a été acquis chez cette souche de *Wolbachia* via le transfert latéral de l'opéron (gène) impliqué dans la biosynthèse de la biotine. Sur le plan de la lutte antivectorielle, cette relation peut constituer un point de vulnérabilité pour *Cimex lectularius* (Nikoh et al. 2014).

Au départ, j'ai ciblé la lignée cellulaire S2 de la drosophile car elle appartient à un hôte naturellement infecté par *Wolbachia* et qu'elle a montré une réceptivité aux autres bactéries de l'ordre des Rickettsiales, tel que *Rickettsia assemboensis* et *Rickettsia felis* (Maina et al. 2016, Luce-Fedrow et al. 2014). Cette lignée cellulaire s'est avérée plus efficace que la lignée C6/36 d'*Aedes albopictus* pour l'isolement et la culture de *Wolbachia*. L'analyse comparative basée sur la taxogénomie, la phylogénie et le métabolisme montre que la *Wolbachia* de *Cimex hemipterius* répond aux critères taxonomiques d'une nouvelle espèce bactérienne, c'est pourquoi, nous lui avons attribué le nom de *Wolbachia massiliensis* sp. nov. Cependant, j'ai

gardé le système de classification en supergroupe/clade, tout en l'attribuant à un nouveau supergroupe T, du fait que le dernier supergroupe décrit était le S (Lefoulon et al. 2020). Il est également important de noter que la relation *Wolbachia*-hôte chez cette espèce de punaise de lit diffère, de manière surprenante, de celle observée chez *Cimex lectularius*, à la fois sur le plan phylogénétique et métabolique. J'ai constaté la complète absence de l'opéron de la biotine et une évolution phylogénétique concordante entre la phylogénie et les autres opérons impliqués dans la biosynthèse des autres vitamine B, ce qui supporte l'hypothèse de l'évolution des *Wolbachia* via le transfert latéral de gènes (Gonza et al. 2009, Hosokawa et al. 2010).

L'approche que j'ai mise en œuvre pour l'isolement, la culture et la caractérisation des *Wolbachia* est détaillée dans **la publication N°1** de cette thèse. Elle peut être utile pour de futures études visant à développer la culture axénique et pour mettre en lumière d'autres mécanismes de cette endosymbiose.

Publication N°1

An Earliest Endosymbiont, *Wolbachia masseliensis* sp. *nov*., Strain PL13 From the Bed Bug (*Cimex hemipterius*), Type strain of a New supergroup T

Younes Laidoudi[1,2], Hacène Medkour[1,2], Mossaab Maaloum[1,3], Mariem Ben-Khedher[1,2], Bernard Davoust[1,2], Florence Fenollar[1,3], Didier Raoult[1,2] and Oleg Mediannikov[1,2]

[1] Aix-Marseille Université, IRD, AP-HM, MEPHI, Marseille, France
[2] IHU–Méditerranée Infection, Marseille, France
[3] Aix-Marseille Université, IRD, AP-HM, SSA, VITROME, Marseille, France

ARTICLE DE RECHERCHE

Soumis à : International Journal of Molecular Sciences

International Journal of
Molecular Sciences

MDPI

Article

An Earliest Endosymbiont, Wolbachia massiliensis sp. nov., Strain PL13 From the Bed Bug (Cimex hemipterius), Type strain of a New supergroup T

Younes Laidoudi1,2, Anthony Levasseur1,2, Hacène Medkour1,2, Mossaab Maaloum3, Mariem BenKhedher1,2, Masse Sambou4,5, Hubert Bassene5, Bernard Davoust1,2, Florence Fenollar1,6, Didier Raoult1,2,6and Oleg Mediannikov1,2*

[1] Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, 13385, Marseille CEDEX 05 ; younes.laidoudi@yahoo.com (Y.L.) ; anthony.levasseur@univ-amu.fr (A.L.) ; hacenevet1990@yahoo.fr (H.M.) ; mariem.ben-khedher@etu.univ-amu.fr (M.B.) ; bernard.davoust@gmail.com (B.D.) ; florence.fenollar@univ-amu.fr (F.F.) ; didier.raoult@gmail.com (D.R.); olegusss1@gmail.com (O.M.)
[2] IHU Méditerranée Infection, Marseille, France
[3] Laboratory of Biology and Health, Faculty of Sciences Ben M'sik, Hassan II University, 7955 Sidi Othmane,Casablanca, Morocco; momossaab@gmail.com (M.M.)
[4] IHU Méditerranée Infection-Vecteurs-Infections Tropicales et Méditerranéennes (VITROME), Marseille, France ; massezorro1@gmail.com (MS).
[5] Vectors-Tropical and Mediterranean Infections (VITROME), Campus International, UCAD-IRD, Dakar, Sénégal ; Hubert.Bassene@ird.fr (H.B.)
[6] Aix-Marseille Univ, IRD, AP-HM, SSA, VITROME, 13385, Marseille CEDEX 05
* Correspondence:Correspondence: olegusss1@gmail.com (O.M)

Abstract: The symbiotic Wolbachia are the most sophisticated mutualistic bacterium among all insect-associated microbiota. Wolbachia-insect relationship fluctuates from the simple facultative/parasitic to an obligate nutritional-mutualistic association as it was the case of the bedbug-Wolbachia from Cimex lectularius. Understanding this association may help in the control of associated arthropods. Genomic data have proven to be reliable tools in resolving some aspects of these symbiotic associations. Although, Wolbachia appear to be fastidious or uncultivated bacteria which strongly limited their study. Here we proposed Drosophila S2 cell line for the isolation and culture model to study Wolbachia strains. We therefore isolated and characterized a novel Wolbachia strain associated with the bedbug Cimex hemipterius, designated as wChem, and proposed Wolbachia masseliensis sp. nov strain wChem-PL13 a type strain of this new species from new supergroup T. Phylogenetically, T-supergroup was close to F and S-supergroups from insects and D-supergroup from filarial nematodes. We determined the 1,291,339-bp genome of wChem, which was the smallest insect-associated Wolbachia genomes. Overall, the wChem genome shared 50% of protein coding genes with the other insect-associated facultative Wolbachia strains. The complete folate and riboflavin biosynthesis pathways were identified in the genome of wChem. However, a diminution of translation factors was observed in the genome of wChem. These findings highlight the diversity of Wolbachia genotypes as well as the Wolbachia-host relationship among Cimicinae subfamily. The wChem provides folate and riboflavin vitamins on which the host depends, while the bacteria had a limited translation mechanism suggesting its strong dependence to its hosts. However, the clear-cut distinction between mutualism and parasitism of the wChem in C. hemipterius cannot be yet ruled out.

Keywords: Wolbachia; Bedbug; Cimex hemipterius; Isolation; Culture; Genomics; B-vitamins

1. Introduction

Bacteria of the genus Wolbachia represent the most successful symbiotic bacteria in the terrestrial ecosystem. Gram-negative bacteria of the family Anaplasmataceae in the order Rickettsiales, they are obligatory intracellular endosymbionts of several invertebrate taxa, Arthropoda and Nematoda. Till now, only one species, Wolbachia pipientis, has been axenically isolated and officially described [1]. Wolbachia are genetically diverse, as are the interactions with their hosts [2–6]. Most of Wolbachia genotypes, representing microbiologically separate species, were never isolated in pure culture. Currently, there is a general consensus to classify all genotypes in monophyletic lineage groups or supergroups from A to R, with a new supergroup "S" recently identified from the pseudoscorpion Atemnus politus [7]. The supergroups C, D and J infect exclusively filarial nematodes (Onchocercidae) [8–10]. Supergroup L exclusively contains plant parasitic nematodes (Pratylenchidae) [11,12]. Wolbachia supergroup F is the only clade composed by strains that infecting arthropods and some infecting filarial nematodes [13,14]. This includes especially hematophagous arthropods, such as biting Diptera and Hemiptera, fleas, lice and parasitic mites [15–26]. Recently, a novel strain of Wolbachia belonging to the supergroup F was isolated in Ixodes scapularis cells from a pool of Ctenocephalides sp. cat fleas [27].

The problems in Wolbachia taxonomy are evident. Different Wolbachia genotypes correspond clearly to different species both genetically and biologically [28]. Genetic distances among Wolbachia supergroups are huge, moreover, the same genotype may infect different insect species. Although attempts to classify Wolbachia in different genera were already done [29], but not widely accepted, mostly because of the difficulties in strain isolation [30]. Until recently, only few Wolbachia strains from clades A and B were known in axenic culture. The recent isolation of Wolbachia from Ctenocephalides felis in tick cell line is a rare example of a successful isolation of Wolbachia strain in cell culture [27].

Five distinct reproductive manipulations are induced in Wolbachia arthropod hosts: cytoplasmic incompatibility (CI), parthenogenesis induction, killing of male, feminisation and meiotic drive, all of which promote its spread by reducing competition for resources from males (a dead-end host) or by imposing an adaptation cost on uninfected females [31–34]. In some cases, Wolbachia form obligate and apparently beneficial relationships with their hosts [35,36]. For blood-feeding Diptera, the CI is the common phenotype [3,17,23]. In addition, Wolbachia has long been of applied interest in biological control for vector-borne disease control, Wolbachia symbiosis can be harnessed for vector control as well as the potential to combine the sterile insect technique and Wolbachia-based approaches for the enhancement of population suppression programs [37]. In filarial nematode diseases such as onchocerciasis and lymphatic filariasis, the use of antibiotics for Wolbachia elimination can safely clear adult worm infections [38]. Furthermore, several control programs releasing Wolbachia-infected Aedes aegypti to reduce the transmission of dengue and other arboviruses [39], because of Wolbachia infections can suppress the dissemination and transmission of pathogens in insects, especially when transinfected into a novel host [40].

Bed bugs are obligatory hematophagous insects with hemimetabolous development from egg to adult through five nymphal stages (instars), each of which requires a blood-meal to molt to the next stage [41]. They have re-emerged over the last decades worldwide where they may cause problems in housing facilities, public facilities, and residential complexes. In economically advanced countries, they are a serious public health. Bed bug infestations have been reported to have physical and psychological effects in humans. In addition, Despite isolation of several pathogens, found in the bed bug body, they have not been confirmed as a vector of pathogens to humans [42,43].

Infections with Wolbachia species of F supergroup seem to be common in the Cimicinae subfamily (Cimex and Oeciacus genera) [41,43]. Wolbachia's relationship with Cimex lectularius presumably evolved from a facultative association to obligate mutualism where the bacteria garner protection and nutrients within their host in exchange for supplementing the host's nutritional needs [20,22,44]. It was suggested new hypotheses about the coordination of Wolbachia growth and regression with its host's physiology and endocrine events [41].

Here, on the basis of taxono-genomic approach, we present the description of Wolbachia massiliensis Strain PL13 (CSURP2929), a new species of the genus Wolbachia belonged to a new Supergroup T, isolated from wild bed bugs Cimex hemipterius from Senegal Its growth condition as well as complete annotated genome are detailed.

2. Materials and Methods

2.1. Source of the bacterium, Inoculum preparation and Isolation

The bacterial strain was isolated from wild Cimex hemipterius collected in Dakar, Senegal. Ten adult bed bug individuals were used to isolate the intracellular bacterium using cell co-culture method using the Schneider 2 cell-line (S2) primarily derived from a culture of the late stage (20–24 hours old) Drosophila melanogaster embryos [45]. This cell line has previously proven to be receptive for several Rickettsiales bacterium such as Rickettsia assemboensis and Rickettsia felis [46,47]. Briefly, adult bugs Cimex hemipterius were rigorously decontaminated by immersing adult bedbug during 5 minutes into 1% of Sodium Hypochlorite solution (Sigma Aldrich) followed by rinsing in sterile water and immersing into 70% ethanol and sterile water rinsing again. Each specimen was manually crashed in 1 mL of Schneider medium (Sigma Aldrich) to generate the bacterial inoculum. Wolbachia cell co-culture was inoculated in the shell vial tubes containing 1 mL of S2 culture as described elsewhere [48,49]. Culture media consisted of a Schneider medium supplemented with 10% of decomplemented Bovine Serum Albumin (BSA) (Sigma Aldrich) and 1% of the combination Penicillin/Streptomycin (Sigma Aldrich) antibiotics to avoid ubiquitous bacterial contamination. The mixture was sterilised using 0.2 µm filtration and was then kept at 4°C until use. The infection was performed using 200 µL of the bacterial inoculum derived from adult C. hemipterius. One hour of centrifugation at 4000 rpm at 28°C was performed to increase Wolbachia-cell adhesion. Shell vials of infected cultures were kept at 28°C. Isolation success was assessed using Diff-Quick™ staining each 7 days followed by sequencing of the 16S RNA gene [50]. During the isolation period, the maintenance of cell culture was performed by a partial renewal of culture medium each 7 days. Shell vials were centrifuged at 3000 rmp at 28°C, then a half of the supernatant (500 µL) was replaced by a fresh medium under sterile conditions.

2.2. Morphological characterization and Scanning electron microscopy

Infected cells were cytocentrifuged for staining with Gimenez and Diff-Quick (Dade Behring, Marburg, Germany) and were then examined under light microscope Leica® DM LB2.

For the electron microscopy, 200 µL of 11 old days PL13-S2 co-culture were centrifuged for 15 minutes at 3000 rmp, then the supernatant was removed and the pellet was fixed using 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 1 hour. After fixation, the pellet was rinsed three times with 0.1M sodium cacodylate (5 minutes each) to remove residual fixative. The graded ethanol concentrations (25% for 5 minutes; 50% for 5 minutes; 70% for 5 minutes; 85% for 5 minutes; 95% for 5 minutes (twice); 100% ethanol for 10 minutes (three times)) was used for sample dehydration. Finally, the pellet was incubated for 5 minutes in an ethanol/Hexamethyldisilazane (HMDS, Sigma Aldrich, USA) (1:2) mixture, then in pure HMDS. The mixture was cytocentrifuged for 5 minutes at 2000 rpm and the glass slide allowed to air dry for 30 minutes before observation. The examination was performed using a TM4000PlusTM (Hitachi, Tokyo, Japan) scanning electron microscope operated at 10kV in BSE mode at magnifications ranging from X200 to X3000.

2.3. Cell co-culture standardization and Wolbachia production

Once the isolation success was confirmed by optical microscopy and 16S rRNA sequencing, the infected cells were transferred into 15 mL cap flasks for the maintenance of isolated strain. Medium changes were performed each 15 days by centrifugation for 15 minutes at 3000 rmp at 28°C, then the supernatant of the old medium was removed and replaced with the same volume of fresh medium. The mixture was subjected to serial gentle repeated pipetting until homogenization, then transferred to sterile cap flasks and maintained at 28°C.

In order to optimize Wolbachia cell co-culture, another arthropod cell line (C6/36) derived from Aedes albopictus mosquitoes. C6/36 cell line(CRL-1660; American Type Culture Collection) was maintained in 15 mL cap flasks containing the Leibowitz-15 medium with L-glutamine and L-amino acids (Gibco), 5% (vol/vol) fetal bovine serum, and 2% (vol/vol) tryptose phosphate (Gibco) at 28°C. One mL of a C6/36 rich cell culture was transferred to bottom tubes 24h prior to the infection. Wolbachia inoculum was obtained from a lysate of the Wolbachia S2 cells following a serial aspiration-injection into 50 mL falcon using a fine needle syringe. The inoculum of 200 µL was used for the infection of C6/36 cells previously prepared in bottom tubes as described above. The receptivity of C6/36 was first checked at day 15 post-infection using Diff-Quick™ staining. Once the infection was confirmed, the infected cells were transferred into 75 mL cap flasks containing 14 mL of 1 old day of C6/36 cell culture.

Bacterial growth was investigated from both infected cells under two different temperatures: 28°C and room temperature. Three cap flasks per each cell-line for each condition were followed for one month on the weekly schedule using the pan-Wolbachia 16S qPCR [All-Wol-16S qPCR] [51]. The repeated measures Analysis of Variance (ANOVA) was used to evaluate the effect of the temperature and the effect of cell line on bacterial growth, while a pairwise comparison using Tukey test was performed to evaluate whether condition is more suitable. Statistical analysis were performed using XLSTAT Addinsoft version 4.1 (XLSTAT 2019: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France). Once, the culture conditions were optimized, the suitable culture conditions (Cell line and temperature) were used to produce three 25 150-cm2 cell culture flasks.

2.4. Purification of the bacterium

S2 cells infected with the bacterium were produced in a total volume of 75mL spread over three 150-cm2 cell culture flasks. The infection rate of 97% was obtained at day 11 post-inoculation with the bacterium. Infected cells were harvested from the three flasks, then were checked for the presence of bacterial and fungal contaminations using both the Diff-Quick™ staining and the bacterial 16S rRNA sequencing. The suspension was subjected to three cycles of sonication of 1 minute at 20 Hz, after which unlysed cells were removed by centrifugation at 500 rpm for 10 minutes. The supernatant containing the bacterium was layered onto a density gradient solution of 15% weight/volume (wt/vol) sucrose in phosphate-buffered saline (PBS). After centrifugation at 9,000 g for 45 minutes at 4°C, the bacterium-containing pellet was resuspended in 2 ml of PBS and carefully layered onto a 20 to 45% (wt/vol in PBS) step density gradient. This gradient was subjected to centrifugation at 9000 rpm for 45 minutes at 4°C; and the bacteria were harvested and washed twice in PBS, resuspended in sterile distilled water in the smallest possible volume, and then frozen at -80°C. At each time point, the pan-Wolbachia 16S qPCR and the Diff-Quick™ staining were performed to assess bacterial load.

2.5. Genome sequencing and de novo assembly

Genomic DNA was extracted from 200 µL of purified bacterium. The extraction was performed using QIAGEN DNA tissues kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. An additional lysis step was applied prior to the extraction procedure using a pre-treatment by lysozyme incubation with buffer G2 and proteinase K for 2 h at 37 °C. The extracted gDNA was eluted in a total volume of 50 µL. Genomic DNA (gDNA) was quantified by a Qubit assay with the high sensitivity kit (Life technologies, Carlsbad, CA, USA); the concentration was equal to 42.86 ng/µL. The DNA was diluted at 1ng as input to prepare the paired end library. The gDNA was barcoded in order to be mixed with other genomic projects with the Nextera Mate Pair sample prep kit (Illumina). The purification on AMPure XP beads (Beckman Coulter Inc) was performed prior to the normalization of the libraries on specific beads according to the Nextera Mate Pair Illumina guide. Automated cluster generation and sequencing run with dual index reads were performed in a single 39-hours run in a 2×251-bp format. Within this run, a total of 190,631 reads were generated and were quality-checked using FastQC, trimmed using Trimmomatic version 0.36.624 and assembled in seventy-eight (78) scaffolds using the SPAdes version 3.5.0 sofware25. The option "careful" was used to reduce the number of mismatches and short indels. Default Nanopore technology (Oxford Nanopore Technologies Ltd.,

United Kingdom) was used by 1D genomic DNA sequencing on the MinION device using the SQK-LSK108 kit. The library was constructed from 1.5 μg of genomic DNA without fragmentation and end repair. Adapters were ligated to both ends of genomic DNA. After purification on AMPure XP beads (Beckman Coulter Inc), the library was quantified by a Qubit assay with the high sensitivity kit (Life technologies) and loaded on the flow cell via the SpotON port. A total of 466 active pores were detected for the sequencing and the workflow WIMP was chosen for sequence analysis. Adapter trimming, quality filtering and error correction of all sequencing raw data analysed here were performed using the Trimmomatic program (version 0.36). Finally, mean read quality was 11.2 (median=11.9). A total of 78,865 reads were generated with a mean length of 1,392.9 (median 762) and an N50 read length of 2,493, which corresponds to 109,847,620 pair bases (pb).

2.6. Comparative genomic analyses and annotation

First, the PL13 strain and eighteen other Wolbachia genomes were annotated using DFAST [52,53]. Numbers of orthologous proteins shared between genomes were visualized using Circos [54]. The following dataset was selected and used for genomic comparisons: wDmel, Wolbachia from Drosophila melanogaster (AE017196) for supergroup A, wPel, Wolbachia from Culex quinquefasciatus (AM999887) for supergroup B, wDimm, Wolbachia from Dirofilaria immitis (CP046578) for supergroup C, wBmal, Wolbachia from Brugia malayi (CP034333) for supergroup D, wFcan, Wolbachia from Folsomia candida (CP015510) for supergroup E, wClec, Wolbachia from Cimex lectularius (AP013028) for supergroup F, wPpe, Wolbachia from Pratylenchus penetrans (MJMG01000000) for supergroup L, wApol, Wolbachia from Atemnus politus (WQMQ00000000) for supergroup S, wCfeJ, Wolbachia from Ctenocephalides felis (CP051157) representing an undescribed supergroup from flea [55]. The circular map of the complete chromosome of PL13 strain was generated using with GCviewer (http://stothard.afns.ualberta.ca/cgview_server/). Annotation, completeness, and contamination values were estimated for the PL13 strain as well as the 9 selected Wolbachia genomes using DFAST [52,53]. The presence of prophage regions was predicted using PHASTER [56]. Orthologous Average Nucleotide Identity (Ortho-ANI) (https://www.ezbiocloud.net/tools/orthoani) [57] was used to evaluate the degree of genomic similarity between strain PL13 and the other Wolbachia genomes. The Pan genome distribution of the PL13 strain and other closely related strains was evaluated using Raory software[58].

Additionally, the pervious dataset was enriched by adding four genomes: wMeg, Wolbachia from Chrysomya megacephala (CP021120) and wDcit, Wolbachia from Diaphorina citri (CP051608) for the clade B, wCtub, Wolbachia from Cruorifilaria tuberocauda (CP046579) for the clade J and wCfelT, Wolbachia from Ctenocephalides felis (CP051156) which is also undescribed to belongs to any supergroup [55]. We also used the Genome-to-Genome Distance Calculator Web service to calculate DNA: Digital DNA hybridization estimates (dDDH) with confidence intervals according to recommended parameters (Formula 2, BLAST)(http://ggdc.dsmz.de/ggdc.php) [59][60]. The probability that an intergenomic distance yielded a DDH larger than 70%, representing a novel species-delimitation threshold [61]. Similarly, the prodigual was used for prediction in the Open Reading Frame (ORF) with the default settings [62]. Deviations in the sequencing regions predicted by ORFs have been excluded. BlastP was used to predict the bacterial proteome (E value of 1e03, coverage of 0.7 and percent identity of 30) according to the Orthological Group (COG) database [63]. In the absence of match within the COG database, the BlastP was performed against the GenBank, Nucleic. Acids database [64]within an E value of 1e03, coverage of 0.7 and 30% of identity. On the other hand, when the length of the sequence is less than 80 amino acids (aa), an E value of 1e05 has been used. The hmmscan analysis tool [65] was used on the domains that are maintained by the PFAM-A and PFAM-B domains. The assigned COGs for each genome were ordered in 26 different categories and were then compared using the Agglomerative Hierarchical Clustering (AHC) analysis. KEGG Orthology (KO) assignments was performed for the 14 Wolbachia genomes using KASS (KEGG Automatic Annotation Server) (http://www.genome.jp/kegg/kaas/) [66]. The KASS analysis was performed using BBH (bi-directional best hit) method. The assigned KO number were ordered in 177 different pathways and were then

assessed using the heat map (OMICS) method. The analysis excluded all pathways that having a variance lower than 0.25. All statistical analysis were performed using XLSTAT Addinsoft version 4.1 (XLSTAT 2019: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France).

2.7. B-vitamins biosynthesis pathway

Genes involved in the biosynthesis of B-vitamins were retrieved from the genome of (AP013028) bedbug-Wolbachia (wClec) of Cimex lectularius. wClec, known to have the most complete B-vitamins biosynthesis profile among insect-associated Wolbachia [67]. For the biotin (vitamin B7), riboflavin (vitamin B2), pyridoxine (vitamin B6), folate (vitamin B9) and thiamine (vitamin B1) synthesis genes present in wClec genome were identified and were then blasted separately against Wolbachia PL13 and the other 12 selected genomes using Blastn. Missing genes from each pathway in the genome wClec were also searched in all other Wolbachia genomes. B-vitamin profile of selected Wolbachia was assessed using the AHC analysis according to the presence/absence of genes. In order to test whether Wolbachia B-vitamins synthesis genes are conserved among Wolbachia supergroups and have the same ancestors, we phylogenetically compared all B-vitamins synthesis genes presumably common within Wolbachia supergroups. This include two datasets based on the following genes: (i) FolC, PdxJ, PdxH and RibB from 13 Wolbachia genomes including wPpe, Wolbachia supergroup L from the earliest Wolbachia host P. penetrans (MJMG01000000) [11]and (ii) FolC, PdxJ, PdxH, RibA, RibB, RibC, RibD, RibE and RibF from the same genome dataset except for wPpe which was excluded because it lacking for the complete gene datasets. Genes from each datasets were aligned using MAFFT [68] and concatenated within Seaview [69].Best fit phylogenetic model was selected for each dataset and the maximum likelihood phylogeny was performed using 1000 bootstraps replicates. Molecular phylogenetic analyses were conducted on Topali v.2 software [70].

2.8. Comparative phylogenies and taxonomy

First, two single locus sequence typing (SLST) phylogenies were performed on the basis of the 16S rDNA and Wolbachia surface protein (WSP) genes. Briefly, a full-length sequence from both genes were retrieved from the annotated genome of the PL13 strain. The 16S sequences were aligned against the representative members of fifteen (A, B, C, D, E, F, H, I, J, K, L, M, N, O, and S) and two undescribed Wolbachia supergroups. While the WSP sequence of the PL13 strain was aligned against the representative members of ten Wolbachia supergroups (A, B, C, D, E, F, J,R and S) and two undescribed supergroups. All alignments were performed using the ClustalW application within Bioedit v.7.2.5. [71]. The Akaike Information Criterion (AIC) option in MEGA6 [72] was used to establish the best nucleotide substitution model for the 16S sequence alignment. The Kimura 2-parameter model (+G) [73] was selected and the maximum likelihood (ML) phylogenetic inference was used with 1000 bootstrap replicates to generate the 16S tree in MEGA6 [72]. The 16 sequences from Ehrlichia chaffeensis (CP007480) and Anaplasma phagocytophilum (CP006617) were used as out groups to root the tree. The WSP phylogeny was inferred using ML method with 1000 bootstrap replicates on IQ-TREE [74] where the most appropriate model of evolution was evaluated by Modelfinder (implemented as functionality of IQ-TREE). The analysis was performed on Galaxy Australia server (https://usegalaxy.org.au/).

Genome-based phylogeny was performed for Wolbachia PL13 and 32 other complete/draft genomes of Wolbachia including 9 known supergroups of Wolbachia: A (CP037426, CP042904, CP042445, CP042444, CP042446, CP001391, AE017196, CP003884 and CP041215), B (CP041924, CP041923, CP031221, CP034335, CP034334, CP003883, CP016430, CP021120 and AM999887), C (CP046578, HE660029 and HG810405), D (CP034333, AE017321 and CP050521), E (CP015510), F (AP013028), J (CP046579 and CP046580), L (MJMG01000000) and S(WQMQ00000000) and two undescribed Wolbachia to belong to any supergroup (CP051156 and CP051157) [50]. The complete genome of Anaplasma phagocytophilum (CP006617) and Ehrlichia chaffeensis (CP007480) were included as outgroups. All genomes were aligned using the global alignment with conserved columns and gaps on Scapper software (https://github.com/tseemann/scapper). The FASTTREE [75]was then builded using ML method. GTR + CAT model was selected and the pseudocounts option was activated.

This latter is recommended for highly gapped/fragmentary sequences[75]. The tree was rooted by turning off maximum-likelihood option (1000 replicates).

Additionally, a multi loci sequence typing (MLST) phylogeny was performed on the basis of ten selected genes including: both the 16S and 23S ribosomal RNAs, DNA-directed RNA polymerase subunit beta (rpoB), 60 kDa chaperonin (GroL), cytochrome c oxidase subunit 1 (CoxA), chromosomal replication initiator protein (DnaA), fructose-bisphosphate aldolase (fbpA), aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B (gatB), transcription termination/antitermination protein (NusA) and gamma-glutamylputrescine synthetase (PuuA). The choice of these loci was conform to the standard loci requirements for an MLST system [76]. These genes were retrieved from the 35 genomes after annotation on DFAST. MAFFT alignment [68] was performed and sequences were then merged using Seaview [69]. ML phylogeny was performed with IQ-TREE [74] using 1000 bootstrap replicates. All phylograms from the SLST, MLST and FASTRTREE phylogenies were edited by iTOL v4 software [77].

3. Results

3.1. Isolation, culture, and description of the bacterium

Four of ten inoculated shell vials produced morphologically identical bacterial strains. Intracellular growth of bacteria were observed beginning from 14th day post inoculation. The 16S sequencing revealed the homogeneity of all isolated strains and were belonged to the genus Wolbachia according to the blast analysis. One strain, designated Wolbachia sp. wChem PL13, was then selected for the following investigations and characterisation. wChem PL13 was best visualised by the Diff-Quick staining while on the Gimenez and Gram staining the bacteria stained poorly, but always appeared to be gram negative. The bacteria appeared as small cocci not connected with each other inside intracellular vacuoles but not in the cytoplasm nor in the nucleus (Figure 1a). Therefore, the infected cells showed several vacuoles of different size according to the bacterial load within these latter (Figure 1a). Heavy infected cells were often disrupted during centrifugation using a Cytospin (Thermo Shandon) centrifuge as revealed by subsequent staining suggesting the fragility and death of S2 cells at hight infection levels (Figure 1b). Meanwhile, the infected cells continued to be able to multiply without there being any obvious cytopathic effect cells. Scanning microscopic examination showed that the bacterium present in the extracellular environment following cell lysis during cytocentrifugation have an average dimension of 570 nm (range: 530 to 615 nm). The bacteria present a regular form of a small cocci (Figure 1c).

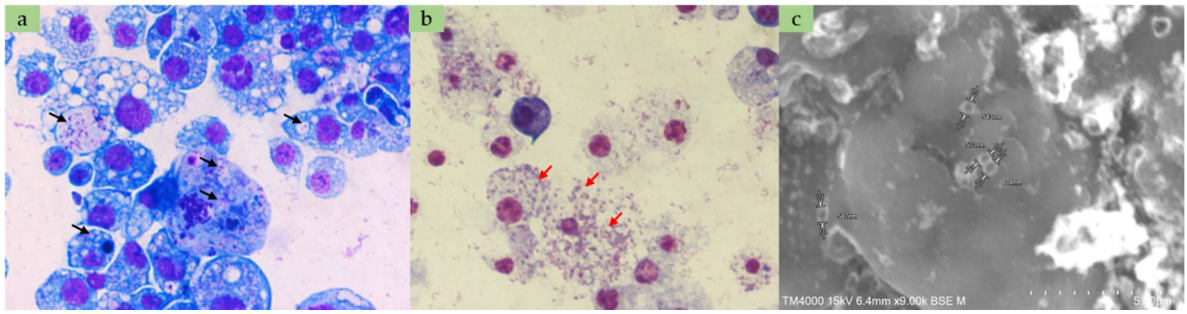


Figure 1. wChem PL13 grown (arrowed) in S2 cell-line. a, b: Diff-Quik staining, x1500 showing the intravacuolar location and bacterial load of wChem PL13 strain respectively. c: scanning microscopic examination of wChem PL13 strain.

The strain wChem PL13 was successfully propagated throughout both S2 and C6/36 cell lines. The bacterial growth was better within S2 cell lines at 28°C according to the qPCR results (Table S1). The difference between S2 and C6/36 cell lines in terms of bacterial load has become significant after three weeks of co-culture, while the difference between culture conditions (temperatures) has become significant after two weeks favourably to cultures maintained at 28°C for both cell lines (Table S1). In

term of speed of growth, the load of the wChem PL13 strain has become significantly detectable after two and three weeks of co-culture with S2 cells maintained at 28°C and room temperature respectively, and after three weeks within C6/36 cells from both conditions (28°C and room temperature) (Table S1).

During the whole processes of the purification, no loss of the bacterium and the sonication step induced only the lysis of cells but not that of the bacteria (Figure S1a), while the gradient density purification provided an integrated bacterium with hight density concentration (Figure S1b). Accordingly, the qPCR results indicated the maintenance of bacterial load during all purification steps. At the end of protocol, the bacteria were highly concentrated which gave the Ct value of 11.23 corresponding to 42.86 ng/µL.

3.2. Genome sequencing, annotation and genomic comparison

De novo assembly based on Illumina and MinION rids (Figure S2) gave a genome sequence from the wChem PL13 constructed by one contig of 1,291,339 with a G+C content of 35.4% (Figure 2a). We identified a total of 1,226 predicted protein-coding genes, in addition to 3 complete rRNA operons, 32 tRNAs and 1 tmRNA. Comparison of these genomic data with those from of the other Wolbachia supergroups showed that the genome of wChem PL13 is close to those encountered in other insects (Table 1). The strain wChem PL13 showed the presence of two prophage regions of 14.5 Kbp and 23.4 Kbp (Figure 2a and S3). Blast analysis revealed that the wChem PL13 prophages shared up to 19% to 58% with an identity ranged from 76.76% to 87.25% with those of other insect-associated Wolbachia such as wDmel, Wolbachia of D. melanogaster from supergroup A (AE017196) and wFcan, Wolbachia of F. candida from supergroup E (CP015510). However, up to 50% of the predicted protein-coding genes from thePL13genome were shared with other Wolbachia supergroups (Figure 2b).

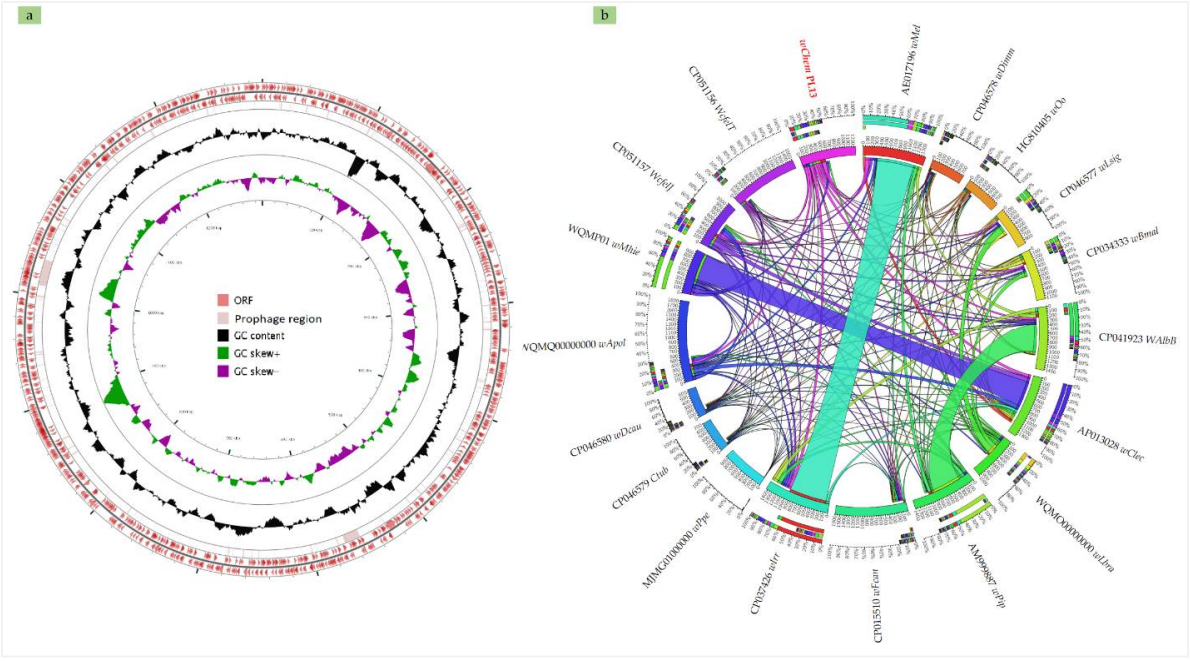


Figure 2. the complete chromosome of the Wolbachia sp. wChem PL13. a: circular map showing the annotation of the whole genome. b: detailed description of the prophage regions in the genome of the wChem PL13 strain.

353

354 **Table 1.** General features of wChem PL13 and other Wolbachia genomes.

Strain information's	***wChem* PL13**	***wClec***	***wDmel***	***wPip***	***wFcan***	***wApol***	***wCfeJ***	***wBmal***	***wDimm***	***wPpe***
Host type				Insects					Nematodes	
Wolbachia host	*C. hemipterius*	*C. lectularius*	*D. melanogaster*	*C. quinquefasciatus*	*F. candida*	*A. politus*	*C. felis*	*B. malayi*	*D. immitis*	*P. penetrans*
Supergroup	**New supergroup "T"**	**F**	**A**	**B**	**E**	**S**	**Undescribed**	**D**	**C**	**L**
Genome features										
Accession Number	CP061738	AP013028	AE017196	AM999887	CP015510	WQMQ00000000	CP051157	CP034333	CP046578	MJMG01000000
Total length (bp)	1,291,339	1,250,060	1,267,782	1,482,455	1,801,626	1,445,964	1,201,647	1,080,064	920,122	975,127
No. of contigs	1	1	1	1	1	373	1	1	1	12
GC content (%)	35.4	36.3	35.2	34.2	34.4	35.6	35.6	34.2	32.7	32.2
N50	1,291,339	1,250,060	1,267,782	1,482,455	1,801,626	5,741	1,201,647	1,080,064	920,122	95,55
Gap ratio (%)	0.326793	0.0	0.0	0.006746	0.0	0.0	0.0	0.0	0.0	0.13547
No. of CDSs	1,194	1,226	1,211	1,395	1,591	1,546	1,045	1,017	709	939
No. of rRNA	3	3	3	3	3	3	3	3	3	3
No. of tRNA	32	34	34	34	35	39	34	34	34	35
No. of CRISPRS	0	0	0	0	0	0	0	0	0	0
Coding ratio (%)	78.5	77.3	81.6	84.4	86.9	66.1	82.3	70.1	70.7	84.8
Completeness (%)	98.00	98.00	98.73	99.45	97.27	95.89	98.36	99.09	98.00	93.32
Contamination (%)	0.36	0.36	0.00	0.00	1.55	19.67	0.36	0.00	0.00	2.73
No. of prophage	2	3	3	4	6	2	0	0	0	1

355

Ortho-ANI values (Figure 3a) ranged from 74.63% with wPpe, Wolbachia supergroup L from P. penetrans (MJMG01000000) to 84.82% with wClec, Wolbachia supergroup F from C. lectularius (AP013028). The pangenome analysis of the wChem PL13 strain showed a total of 11,402 clusters genes distributed as follows: (Core genes = 0), (Soft core genes = 0), (Shell genes = 210) and (Cloud genes = 11192) respectively. The Ortho-ANI and the pangenome trees were clearly congruent (Figure 3a and b), where the wChem PL13 strain clustered with wApolK5, Wolbachia supergroup S from A. politus (WQMQ00000000), wClec, Wolbachia supergroup F from C. lectularius (AP013028) and wCfeJ, an undescribedWolbachia supergroup from C. felis (CP051157).

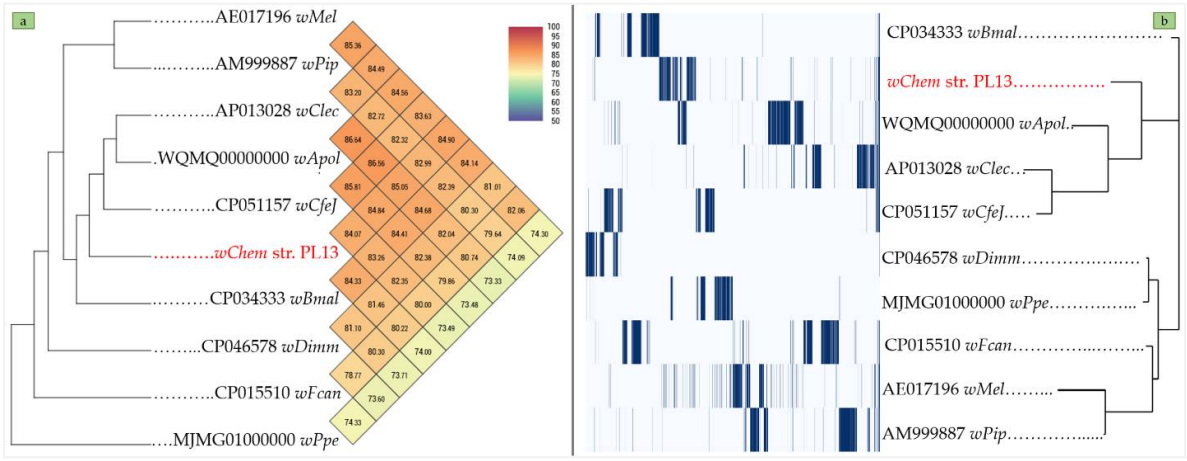


Figure 3. a. heatmap generated with Ortho-ANI values between the wChem PL13 strain and the other Wolbachia supergroups. b. pan-genome analysis of wChem PL13 strain based on the maximum likelihood tree from the accessory genome elements (right). The presence (blue) and absence (white) of accessory genome elements are presented on the left matrix.

Genomic comparaison of the wChem PL13 strain with the other Wolbachia supergroups using Digital DNA-DNA hybridization values (dDDH) are reported in Table 2. For the strain PL13, these values ranged from 19.8% with Ctub, Wolbachia supergroup J from C. tuberocauda (CP046579) to 30% with wApolK5, Wolbachia supergroup S from A. politus (WQMQ00000000).

Table 2. dDDH values of the wChem PL13 comparatively to the other Wolbachia supergroups using the formula 2.

Strain	Wolbachia host	Accession Number	DDH	Distance	Prob. DDH >= 70%	G+C difference	Model C.I.
wPpe	*P. penetrans*	MJMG01000000	19.8	0.2216	0	3.21	[17.6 - 22.2%]
Ctub	*C. tuberocauda*	CP046579	23	0.1901	0	3.09	[20.7 - 25.5%]
WCfelT	*C. felis*	CP051156	23.6	0.1853	0	0.19	[21.3 - 26%]
wFcan	*F. candida*	CP015510	24.1	0.1809	0.01	1.02	[21.8 - 26.6%]
wDimm	*D. immitis*	CP046578	24.8	0.1757	0.01	2.67	[22.5 - 27.3%]
wCmeg	*C. megacephala*	CP021120	27	0.1599	0.03	1.42	[24.7 - 29.5%]
wDcit	*D. citri*	CP051608	27	0.1604	0.03	1.38	[24.6 - 29.4%]
wPip	*C. quinquefasciatus*	AM999887	27.1	0.1598	0.03	1.18	[24.7 - 29.5%]
wCfeJ	*C. felis*	CP051157	27.8	0.155	0.04	0.2	[25.4 - 30.3%]
wBmal	*B. malayi*	CP034333	28.4	0.1512	0.05	1.19	[26 - 30.9%]
wDmel	*D. melanogaster*	AE017196	29.3	0.1462	0.08	0.14	[26.9 - 31.8%]
wClec	*C. lectularius*	AP013028	29.3	0.1462	0.08	0.88	[26.9 - 31.8%]
wApolK5	*A. politus*	WQMQ00000000	30	0.1423	0.1	0.23	[27.6 - 32.5%]

3.3. Metabolic pathway comparison

Overall, cluster of orthologous groups category composition (Figure 4A, Table S2) as well as KEGG pathways (Figure 4B, Table S3) of the wChem PL13 strain were similar to those of other insect-associated Wolbachia from A and B supergroups, indicating basically similarmetabolic capacities among these different Wolbachia strains. As any other insect-associated Wolbachia, the genome from the wChem PL13 strain differed from nematode-associated Wolbachia by the presence of replication, recombination and repair [L], Signal transduction mechanisms [T] and Mobilome: prophages, transposons [X]. However, the wChem PL13 genome revealed a diminutive number of genes involved in the translations [J] category (Figure 4A).

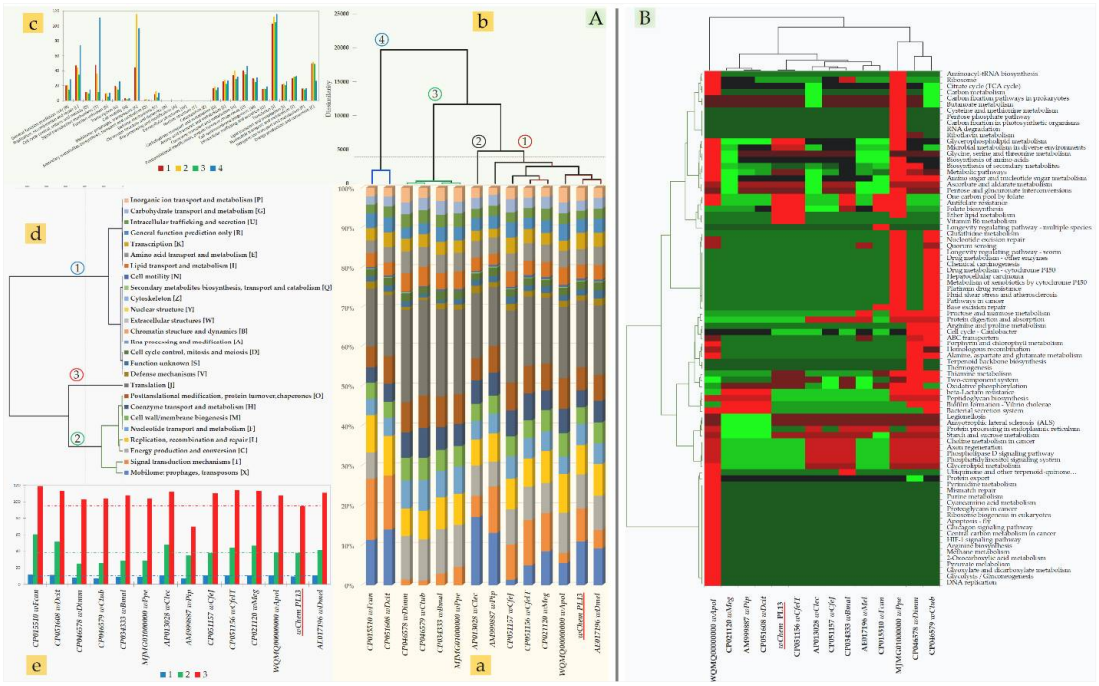


Figure 4. Comparative genomic analyses based on gene categories and metabolic pathways of the wChem PL13 (underlined in red) and other Wolbachia genomes. A.a.3-D stacked column representing COG profiles of the PL13 and the other Wolbachia strains, b. Hierarchical clustering of Wolbachia strains according to their COGs profiles c. Clustered columns comparing the numbers of COG categories of each Wolbachia clusters. c. Hierarchical clustering of COG categories among Wolbachia strains and d. Clustered columns comparing the numbers of each COG cluster among Wolbachia strains. B. OMICs analysis of KEGG pathways showing the position of metabolic profile of the wChem PL13 strain among the other Wolbachia supergroups.

3.4. B-vitamin synthesis patterns in the wChem PL13 and other Wolbachia genomes

The inspection of wChem PL13 genome revealed B-vitamins synthetic pathways commonly present in the most Wolbachia genomes. This include the complete pathway for riboflavin (vitamin B2) and for folate (vitamin B9) with a partial pathway for both pyridoxine (vitamin B6) and thiamine (vitamin B1). Unlike Wolbachia of C. lectularius, the biotin (vitamin B7) biosynthesis pathway was completely absent in the genome of the wChem PL13 strain (Figure 5a).

Both datasets based on gene involved in B-vitamins biosynthesis produced a close topology and very similar posterior bootstrap values. Insect-associated Wolbachia from the A, B and E supergroups as well as the supergroup L from the non-filarial nematode had an earliest divergence compared to the filarial and the other insect-associated Wolbachia. However, the wChem PL13 strain and the supergroup T appeared to be a monophyletic sister with the clade regrouping the supergroup F from C. lectelarius and C, D and J supergroups from filarial nematodes (Figure 5b), suggesting a less older B-vitamins biosynthesis genes compared to those from the other insect-associated Wolbachia (e.g. A, B and E supergroups) genomes.

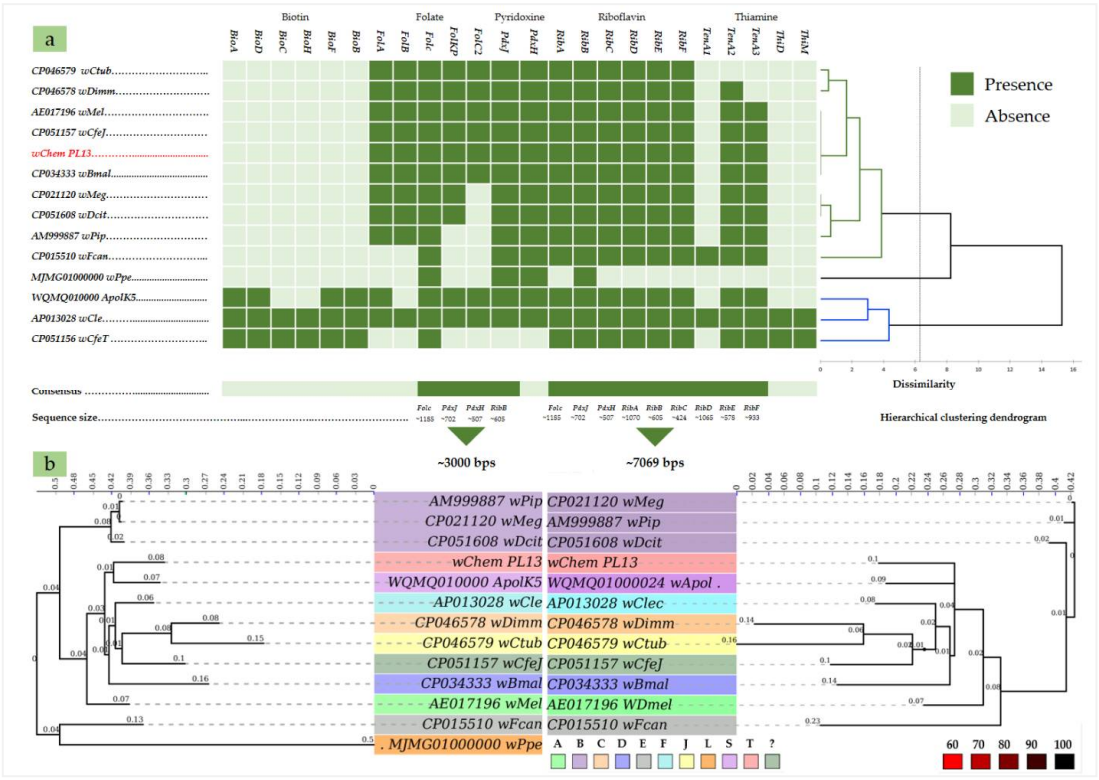


Figure 5. a.Comparative analysis of B-vitamins biosynthesis pathways of the wChem PL13 strain and the other Wolbachia strains. a. Matrix based on the presence/absence of B-vitamins biosynthesis genes from the wChem PL13 and the other Wolbachia genomes. The matrix was organized according to the hierarchical clustering (AHC) of B-vitamins profile among Wolbachia strains (left cladogram). b. Maximum likelihood phylogenies based on 3000 bps (right phylogram) and 7069 bps (left phylogram) using respectively K81uf (+G) [78] and GTR (+G) [79] substitution models. Likelihoods values were -16949.78 and -36573.10 respectively. Values above branches indicate the length of each branch's, while the axis showed the global distance observed throughout the trees. Colour codes indicating the Wolbachia supergroup (label) and the bootstraps percent's (branches).

3.5. Comparative Phylogenies and Placement of Wolbachia sp. Strain wChem PL13 in the Wolbachia T Supergroup

Together, the SLST based on the 16S and the WSP genes, the MLST based on the ten selected genes (16S, 23S, GroL, rpoB, gatB, coxA, dnaA, fbpA, puuA and nusA) as well as the genome-based phylogeny allowed the comparison of the wChem PL13 strain with all known Wolbachia supergroups except for G, Q and P supergroups, where the suitable dataset were not available. However, the blast comparison of the 16S gene from the wChem PL13 strain with Wolbachia of Diaea sp. (AY486069) supergroup G [80], Wolbachia of Torotrogla cardueli (KP114100) supergroup Q and Wolbachia of Torotrogla merulae (KP114099) supergroup P [81], revealed an identity-query cover of 97.77%-53%, 97.72%-41% and 98.54%-41% respectively, which is lower than the 98.7% threshold used to discriminate bacterial species [82].

All cladograms constructed from the SLSTs (Figure S4 a and b), MLST (Figure 6a) and genome-based phylogeny (Figure 6b) supported the divergence of the wChem PL13 strain from known Wolbachia supergroups. Notably, both MLST and genome-based phylogenies produced similar topologies within the same clade and very similar bootstrap values. The only topology differences between trees based on MLST and the Wolbachia whole genome datasets were the varying positions of wCfeJ, an undescribed Wolbachia supergroup from C. felis (CP051157) and wApolK5, Wolbachia supergroup S from A. politus (WQMQ00000000), but otherwise no conflicts in Wolbachia clade topologies were observed (Figure 6 a and b).

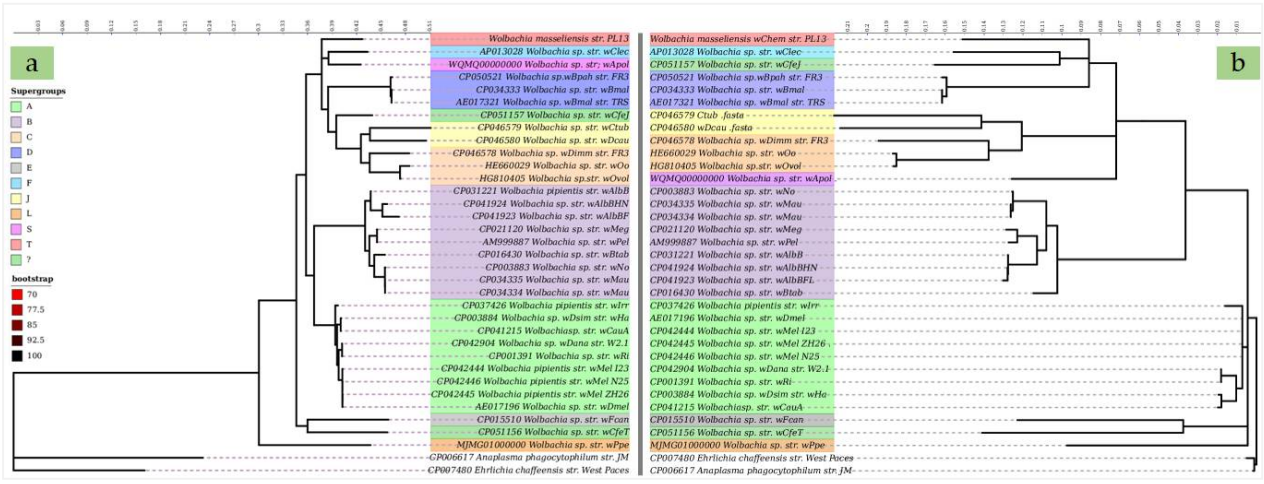


Figure 6. Comparative phylogenies showing the position of the wChem PL13 strain among the other Wolbachia supergroups. a. IQTREE based on ML method with 1000 bootstraps from the concatenated ten selected genes using GTR (+F+R3) substitution model [79]. Outgroup taxons "Ehrlichia chaffeensis (CP007480) and Anaplasma phagocytophilum (CP006617)" are drawn at root. Log-likelihood of consensus tree is -88934.819989.b. Genome based phylogeny generated using the FastTree Version 2.1.10, double precision (No SSE3) [75]. The tree was rooted using Jukes-Cantor Joins model with 1000 local boots. The nearest-neighbor interchange (NNI) and the subtree pruning and regrafting (SPR) (2 rounds range 10) were used for the tree rearrangement. The TopHits was 1.00*sqrtN.

3.6. Description of Wolbachia massiliensis sp. nov.

From all descriptive results taken together, the wChem-PL13 isolated from the bedbug C. hemipterius strain constitutes a divergent Wolbachia strain. Genotypic profile based on the 16S and 23S rRNA, WSP, rpoB, GroL, CoxA, DnaA, fbpA, Asn/Gln, gatB, NusA and PuuA as well as the taxono-genomic features delineated a distinct species, clearly different from all other recognized Wolbachia strains for which we propose the name Wolbachia masseliensis sp. nov.

Wolbachia masseliensis (mas.si' li.en.sis. L. gen. adj. massiliensis, from Massilia, the Latin name of Marseille, France, where the organism was first grown, identified and characterized). Since the current Wolbachia supergroup classification system is yet be revisited [30], we maintain the notion of supergroup at a strain level and we propose a new supergroup T with a type strain Wolbachia masseliensis sp. nov. strain wChem-PL13. The known host of this bacterium is Cimex hemipterius, a wild strain from Senegal. This isolate has been deposited in the strain collection CSUR (Collection de Souches de l'Unité des Rickettsies WDCM 875) under the accession number CSURP2929. The complete genome sequence of W. masseliensis is available in GenBank: Bio Project PRJNA663644; Bio Sample: SAMN16175503 and genome accession number CP061738.

The cells are best visualised by the Diff-Quick staining and appear to be gram negative, small cocci not connected with each other. The bacteria are obligate intracellular and occur inside vacuoles of eukaryotic cells (A. albopictus and D. melanogaster). The bacteria grow in S2-cell line at 28°C in Schneider medium supplemented with 10% of decomplemented Bovine Serum Albumin (BSA) and 1% of the combination Penicillin/Streptomycin antibiotics.

4. Discussion

In the present study we demonstrate the possibility to use S2 cells for the isolation and maintenance of Wolbachia. Our data demonstrated the susceptibility of two arthropod cells derived from D. melanogaster and A. albopictus mosquito, arthropods naturally infected with well-known Wolbachia supergroup A and B, respectively. However, the best bacterial growth was obtained after two weeks on Drosophila S2 cell lines. Several cell lines were previously used in the isolation and/or cultivation of Wolbachia bacterium including the Aa23 mosquito cell line [83,84], C6/36 cells, another

A. albopictus cell line and the human embryonic lung (HEL) fibroblast monolayers [84] for wALB13, Wolbachia supergroup B from A. albopictus. Different cell lines derived from Ixodes scapularis and I. Ricinus as well as well as the A. albopictus cells (AeAl-2) were used for the propagation of three Wolbachia strains wStri, supergroup A, wAlbB supergroup B and wCfeF supergroup F from Laodelphax striatellus, A. albopictus and C. felis respectively[27].

Despite the successful propagation of different Wolbachia supergroups on different mammalian and insects cell lines, there are no standardized cell line for the co-culture of Wolbachia. Our data showed that the infection of up to 97% S2 cells with Wolbachia from C. hemipterius occurred at 11 days which consist with the results previously obtained on Aa23 mosquito cells, naturally found infected with Wolbachia [83]. Since the ability of the S2 cell line to provide a similar Wolbachia growth to that obtained on naturally infected cell line, we propose S2 cell line as standard line for Wolbachia culture.

It is clear from our results that culture temperature affects the growth of Wolbachia, where the best growth was always at 28°C, the adequate temperature for S2 and C6/36 cell lines. However, several studies shown that the in vivo growth of Wolbachia was always in line with the culture temperature of the cell line which varied from 28°C to 37°C [3,27,83,84]. Recently, it was demonstrated that changes to host temperature preference do not alter bacterial load of several A and B-supergroup Wolbachia strains. However, hosts infected with A-group Wolbachia strains prefer cooler temperatures while those infected with B-group Wolbachia strains prefer a warmer temperature, suggesting that Wolbachia strains are differently involved in the host-thermoregulation [85].

Recently, an efficient genome sequencing approach based on probe hybridization enrichment was developed to provide Wolbachia genomes directly from their hosts [86]. However, the application of the approach was strongly limited by the amount of bacteria from their hosts, as it was the cases of wApolK5, Wolbachia supergroup S from A. politus (WQMQ00000000) and wLbra, Wolbachia supergroup D from Litomosoides brasiliensis (WQMO00000000) [7,87]. In addition to the manipulation of the bacterium, the standardized protocol for the isolation, culture as well as the purification of Wolbachia herein we described, leading to easily obtain enough bacterial DNA which facilitate the genome sequencing.

We obtained the complete genome of wChem PL13 strain, W. massiliensis, a type strain of new supergroup T from C. hemipterius (1,250,060 bp long) which closely mimic the size of wCle, Wolbachia supergroup F from C. lectelarius (1,291,339) and wDmel, Wolbachia supergroup A from D. melanogaster (1,267,782) genomes. However, it seems to be the smaller complete genome from insect-associated Wolbachia since the size of complete insect-associated Wolbachia genomes ranged between 1, 133,809 bp long and 1,801,626 bp long [6,88]. While it was clearly bigger than those associated to nematodes where the size ranged from 920,122 bp long to 1,080,064 bp long [87,89]. Furthermore, we noted the presence of two sequences coding for phage-like proteins, such as portal, coat transposons, and integrase proteins (Figure S3).Wolbachia phage-like proteins were mostly identified in insect-associated Wolbachia and in wPpe, Wolbachia supergroup L from plant parasitic nematode (MJMG01000000), while they were completely absent in filarial-associated Wolbachia (Table 1) suggesting Wolbachia bacteriophage (WO) infections from the environment of their hosts. Furthermore, the molecular analysis of the prophage coding sequences from W. massiliensis wChem PL13, revealed a partial similarity with those encountered in wDmel, Wolbachia of D. melanogaster from supergroup A (AE017196) and wFcan, Wolbachia of F. candida from supergroup E (CP015510). Wolbachia-bacteriophage WO relationship was molecularly studied in wasps community [90]. Authors were noted the absence of congruence between WO and host Wolbachia as well as WO and insect host, suggesting that the phage WO exchanged frequently and independently within the closed syconium [90].

The genome of W. massiliensis, wChem PL13 strain from the bedbug C. hemipterius revealed similar metabolic capacities among the parasitic insect-associated Wolbachia from A and B-supergroups. By contrast, the mutualistic bedbug-Wolbachia supergroup F from C. lectilarius[67] closed to the other mutualistic Wolbachia supergroups [89] (Figure 4). This emphasis the diversity of

Wolbachia-host relationship among the bedbugs. Except for the translation COG category [J] which appears to be reduced in the genome of W. massiliensis suggesting a strong dependence to its host. Although it is difficult to conclude about the provision of nutritional elements to the host by W. massiliensis as long as the biotin pathway was completely absent. The biotin appears to be rare among Wolbachia supergroups and was detected only in few insect-associated Wolbachia [7]. The complete Wolbachia biosynthesis pathway for the biotin was firstly detected in the bedbug Wolbachia of C. lectilarius wClec. In addition to the biotin, wClec participates in the host fitness by producing B-vitamins [67,91]. The biotin was latter well studied in the community of Cimex and Paracimex arthropods using gene-specific PCRs [91]. Authors repported the foncional biotin pathways in at leat 10 out of 15 studied genera but not in C. hemipterius [91]. The absence of Wolbachia-biotin production in the bedbug C. hemiterius could be encompenssed by the other symbiotic bacterium. The genitic study of the origin of biotin operons demonstrated that they were acquired via lateral gene transfer presumably from a coinfecting endosymbiont Cardinium or Rickettsia [67]. The congruence between the phylogenies of B-vitamins operons (i. e. riboflavin, folate and pyridoxine) consistently exhibited similar evolutionary patterns with Wolbachia phylogeny. Consequently, it is conceivable, although speculative, B-vitamins synthesis genes are originated from the other symbiotic bacterium within their hosts as it was the case of biotin and thiamine from the bedbug-Wolbachia of C. lectelarius and the obligate symbiont Wigglesworthia glossinidia of tsetse flies [67,92].

5. Conclusion

Our results emphasize the usefulness of the S2 cells as a suitable line for the isolation and the propagation of Wolbachia. Thereby, the standardized procedure herein we proposed provided sufficient material for genome sequencing and other manipulation. This may help to resolve problems related to the direct sequencing of wolbachial genomes from their hosts [7,86] as well as to cultivate the previously uncultivated Wolbachia (e.g. filaria-associated Wolbachia) since the S2 line allowed the successful culture of the PL13 strain in record time (11 days) despite the reduction of translational machinery of this bacteria.

The strain seems to be appeared as nutritional-mutualistic and parasitic endosymbiont, because its translation ability is reduced but provided B-vitamins (folate and riboflavin), necessary metabolites for the fitness of its host [91]. These features provide the platform for the feature research to understand Wolbachia-host interaction. By combining bacterial isolation and taxo-genomic descriptions may ultimately assist in the quest to classify Wolbachia in multiple species as is the case of the other Rickettsiales bacterium such as Rickettsia spp. Anaplasma spp., and Ehrlichia spp.

Supplementary Materials: Table S1: Weekly follow-up of bacterial growth using qPCR Ct values. Table S2: Number of genes associated with the 25 general clusters of orthologous groups (COG) functional categories of Wolbachia massiliensis and other Wolbachia genomes. Table S3: Number of genes associated with metabolic pathways of Wolbachia massiliensis and other Wolbachia strains according to the Kyoto Encyclopedia of Genes and Genomes (KEEG). Figure S1: Microscopic examination of sonicated S2 cells infected with Wolbachia massiliensis, a. before purification, b. after purification. c and d. assessment of bacterial load using qPCR. Figure S2: Representative output describing the results of the de novo assembly of the Wolbachia massiliensis genome based on MinION and Illumina reads. Figure S3: Identification and annotation of Wolbachia prophage regions from the Wolbachia massiliensis genome using PHASTER (PHAge Search Tool Enhanced Release) server. Figure S4: Comparative single locus sequence typing (SLST) phylogenies showing the position of Wolbachia massiliensis throughout Wolbachia supergroups. Phylograms were generated using the maximum likelihood (ML) inferences with 1000 replicates based on a. the Kimura 2-parameter model (+G) from the 16S rRNA gene and IQ-TREE from the WSP gene.

Author Contributions: Y.L., BD., FF. and O.M. designed the study; Y.L., performed the lab work; YL., MB., MM and AL., genomic analysis; MS., HB. and OM. field investigations; YL., H.M., F.F., D.R., and OM., writing—review and editing the article. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Institut Hospitalo-Universitaire Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: We are in debt to Maxence Aubadie-Ladrix for technical assistance and Terras Jerome for taking the scanning electron microscope photographs. We are grateful for Hitachi team of Japan (Hitachi High-Technologies Corporation, Science & Medical Systems Business Group 24-14, Tokyo, Japan) for the installation of a TM4000 microscope at the IHU Méditerranée Infection.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hertig, B.Y.M.; Ph, D. XXVIII OCTOBER , 1936 No . 4 THE RICKETTSIA , WOLBACHIA PIPIENT1S (GEN . ET SP . N .) AND ASSOCIATED INCLUSIONS OF THE MOSQUITO , CULEX PIPIENS The Rickettsia , Wolbachia pipientis. 1936, XXVIII.
2. Zhou, W.; Neill, S.O. Phylogeny and PCR-based classi ® cation of Wolbachia strains using wsp gene sequences. 1998.
3. Khoo, J.J.; Kurtti, T.J.; Husin, N.A.; Beliavskaia, A.; Lim, F.S.; Mustika, M.; Zulkifli, S.; Al-khafaji, A.M.; Hartley, C.; Darby, A.C.; et al. Isolation and Propagation of Laboratory Strains and a Novel Flea-Derived Field Strain of Wolbachia in Tick Cell Lines. 1980, 1–19.
4. Baldo, L.; Hotopp, J.C.D.; Jolley, K.A.; Bordenstein, S.R.; Biber, S.A.; Choudhury, R.R.; Hayashi, C.; Maiden, M.C.J.; Werren, J.H. Multilocus Sequence Typing System for the Endosymbiont Wolbachia pipientis . 2006, 72, 7098–7110, doi:10.1128/AEM.00731-06.
5. Comandatore, F.; Cordaux, R.; Bandi, C.; Blaxter, M.; Darby, A.; Makepeace, B.L.; Montagna, M.; Sassera, D. Supergroup C Wolbachia , mutualist symbionts of filarial nematodes , have a distinct genome structure. 2015.
6. Kampfraath, A.A.; Klasson, L.; Anvar, S.Y.; Vossen, R.H.A.M.; Roelofs, D.; Kraaijeveld, K.; Ellers, J. Genome expansion of an obligate parthenogenesis-associated Wolbachia poses an exception to the symbiont reduction model. 2019, 1–14.
7. Lefoulon, E.; Clark, T.; Borveto, F.; Perriat-sanguinet, M.; Moulia, C.; Slatko, B.E.; Gavotte, L. Pseudoscorpion Wolbachia symbionts : diversity and evidence for a new supergroup S. 2020, 1–15.
8. Bandi, C.; Anderson, T.J.C.; Genchi, C.; Blaxter, M.L.; Celoria, V. Phylogeny of Wolbachia in ® larial nematodes. 1998.
9. Casiraghi, M.; Bain, O.; Guerrero, R.; Martin, C.; Pocacqua, V.; Gardner, S.L.; Franceschi, A.; Bandi, C. Mapping the presence of Wolbachia pipientis on the phylogeny of filarial nematodes: evidence for symbiont loss during evolution. Int. J. Parasitol. 2004, 34, 191–203.
10. Lefoulon, E.; Bain, O.; Makepeace, B.L.; d'Haese, C.; Uni, S.; Martin, C.; Gavotte, L. Breakdown of coevolution between symbiotic bacteria Wolbachia and their filarial hosts. PeerJ 2016, 4, e1840.
11. Brown, A.M.V.; Wasala, S.K.; Howe, D.K.; Peetz, A.B.; Zasada, I.A.; Denver, D.R. Genomic evidence for plant-parasitic nematodes as the earliest Wolbachia hosts. Sci. Rep. 2016, 6, 1–14, doi:10.1038/srep34955.
12. Haegeman, A.; Vanholme, B.; Jacob, J.; Vandekerckhove, T.T.M.; Claeys, M.; Borgonie, G.; Gheysen, G. An endosymbiotic bacterium in a plant-parasitic nematode: member of a new Wolbachia supergroup. Int. J. Parasitol. 2009, 39, 1045–1054.
13. Ferri, E.; Bain, O.; Barbuto, M.; Martin, C.; Lo, N.; Uni, S.; Landmann, F.; Baccei, S.G.; Guerrero, R.; de Souza Lima, S. New insights into the evolution of Wolbachia infections in filarial nematodes inferred from a large range of screened species. PLoS One 2011, 6, e20843.
14. Lefoulon, E.; Gavotte, L.; Junker, K.; Barbuto, M.; Uni, S.; Landmann, F.; Laaksonen, S.; Saari, S.; Nikander, S.; Souza, S. De; et al. A new type F Wolbachia from Splendidofilariinae (Onchocercidae) supports the recent emergence of this supergroup q. Int. J. Parasitol. 2012, doi:10.1016/j.ijpara.2012.09.004.
15. Fischer, P.; Schmetz, C.; Bandi, C.; Bonow, I.; Mand, S.; Fischer, K.; Dietrich, W.B. Tunga penetrans : molecular identification of Wolbachia endobacteria and their recognition by antibodies against proteins of endobacteria from filarial parasites q. 2003, 102, 201–211, doi:10.1016/S0014-4894(03)00058-4.
16. Gorham, C.H.; Fang, Q.Q.; Durden, L.A. Wolbachia endosymbionts in fleas (Siphonaptera). J. Parasitol. 2003, 89, 283–289.
17. Sicard, M.; Bonneau, M. ScienceDirect Wolbachia prevalence , diversity , and ability to induce cytoplasmic incompatibility in mosquitoes ` ne Weill. 2019, 12–20, doi:10.1016/j.cois.2019.02.005.

18. Driscoll, T.P.; Verhoeve, V.I.; Gillespie, J.J.; Johnston, J.S.; Guillotte, M.L.; Rennoll-bankert, K.E.; Rahman, M.S.; Hagen, D.; Elsik, C.G.; Macaluso, K.R.; et al. A chromosome-level assembly of the cat flea genome uncovers rampant gene duplication and genome size plasticity. 2020, 1–19.
19. Dittmar, K.; Whiting, M.F.; Dittmar, K.; Whiting, M.F. new Wolbachia endosymbionts from nearctic and neotropical fleas (siphonaptera) new Wolbachia endosymbionts from nearctic and neotropical fleas (siphonaptera). 2004, 90, 953–957.
20. Gonza, G.; Santos, M.F.B.; Solano, J.; Sousa, O.; Moreno, N.; Windsor, D.; Ying, A.; Vilchez, S.; Osuna, A. Detection of Wolbachia Bacteria in Multiple Organs and Feces of the Triatomine Insect Rhodnius pallescens (Hemiptera , Reduviidae) . 2009, 75, 547–550, doi:10.1128/AEM.01665-08.
21. Crainey, J.L.; Wilson, M.D.; Post, R.J. Phylogenetically distinct Wolbachia gene and pseudogene sequences obtained from the African onchocerciasis vector Simulium squamosum q. Int. J. Parasitol. 2010, 40, 569–578, doi:10.1016/j.ijpara.2009.10.017.
22. Hosokawa, T.; Koga, R.; Kikuchi, Y.; Meng, X.; Fukatsu, T. Wolbachia as a bacteriocyte-associated nutritional mutualist. 2010, 107, doi:10.1073/pnas.0911476107.
23. Doudoumis, V.; Alam, U.; Aksoy, E.; Abd-alla, A.M.M.; Tsiamis, G.; Brelsfoard, C.; Aksoy, S.; Bourtzis, K. Tsetse- Wolbachia symbiosis : Comes of age and has great potential for pest and disease control. J. Invertebr. Pathol. 2013, doi:10.1016/j.jip.2012.05.010.
24. Tay, S.T. Scientific Note Wolbachia endosymbionts , Rickettsia felis and Bartonella species , in Ctenocephalides felis fleas in a tropical region. 2013, 59, 2011–2013.
25. Chaisiri, K.; Mcgarry, J.W.; Morand, S.; Makepeace, B.L. Symbiosis in an overlooked microcosm : a systematic review of the bacterial fl ora of mites. 2015, 1152–1162, doi:10.1017/S0031182015000530.
26. Onder, Z.; Ciloglu, A.; Duzlu, O.; Yildirim, A.; Okur, M.; Yetismis, G.; Inci, A. Molecular detection and identification of Wolbachia endosymbiont in fleas (Insecta : Siphonaptera). 2019.
27. Khoo, J.J.; Kurtti, T.J.; Husin, N.A.; Beliavskaia, A.; Lim, F.S.; Zulkifli, M.M.S.; Al-Khafaji, A.M.; Hartley, C.; Darby, A.C.; Hughes, G.L.; et al. Isolation and propagation of laboratory strains and a novel flea-derived field strain of Wolbachia in tick cell lines. Microorganisms 2020, 8, doi:10.3390/microorganisms8070988.
28. Rosselló-Mora, R.; Amann, R. The species concept for prokaryotes. FEMS Microbiol. Rev. 2001, 25, doi:10.1111/j.1574-6976.2001.tb00571.x.
29. Ramírez-Puebla, S.T.; Servín-Garcidueñas, L.E.; Ormeño-Orrillo, E.; Vera-Ponce de León, A.; Rosenblueth, M.; Delaye, L.; Martínez, J.; Martínez-Romero, E. Species in Wolbachia? Proposal for the designation of "Candidatus Wolbachia bourtzisii", "Candidatus Wolbachia onchocercicola", "Candidatus Wolbachia blaxteri", "Candidatus Wolbachia brugii", "Candidatus Wolbachia taylori", "Candidatus Wolbachia collembolicola" and "Candidatus Wolbachia multihospitum" for the different species within Wolbachia supergroups. Syst. Appl. Microbiol. 2015, 38, doi:10.1016/j.syapm.2015.05.005.
30. Lindsey, A.R.I.; Bordenstein, S.R.; Newton, I.L.G.; Rasgon, J.L. Wolbachia pipientis should not be split into multiple species: A response to Ramírez-Puebla et al., "Species in Wolbachia? Proposal for the designation of 'Candidatus Wolbachia bourtzisii', 'Candidatus Wolbachia onchocercicola', 'Candidatus Wolbachia blaxteri', 'Candidatus Wolbachia brugii', 'Candidatus Wolbachia taylori', 'Candidatus Wolbachia collembolicola' and 'Candidatus Wolbachia multihospitum' for the different species within Wolbachia supergroups." Syst. Appl. Microbiol. 2016, 39, doi:10.1016/j.syapm.2016.03.001.
31. Weeks, A.R.; Breeuwer, J.A.J. Wolbachia -induced parthenogenesis in a genus of phytophagous mites. 2001, 2245–2251, doi:10.1098/rspb.2001.1797.
32. Hornett, E.A.; Duplouy, A.M.R.; Davies, N.; Roderick, G.K.; Wedell, N.; Hurst, G.D.D.; Charlat, S. You can ' t keep a good parasite down : evolution of a male-killer suppressor uncovers cytoplasmic incompatibility. 2008, 1258–1263, doi:10.1111/j.1558-5646.2008.00353.x.
33. Si, S.; Werren, J.H.; Baldo, L.; Clark, M.E. E b Wolbachia : master manipulators of invertebrate biology., doi:10.1038/nrmicro1969.
34. Kern, P.; Cook, J.M.; Kageyama, D.; Riegler, M. Double trouble : combined action of meiotic drive and Wolbachia feminization in Eurema butterflies. 2015.
35. Fenn, K.; Blaxter, M. Wolbachia genomes: revealing the biology of parasitism and mutualism. Trends Parasitol. 2006, 22, 60–65.
36. Pike, N.; Kingcombe, R. Antibiotic treatment leads to the elimination of Wolbachia endosymbionts and sterility in the diplodiploid collembolan Folsomia candida. 2009, 6, 1–6, doi:10.1186/1741-7007-7-54.

37. Bourtzis, K.; Dobson, S.L.; Xi, Z.; Rasgon, J.L.; Calvitti, M.; Moreira, L.A.; Bossin, H.C.; Moretti, R.; Anthony, L.; Hughes, G.L.; et al. Acta Tropica Harnessing mosquito – Wolbachia symbiosis for vector and disease control. Acta Trop. 2014, 132, S150–S163, doi:10.1016/j.actatropica.2013.11.004.
38. Bouchery, T.; Lefoulon, E.; Karadjian, G.; Nieguitsila, A.; Martin, C. The symbiotic role of Wolbachia in Onchocercidae and its impact on filariasis. Clin. Microbiol. Infect. 2013, 19, 131–140, doi:10.1111/1469-0691.12069.
39. Kamtchum-tatuene, J.; Makepeace, B.L.; Benjamin, L.; Baylis, M.; Solomon, T. The potential role of Wolbachia in controlling the transmission of emerging human arboviral infections. 2017, 30, doi:10.1097/QCO.0000000000000342.
40. Hughes, G.L.; Rasgon, J.L. NIH Public Access. 2015, 23, 141–151, doi:10.1111/imb.12066.Transinfection.
41. Fisher, M.L.; Watson, D.W.; Osborne, J.A.; Mochizuki, H.; Breen, M.; Schal, C. Growth kinetics of endosymbiont Wolbachia in the common bed bug , Cimex lectularius. Sci. Rep. 2018, 1–9, doi:10.1038/s41598-018-29682-2.
42. Meriweather, M.; Matthews, S.; Rio, R.; Baucom, R.S. A 454 Survey Reveals the Community Composition and Core Microbiome of the Common Bed Bug (Cimex lectularius) across an Urban Landscape. 2013, 8, doi:10.1371/journal.pone.0061465.
43. Akhoundi, M.; Cannet, A.; Loubatier, C.; Berenger, J.; Izri, A.; Marty, P.; Delaunay, P. Molecular characterization of Wolbachia infection in bed bugs (Cimex lectularius) collected from several localities in France. 2016, doi:10.1051/parasite/2016031.
44. Riboflavin Provisioning Underlies Wolbachia ' s Fitness Contribution to. 2015, 6, 1–8, doi:10.1128/mBio.01732-15.Editor.
45. Schneider, I. Cell lines derived from late embryonic stages of Drosophila melanogaster. J. Embryol. Exp. Morphol. 1972, 27.
46. Maina, A.N.; Luce-Fedrow, A.; Omulo, S.; Hang, J.; Chan, T.C.; Ade, F.; Jima, D.D.; Ogola, E.; Ge, H.; Breiman, R.F.; et al. Isolation and characterization of a novel Rickettsia species (Rickettsia asembonensis sp. nov.) obtained from cat fleas (Ctenocephalides felis). Int. J. Syst. Evol. Microbiol. 2016, 66, doi:10.1099/ijsem.0.001382.
47. Luce-Fedrow, A.; Macaluso, K.R.; Richards, A.L. Growth of rickettsia felis in Drosophila melanogaster S2 cells. Vector-Borne Zoonotic Dis. 2014, 14, doi:10.1089/vbz.2013.1370.
48. Mediannikov, O.; Matsumoto, K.; Samoylenko, I.; Drancourt, M.; Roux, V.; Rydkina, E.; Davoust, B.; Tarasevich, I.; Brouqui, P.; Fournier, P.E. Rickettsia raoultii sp. nov., a spotted fever group rickettsia associated with Dermacentor ticks in Europe and Russia. Int. J. Syst. Evol. Microbiol. 2008, 58, doi:10.1099/ijs.0.64952-0.
49. Diop, A.; Barker, S.C.; Eberhard, M.; Barker, D.; Nguyen, T.T.; Di Pinto, F.; Raoult, D.; Mediannikov, O. Rickettsia fournieri sp. nov., a novel spotted fever group rickettsia from argas lagenoplastis ticks in australia. Int. J. Syst. Evol. Microbiol. 2018, 68, doi:10.1099/ijsem.0.003057.
50. Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 1991, 173, doi:10.1128/jb.173.2.697-703.1991.
51. Younes Laidoudi, Bernard Davoust, Marie Varloud, El Hadji Amadou Niang, Florence Fenollar, O.M. Development of a multiplex qPCR-based approach for the diagnosis of Dirofilaria immitis, D. repens and Acanthocheilonema reconditum. Parasit. Vectors 2020, 13, doi:Parasites & Vectors.
52. Tanizawa, Y.; Fujisawa, T.; Kaminuma, E.; Nakamura, Y.; Arita, M. DFAST and DAGA: Web-based integrated genome annotation tools and resources. Biosci. Microbiota, Food Heal. 2016, 35, doi:10.12938/bmfh.16-003.
53. Tanizawa, Y.; Fujisawa, T.; Nakamura, Y. DFAST: A flexible prokaryotic genome annotation pipeline for faster genome publication. Bioinformatics 2018, 34, doi:10.1093/bioinformatics/btx713.
54. Krzywinski, M.; Schein, J.; Birol, I.; Connors, J.; Gascoyne, R.; Horsman, D.; Jones, S.J.; Marra, M.A. Circos: An information aesthetic for comparative genomics. Genome Res. 2009, 19, doi:10.1101/gr.092759.109.
55. Driscoll, T.P.; Verhoeve, V.I.; Gillespie, J.J.; Johnston, J.S.; Guillotte, M.L.; Rennoll-Bankert, K.E.; Rahman, M.S.; Hagen, D.; Elsik, C.G.; Macaluso, K.R.; et al. Cat fleas in flux: Rampant gene duplication, genome size plasticity, and paradoxical Wolbachia infection. bioRxiv 2020, 2020.04.14.038018, doi:10.1101/2020.04.14.038018.
56. Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T.; Pon, A.; Liang, Y.; Wishart, D.S. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res. 2016, 44, doi:10.1093/nar/gkw387.

735 57. Lee, I.; Kim, Y.O.; Park, S.C.; Chun, J. OrthoANI: An improved algorithm and software for calculating
736 average nucleotide identity. Int. J. Syst. Evol. Microbiol. 2016, 66, doi:10.1099/ijsem.0.000760.
737 58. Page, A.J.; Cummins, C.A.; Hunt, M.; Wong, V.K.; Reuter, S.; Holden, M.T.G.; Fookes, M.; Falush, D.; Keane,
738 J.A.; Parkhill, J. Roary: Rapid large-scale prokaryote pan genome analysis. Bioinformatics 2015, 31,
739 doi:10.1093/bioinformatics/btv421.
740 59. Auch, A.F.; von Jan, M.; Klenk, H.P.; Göker, M. Digital DNA-DNA hybridization for microbial species
741 delineation by means of genome-to-genome sequence comparison. Stand. Genomic Sci. 2010, 2,
742 doi:10.4056/sigs.531120.
743 60. Auch, A.F.; Klenk, H.P.; Göker, M. Standard operating procedure for calculating genome-to-genome
744 distances based on high-scoring segment pairs. Stand. Genomic Sci. 2010, 2, doi:10.4056/sigs.541628.
745 61. Meier-Kolthoff, J.P.; Auch, A.F.; Klenk, H.P.; Göker, M. Genome sequence-based species delimitation with
746 confidence intervals and improved distance functions. BMC Bioinformatics 2013, doi:10.1186/1471-2105-
747 14-60.
748 62. Hyatt, D.; Chen, G.L.; LoCascio, P.F.; Land, M.L.; Larimer, F.W.; Hauser, L.J. Prodigal: Prokaryotic gene
749 recognition and translation initiation site identification. BMC Bioinformatics 2010, 11, doi:10.1186/1471-
750 2105-11-119.
751 63. Tatusov, R.L.; Galperin, M.Y.; Natale, D.A.; Koonin, E. V. The COG database: A tool for genome-scale
752 analysis of protein functions and evolution. Nucleic Acids Res. 2000, 28.
753 64. Clark, K.; Karsch-Mizrachi, I.; Lipman, D.J.; Ostell, J.; Sayers, E.W. GenBank. Nucleic Acids Res. 2016, 44,
754 doi:10.1093/nar/gkv1276.
755 65. Finn, R.D.; Clements, J.; Eddy, S.R. HMMER web server: Interactive sequence similarity searching. Nucleic
756 Acids Res. 2011, 39, doi:10.1093/nar/gkr367.
757 66. Moriya, Y.; Itoh, M.; Okuda, S.; Yoshizawa, A.C.; Kanehisa, M. KAAS: An automatic genome annotation
758 and pathway reconstruction server. Nucleic Acids Res. 2007, 35, doi:10.1093/nar/gkm321.
759 67. Nikoh, N.; Hosokawa, T.; Moriyama, M.; Oshima, K.; Hattori, M.; Fukatsu, T. Evolutionary origin of insect-
760 Wolbachia nutritional mutualism. Proc. Natl. Acad. Sci. U. S. A. 2014, 111, doi:10.1073/pnas.1409284111.
761 68. Katoh, K.; Misawa, K.; Kuma, K.I.; Miyata, T. MAFFT: A novel method for rapid multiple sequence
762 alignment based on fast Fourier transform. Nucleic Acids Res. 2002, 30, doi:10.1093/nar/gkf436.
763 69. Gouy, M.; Guindon, S.; Gascuel, O. Sea view version 4: A multiplatform graphical user interface for
764 sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 2010, 27, doi:10.1093/molbev/msp259.
765 70. Milne, I.; Lindner, D.; Bayer, M.; Husmeier, D.; Mcguire, G.; Marshall, D.F.; Wright, F. TOPALi v2: A rich
766 graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core
767 desktops. Bioinformatics 2009, 25, 126–127, doi:10.1093/bioinformatics/btn575.
768 71. Hall, T.; Biosciences, I.; Carlsbad, C. BioEdit: An important software for molecular biology. GERF Bull.
769 Biosci. 2011, doi:10.1002/prot.24632.
770 72. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics
771 analysis version 6.0. Mol. Biol. Evol. 2013, doi:10.1093/molbev/mst197.
772 73. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative
773 studies of nucleotide sequences. J. Mol. Evol. 1980, doi:10.1007/BF01731581.
774 74. Nguyen, L.T.; Schmidt, H.A.; Von Haeseler, A.; Minh, B.Q. IQ-TREE: A fast and effective stochastic
775 algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 2015, 32,
776 doi:10.1093/molbev/msu300.
777 75. Price, M.N.; Dehal, P.S.; Arkin, A.P. FastTree 2 - Approximately maximum-likelihood trees for large
778 alignments. PLoS One 2010, 5, doi:10.1371/journal.pone.0009490.
779 76. Lorenzo-Carballa, M.O.; Torres-Cambas, Y.; Heaton, K.; Hurst, G.D.D.; Charlat, S.; Sherratt, T.N.; Van
780 Gossum, H.; Cordero-Rivera, A.; Beatty, C.D. Widespread Wolbachia infection in an insular radiation of
781 damselflies (Odonata, Coenagrionidae). Sci. Rep. 2019, 9, doi:10.1038/s41598-019-47954-3.
782 77. Letunic, I.; Bork, P. Interactive Tree of Life (iTOL) v4: Recent updates and new developments. Nucleic
783 Acids Res. 2019, 47, doi:10.1093/nar/gkz239.
784 78. Kimura, M. Estimation of evolutionary distances between homologous nucleotide sequences. Proc. Natl.
785 Acad. Sci. U. S. A. 1981, doi:10.1073/pnas.78.1.454.
786 79. Waddell, P.J.; Steel, M.A. General Time-Reversible Distances with Unequal Rates across Sites: Mixing Γ and
787 Inverse Gaussian Distributions with Invariant Sites. Mol. Phylogenet. Evol. 1997,
788 doi:10.1006/mpev.1997.0452.

80. Bordenstein, S.; Rosengaus, R.B. Discovery of a novel Wolbachia supergroup in Isoptera. Curr. Microbiol. 2005, 51, 393–398.
81. Glowska, E.; Dragun-Damian, A.; Dabert, M.; Gerth, M. New Wolbachia supergroups detected in quill mites (Acari: Syringophilidae). Infect. Genet. Evol. 2015, 30, 140–146.
82. Erko, S.; Ebers, J. Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today 2006, 33.
83. O'Neill, S.L.; M. Pettigrew, M.; Sinkins, S.P.; Braig, H.R.; Andreadis, T.G.; Tesh, R.B. In vitro cultivation of Wolbachia pipientis in an Aedes albopictus cell line. Insect Mol. Biol. 1997, 6, 33–39.
84. Fenollar, F.; La Scola, B.; Inokuma, H.; Dumler, J.S.; Taylor, M.J.; Raoult, D. Culture and phenotypic characterization of a Wolbachia pipientis isolate. J. Clin. Microbiol. 2003, 41, 5434–5441.
85. Hague, M.T.J.; Caldwell, C.N.; Cooper, B.S. Divergent effects of Wolbachia on host temperature preference. 2020, 59812.
86. Lefoulon, E.; Vaisman, N.; Frydman, H.M.; Sun, L.; Foster, J.M.; Slatko, B.E. Large Enriched Fragment Targeted Sequencing (LEFT-SEQ) Applied to Capture of Wolbachia Genomes. Sci. Rep. 2019, 9, doi:10.1038/s41598-019-42454-w.
87. Lefoulon, E.; Clark, T.; Guerrero, R.; Cañizales, I.; Cardenas-Callirgos, J.M.; Junker, K.; Vallarino-Lhermitte, N.; Makepeace, B.L.; Darby, A.C.; Foster, J.M.; et al. Diminutive, degraded but dissimilar: Wolbachia genomes from filarial nematodes do not conform to a single paradigm. 2020, 3, 1–45, doi:10.1101/2020.06.18.160200.
88. Lindsey, A.R.I.; Werren, J.H.; Richards, S.; Stouthamer, R. Comparative genomics of a parthenogenesis-inducing wolbachia symbiont. G3 Genes, Genomes, Genet. 2016, 6, doi:10.1534/g3.116.028449.
89. Foster, J.; Ganatra, M.; Kamal, I.; Ware, J.; Makarova, K.; Ivanova, N.; Bhattacharyya, A.; Kapatral, V.; Kumar, S.; Posfai, J. The Wolbachia genome of Brugia malayi: endosymbiont evolution within a human pathogenic nematode. PLos biol 2005, 3, e121.
90. Wang, N.; Jia, S.; Xu, H.; Liu, Y.; Huang, D. Multiple horizontal transfers of bacteriophage WO and host wolbachia in fig wasps in a closed community. Front. Microbiol. 2016, 7, doi:10.3389/fmicb.2016.00136.
91. Balvín, O.; Roth, S.; Talbot, B.; Reinhardt, K. Co-speciation in bedbug Wolbachia parallel the pattern in nematode hosts. Sci. Rep. 2018, 8, doi:10.1038/s41598-018-25545-y.
92. Akman, L.; Yamashita, A.; Watanabe, H.; Oshima, K.; Shiba, T.; Hattori, M.; Aksoy, S. Genome sequence of the endocellular obligate symbiont of tsetse flies, Wigglesworthia glossinidia. Nat. Genet. 2002, 32, doi:10.1038/ng986.

Chapitre 2 :

Diagnostic et caractérisation des nématodoses vectorielles

2.1 : Approches diagnostiques des filarioses

Préambule

Les filarioses canines revêtent différents aspects en termes de vecteurs, de pathogénie, de physiopathogénie, d'épidémiologie et de risque zoonotique. L'hôte canin est le réservoir naturel de plusieurs espèces telles que *Dirofilaria immitis*, *Dirofilaria repens*, *Acanthocheilonema* (*Dipetalonema*) *reconditum*, *Acanthocheilonema dracunculoides*, *Cercopithifilaria grassi*, *Cercopithifilaria bainae* (sp. I), *Cercopithifilaria* sp. II, *Brugia malayi*, *Brugia pahangi* et *Onchocerca lupi* (Anderson 2000, Ravindran et al. 2014, Latrofa et al. 2018). A l'exception de *Cercopithifilaria* spp. et d'*Acanthocheilonema dracunculoides*, toutes les autres espèces ont un pouvoir zoonotique (Otranto et al. 2013). Leur importance diffère d'une région à l'autre, ainsi, en Asie, le chien peut constituer un réservoir des redoutables filarioses lymphatiques humaines dues à *Brugia* spp. Aux Etats-Unis, la dirofilariose cardiopulmonaire, à *Dirofilaria immitis*, constitue la parasitose grave, la plus fréquente dans la population canine (Bowman et al. 2016). L'émergence de toutes les espèces citées, à l'exception des *Brugia* spp., a été, récemment, observée dans tout le Bassin méditerranéen, exposant de ce fait la population humaine et canine à un risque zoonotique (Tahir et al. 2019).

Cependant, ces parasitoses sont encore complexes à comprendre. La mise en œuvre de leur diagnostic diffère d'un parasite à l'autre et d'un hôte à un autre. Généralement, le diagnostic de l'infection filarienne repose, essentiellement, sur l'identification morphologique ou moléculaire (PCR et séquençage) des larves L1, appelées microfilaires. De ce point de vue, les filarioses canines peuvent être classées en deux principaux groupes en fonction de la localisation de leur forme larvaire chez l'hôte canin : (I) des espèces produisant des microfilaires sanguinoles tel que *Dirofilaria* spp. *Acanthocheilonema* spp. et *Brugia* spp. et (II) des espèces dont les microfilaires se localisent dans le derme et le tissu conjonctif, incluant, principalement, *Cercopithifilaria* spp. et *Onchocerca lupi* (zoonotique).

J’ai été amené, au départ, à diagnostiquer la dirofilariose chez des chiens militaires, en France, dans le cadre d’un suivi épidémiologique et de l’évaluation de l’efficacité de traitements préventifs antiparasitaires. A ce sujet, j’ai été confronté à plusieurs problèmes liés au diagnostic de ces parasitoses que l’on peut résumer, principalement, dans les points suivants :

I. Difficultés en termes de temps pour l’interprétation de l’examen parasitologique destiné à l’identification morphologique et au dénombrement des microfilaires.
II. Incohérence entre l’examen parasitologique et la détection des antigènes de *Dirofilaria immitis*.
III. Difficulté d’accès aux prélèvements cutanés pour le diagnostic des filarioses à microfilaires cutanées.
IV. Absence d’outils moléculaires spécifiques, et inefficacité des systèmes PCR générique pour la détection des co-infections et des infections occultes (c’est-à-dire avec présence de parasites adultes et absence de microfilarémie).
V. Absence d’outils moléculaires pour le dépistage de l’ADN filarien à titre exploratoire.

Pour faire face à ces contraintes, je me suis d’abord intéressé à résoudre par biologie moléculaire les problèmes liés au diagnostic des filarioses canines dont la forme larvaire est dans le sang, particulièrement, les espèces (*Dirofilaria* spp. et *Acanthocheilonema reconditum*), les plus répandues en Europe. Parallèlement, des auteurs, des quatre coins du monde, ont rapporté, à plusieurs reprises, la présence dans le sang de chiens filariens d’ADN de *Wolbachia* infectant les filaires, ce qui nous a amené à sélectionner les *Wolbachia* comme cible supplémentaire pour le diagnostic des filarioses canine. J’ai ainsi mis en œuvre une approche moléculaire combinant la détection de l’ADN filarien à celui des *Wolbachia,* en utilisant, principalement, la technologie TaqMan, basée sur une détection multiple, via des PCR quantitatives multiplexes (qPCR). J’ai conçu des qPCRs destinées à explorer la présence des

filaires et de leurs *Wolbachia* dans le sang, sans spécificité particulière, parallèlement à deux autres qPCRs, de type multiplexe, dont l'une est ciblée, simultanément, en une seule réaction, pour l'ADN de *Dirofilaria immitis, Dirofilaria repens* et d'*Acanthocheilonema reconditum* et l'autre pour la détection simultanée, d'une manière spécifique, de l'ADN des *Wolbachia* associées à *Dirofilaria immitis* et *Dirofilaria repens*. De plus, j'ai également développé un jeu d'amorces destiné à l'amplification et au séquençage d'une séquence du gène *cox 1* (cytochrome oxydase sous unité I) des filaires et un autre système ciblant le gène *ftsZ* (mutant Z thermosensible filamenteux) des *Wolbachia,* afin de compléter notre approche dans le cas d'infections par des espèces autres que celles susmentionnées. A cela, s'ajoute une autre et dernière duplex qPCR destinée à détecter et différencier l'ADN de *Dirofilaria immitis* et d'*Angiostrongylus vasorum*, deux nématodes affectant le cœur et les poumons du chien.

Tous les paramètres des systèmes PCR conçus, à savoir la sensibilité (Se), la spécificité (Sp) et les limites de détection ont été validés *in silico* et *in vitro,* en utilisant un panel d'ADN connus. Ils ont ensuite été validés avec un panel de 168 échantillons de sang de chiens, infectés ou non, par des filaires. Parallèlement, j'ai évalué l'apport diagnostic de la détection des antigènes de *Dirofilaria immitis,* avec ou sans chauffage des sérums, comme cela a été proposé dans certaines études dans le but d'améliorer la sensibilité du test. Il s'est avéré que la limite de détection des microfilaires (mfs) sanguicoles des différentes espèces est d'environ 5×10^{-1} et 1,5 × 10−4 mfs/ml pour l'ADN des filaires et des *Wolbachia,* respectivement, avec des coefficients d'ajustement supérieur à 0,99. Il est important de mentionner que l'utilisation des *Wolbachia* comme cible supplémentaire dans le diagnostic de la filariose a permis, non seulement, d'améliorer le dépistage, mais aussi de détecter les infections occultes. Globalement, l'approche diagnostique, combinant la détection spécifique des filaires et de leurs *Wolbachia,* a montré 100% de sensibilité et 99,3% de spécificité. Différemment, le test de détection des antigènes de *Dirofilaria immitis* a montré une sensibilité de 65,5% et 100% de spécificité. En revanche, le

chauffage des sérums avant le dépistage antigénique de *D. immitis* a montré une sensibilité de 100% avec une très mauvaise spécificité de 34,5%. Ainsi, nous déconseillions, fortement, l'usage du chauffage des sérums avant le dépistage antigénique. Nous proposons, en parallèle, une approche moléculaire, à la fois, pour la surveillance épidémiologique, le diagnostic, et l'évaluation de l'efficacité des traitements anti-filariens et anti-*Wolbachia*. En outre, les chercheurs peuvent adapter notre combinaison en fonction de leur recherche scientifique. Par exemple, ils peuvent utiliser la qPCR destinée à détecter *Angiostrongylus vasorum* en tant que simplex pour dépister la présence du parasite dans des matières fécales ou chez un hôte intermédiaire (**Publication N°2**).

Dans un deuxième temps, j'ai porté mon attention sur les filaires à microfilaires dermiques du genre *Cercopithifilaria* en concevant deux autres multiplex qPCRs, dont l'une destinée à explorer la présence de l'ADN filarien avec la possibilité d'identifier celui de *Cercopithifilaria grassi* de manière spécifique, et la deuxième pour détecter et discriminer entre les ADNs de *Cercopithifilaria bainae* (sp. I) et de *Cercopithifilaria* sp. II. En suivant un protocole de conception et de validation de qPCR triplex, je l'ai proposé pour la détection de *Dirofilaria immitis, Dirofilaria repens* et d'*Acanthocheilonema reconditum*, comme étant une approche diagnostique globale des filarioses canines. L'utilisation de ce protocole a été ensuite affinée avec l'exploitation de tiques infestant les chiens. Ces ectoparasites, connus comme vecteurs des cercopithifilaires, se gorgent, également, de sang microfilarémique dans le cas des chiens infectés par des espèces sanguicoles. L'approche diagnostique a été validée sur un panel d'échantillons sang/tiques prélevés sur une population de 72 chiens d'Algérie. Il s'est avéré, qu'en utilisant les tiques gorgées comme échantillons biologiques destinés au diagnostic des filarioses canines, l'approche moléculaire combinant les trois systèmes de qPCR a montré une spécificité et une sensibilité de 100%, à la différence de l'examen du sang qui ne permet que l'exploration des filaires sanguicoles, ce qui altère la valeur de la sensibilité (89,47%) du

diagnostic proposé. De plus, cette approche a démontré son utilité, non seulement, pour le diagnostic, mais aussi pour la visibilité de la forte interaction de ces parasitoses canines, en zone endémique. Ceci nous a également permis de rapporter la prévalence élevée de *Dirofilaria immitis* et, pour la première fois, de *Dirofilaria repens*, d'*Acanthocheilonema reconditum*, de *Cercopithifilaria bainae* et de *Cercopithifilaria* sp. II, chez les chiens d'Algérie. En outre, les chercheurs peuvent toujours adapter ces nouveaux outils moléculaires, en fonction de la nature des échantillons (sang, peau ou tiques gorgées) et de leurs études (**Publication N°3**).

Du fait que dans les cliniques vétérinaires, il est rarement possible de mettre en œuvre des techniques de biologie moléculaire, l'approche que j'ai proposée pour le diagnostic des filarioses canines, repose sur des techniques qui, malgré leurs performances élevées, sont, plus ou moins réservées aux domaines de la recherche et de la surveillance épidémiologique. Par conséquent, le diagnostic courant de ces parasitoses nécessite des améliorations. Dans le cadre du développement de nouveaux tests spécifiques et fiables, nous avons, dans un premier temps, essayé de mettre au point un modèle expérimental de la dirofilariose à *Dirofilaria immitis*. Jusqu'à présent, plusieurs modèles expérimentaux ont été mis en œuvre, notamment, pour étudier les filarioses humaines. On peut citer le modèle murin (*Litomosoides sigmodontis*) de *Loa loa* et le modèle gerbille de *Brugia malayi*. En revanche, en dehors des modèles canins, aucun modèle n'a été publié pour *D. immitis*, ce qui entrave l'avancée des connaissances sur cette parasitose. De plus, la reproduction expérimentale du cycle complet de la dirofilariose offrirait la possibilité d'évaluer correctement l'efficacité de traitements préventifs et curatifs. D'abord, j'ai mis en œuvre un modèle expérimental d'infection d'*Aedes albopictus* par le sang canin microfilarémique. Ce dernier a été récolté, dans des tubes citratés et à EDTA, sur des chiens d'Algérie diagnostiqués microfilarémiques sur place, et avant l'administration de tout traitement. Il a été, ensuite, transporté (à la température de 22°C sous agitation) et utilisé à différentes concentrations pour le nourrissage d'un élevage d'adultes (4 à 5 jours post-éclosion)

d'*Aedes albopictus*, préalablement réalisé au sein de l'insectarium de l'IHU. Nos résultats montrent que seul, le sang citraté a permis un engorgement significatif des moustiques. La dose infectante permettant le développement de la forme infectante (L3) de *D. immitis* au bout de 14 jours sans qu'il y ait une mortalité significative des moustiques est de 35 microfilaires par 20 µL de sang microfilarémique. Au-delà de cette dose, la mortalité des moustiques devient significative à partir de 12h post-infection et atteint 97% pour 80-90 microfilaires par 20 µL. Cela montre la fragilité d'*Aedes albopictus* vis-à-vis des microfilaires de *D. immitis* à la différence d'*Aedes aegypti* qui tolère bien cette dose. Dans un deuxième temps, j'ai procédé à l'infection de trois espèces différentes d'animaux de laboratoire : un lapin (*Oryctolagus cuniculus*), deux cobayes « cochons d'Inde » (*Cavia porcellus*) et deux gerbilles mongoliennes (*Meriones unguiculatus*). Afin de s'assurer de l'inoculation des larves infectantes, deux types d'infections ont été effectués : (i) d'abord, les animaux ont été exposés aux piqures de moustiques au stade infecté/infectant (hébergeant la forme L3 dans le compartiment buccale), et (ii) puis, par injection sous cutanée de larves L3 récupérées à partir de moustiques infectés. En dehors d'une forte réaction inflammatoire aux endroits des piqures/injections, aucun des animaux n'a développé la maladie au cours des neuf mois de suivi. Celui-ci a été réalisé à la fois par biologie moléculaire et par la recherche sérologique d'antigènes de *D. immitis* ainsi que lors de l'autopsie finale des animaux. Nous en avons conclu que, malheureusement, ces hôtes ne sont pas réceptifs à *Dirofilaria immitis*.

Finalement, en plus de la valeur diagnostique de la détection supplémentaire des *Wolbachia* lors d'une infection filarienne, la surveillance de leur cinétique chez l'hôte infecté s'avère de plus en plus nécessaire. Par exemple, l'élimination des *Wolbachia* chez *Onchocerca volvulus*, via une antibiothérapie lors de co-infection par *Loa loa* est nécessaire avant tout traitement filaricide (Makepeace and Tanya 2016). Ce phénomène a été, récemment, rapporté chez le chien massivement infesté par *Dirofilaria repens*, où le non-respect de cette étape a

conduit à sa mort (Wysmołek, Klockiewicz and Sobczak-filipiak 2020). L'insuffisance des outils moléculaires disponibles de dépistage des nématodes, en particulier des filaires, ainsi que de leurs *Wolbachia,* nous a donc amené à mettre en œuvre de nouvelles techniques moléculaires pratiques dans la détection, l'identification, le diagnostic et le suivi des traitements.

Au cours de la détection par qPCR multiplexe, les systèmes qui se réfèrent à l'amplification de plus d'une cible, en utilisant un seul jeu d'amorces avec différentes sondes spécifiques, sont les plus appropriés. Cela minimise l'interférence et l'inhibition de la réaction. Cependant, le développement d'un tel système est souvent limité aux mêmes gènes des espèces ou sous-espèces, génétiquement proches. Convaincu de l'importance de la détection simultanée de l'ADN filarien et de celui des *Wolbachia,* au cours de l'infection filarienne, j'ai conçu à partir du gène codant pour la grande sous-unité ribosomale des filaires (28S) et de son homologue chez les bactéries *Wolbachia* (23S) un seul jeu d'amorces universelles capable d'amplifier un fragment d'environ 150 paires de bases des deux gènes à la fois, et des sondes spécifiques pour chaque cible (filaire et *Wolbachia*) (Figure 1). De plus, la même approche, ciblant les mêmes gènes, a été adoptée pour la mise en œuvre d'un autre système duplex pour la détection simultanée de *Dirofilaria immitis* et de sa *Wolbachia* d'une manière spécifique (Figure 2). D'un autre côté, j'ai également proposé une qPCR basée sur le 5S, un gène conservé au sein des nématodes, afin d'explorer leur ADN dans les échantillons biologiques. Finalement, afin d'achever l'identification du génotype, j'ai conçu douze systèmes de PCR standard (Tableau 1, Figure 3), dont six ciblant, spécifiquement, les gènes suivants : protéines du choc thermique (*Hsp-70* et *Hsp-90*), chaîne lourde de myosine (*MoyoHC*), bêta-tubuline, collagène cuticulaire et 5S des filaires. Six autres PCR ciblant : le 18S, le fragment complet des espaceurs internes transcrits (*Internal Transcribed Spacer -ITS1-5.8S-ITS2*), le 28S, le 12S, le 16S et la sous-unité du cytochrome oxydase I (cox-1) des nématodes incluant les filaires, ont également été conçues. En plus de la qPCR 5S, ces systèmes de PCR ciblant les nématodes, ont déjà prouvé

leur efficience à détecter et caractériser les nématodes dans nos études détaillées dans le chapitre suivant (**Publications N°4 et 5**).

Tous les systèmes conçus dans le cadre de cette étude, ont été validés *in silico* et *in vitro*, selon la procédure susmentionnée, et seront publiés dans un article de recherche récapitulatif dont la rédaction est en cours.

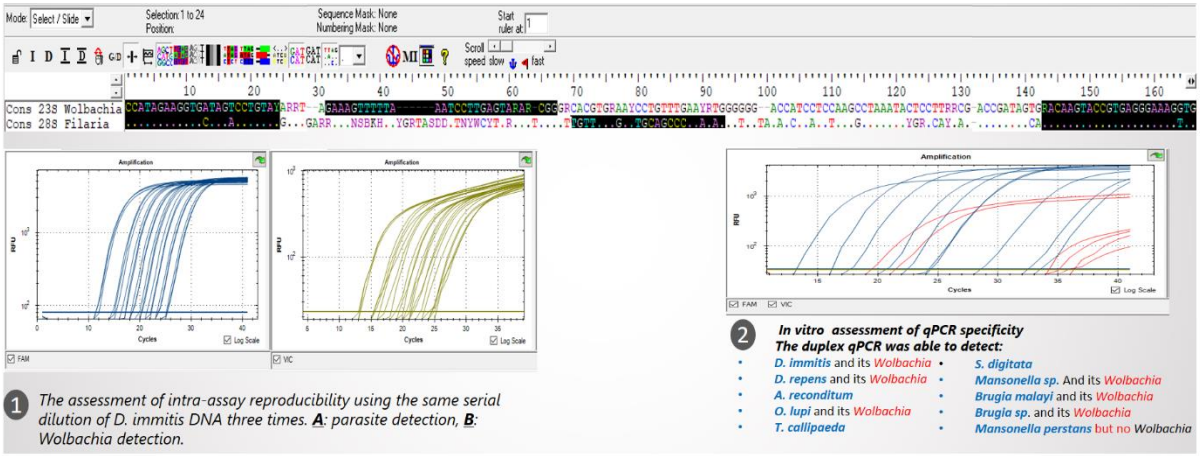


Figure 1 : Désignation et validation *in vitro* de la duplex qPCR ciblant les filaires et leur *Wolbachia*.

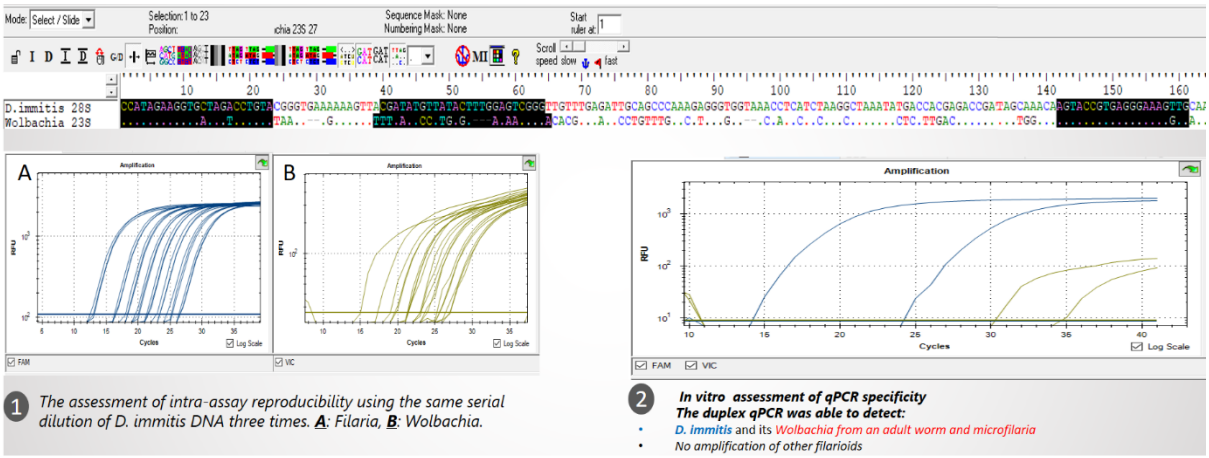


Figure 2 : Désignation et validation *in vitro* de la duplex qPCR ciblant spécifiquement *Dirofilaria immitis* et sa *Wolbachia*.

Tableau 1 : Systèmes de PCR proposés pour le génotypage des nématodes et des filaires.

Gène	Nom des amorces	Séquence d'amorce	Taille (bp)	Température (°C)	Temps d'élongation	Utilisation
Hsp-70	Fwd-Hsp70-Fil-348 Rwd-Hsp70-Fil-1078	GCTATTGCYTAYGGTYTRGACA GCTTTYTCAACYGGATCCAT	**730**	**47,5°C**	**1'**	**PCR+Séquençage**
Hsp-90	Fwd-NemIII-248 Rwd-NemIII-1196	TGGACACAGGGATTGGTATG GAAATGTTCAACGGCAAGTC	**948**	**55°C**	**1'**	**PCR+Séquençage**
	dgFwd-NemIII-248 dgRwd-NemIII-1131	TGGACACDGGDATTGGWATG CARCTCGTCRCAATTCTCCA	**883**	**50°C**	**1'**	**Hemi-nested PCR+Séquençage**
MoyoHC	Fwd-MyoHC-1837-C Rwd-MyoHC-2555-C	AAGACACGGCTTAACAGTGAAAA TCGYTGTGCRTTGTCCACTT	**718**	**51°C**	**1'**	**PCR+Séquençage**
Bêta-tubulin	Fwd-385 Rwd-1507	GGATGCGAYTGTCTTCAGG TGCTGCCATCATATTTTTGG	**1122**	**53°C**	**1'30''**	**PCR+Séquençage**
	Fwd-1496 Rwd-2412	ATGATGGCAGCATGTGATCC ATTCCGATTCACCTTCCTGA	**916**	**58°C**	**1'**	**PCR+Séquençage**
cuticular collagen	Fwd-CAg-10 Rwd-CAg-577	GAATCATCGCGTTCAGCACT GGACCTGCTTCTCCTTGATC	**567**	**50°C**	**1'**	**PCR+Séquençage**
5S	Fwd.5S.Nem.11 Rwd.5S.Nem.834	CACGTTGAAAGCACGACATC GGACGAGATGTCGTGCTTTC	**823**	**55°C**	**1'**	**PCR+Séquençage**
18S	Fwd-75 (Fwd-Nem-18S-523) Rwd-rRNA-Nem-1825	CGGTTATCGGAATGGGTACA GGTTCAAGCCACTGCGATTAA	**1302**	**55°C**	**1'30''**	**PCR+Séquençage**
	Fwd-18S-157 (Fwd-18S-Nem-610) Rwd-18S-1242 (Rwd-Nem-18S-1720)	TCCAGCTCTCAAAGTGTATATCG GGGCGGTGTGTACAAAGG	**1110**	**55°C**	**1'30''**	**Nested PCR+Séquençage**
	Fwdwd-rRNA-Nem-945 Rwd-18S-592 (Rwd-18S-Nem-1062)	GAGAGGTGAAATTCTTGGACCGT TATCTGATCGCCTTCGAACC	//	//	//	**Séquençage**
ITS1-5.8S-ITS2	Fwd-ITS-57 Rwd-ITS-1495	GAACCTGCGGAAGGATCA AGTTTCTTTTCCTCCGCTTAGTT	**1495**		**1'30''**	**PCR+Séquençage**
	Fwd-ITS-57 Rwd-ITS-794	GAACCTGCGGAAGGATCA GCTAGCTGCGTTCTTCATCG	**737**	**58°C**	**1'**	**Hemi-nested PCR+Séquençage**
	Fwd-ITS-793 Rwd-ITS-1495	TCGATGAAGAACGCAGCTA AGTTTCTTTTCCTCCGCTTAGTT	**702**		**1'**	**Hemi-nested PCR+Séquençage**
28S	Fwd-28S-Nem-4236 Rwd-28S-Nem-5793	ATCYGACCCGTCTTGAAAC GCCCAGTCCTTAGAGCCAAT	**1557**	**58°C**	**1'30''**	**PCR+Séquençage**
	Fwd-28S-Nem-5166 Rwd-28S-Nem-5793	GCCGCAACGAGTAAGAGG GCCCAGTCCTTAGAGCCAAT	**627**	**58°C**	**1'**	**PCR+Séquençage**
12S	Fwd-12S-Nem-1 Rwd-12S-Nem-681	AAGTTTGATTTTGGTTTTGGTTG CCATTGACGGATGGTTTGTA	**680**	**58°C**	**1'**	**PCR+Séquençage**
	Fwd-12S-Nem-110 Rwd-12S-Nem-681	TCCAGAATAATCGGCTATACATTTT CCATTGACGGATGGTTTGTA	**571**	**58°C**	**1'**	**PCR+Séquençage**
16S	Fwd-16S-Nem-488 Rwd-16S-Nem-918	GCAGCCTTAGCGTGATGG TAAACCGCTCTGTCTCACGA	**430**	**58°C**	**1'**	**PCR+Séquençage**
COI	Fwd.COI.Nem.257 dg.Fwd.COI.Nem.257 dg.Rwd.COI.Nem.1325	TTGGTGGTTTTGGAAATTGG TTGGKGGTTTTGGWAATTGG CCAGCAAAATGCAWAGGAAAA	**1069**	**52°C**	**1'30"**	**PCR+Séquençage**
	dg.Fwd.COI.Nem.663	GGATCGTAATTTKAATACTTCTTTTT	**663**		**1'**	

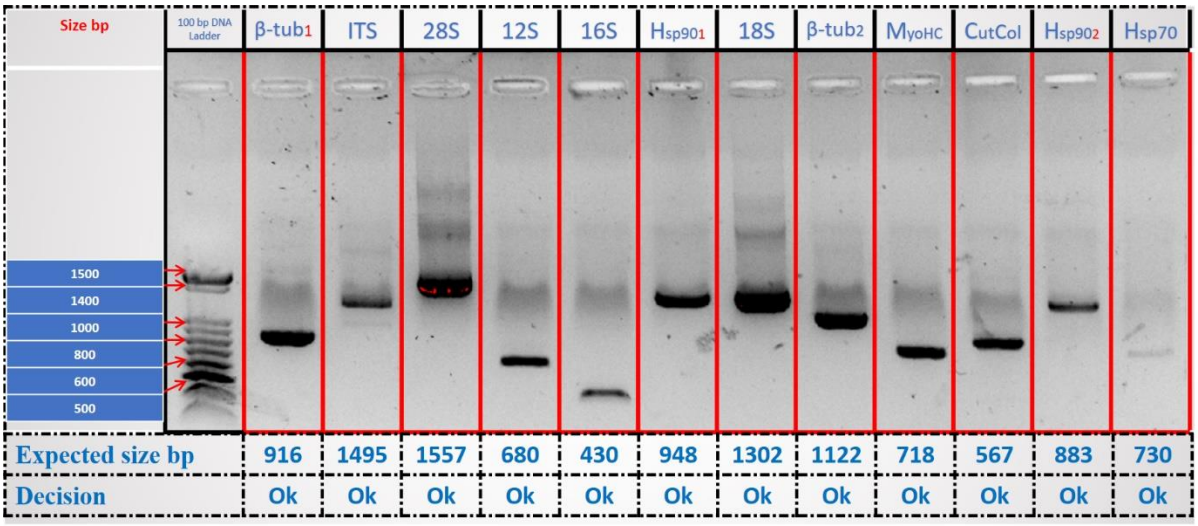


Figure 3 : Validation *in vitro* des systèmes proposés pour le génotypage des filaires et/ou des nématodes, en utilisant l'ADN de *Dirofilaria immitis*.

Publication N°2

Development of a multiplex qPCR-based approach for the diagnosis of *Dirofilaria immitis, D. repens* and *Acanthocheilonema reconditum*

Younes Laidoudi[1,2], Bernard Davoust[1,2], Marie Varloud[3], El Hadji Amadou Niang[1,2], Florence Fenollar[2,4] and Oleg Mediannikov[1,2*]

[1]Microbes, Evolution, Phylogeny and Infection (MEPHI), UMR Aix-Marseille University, IRD, APHM, IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France
[2]IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France
[3] Ceva Santé Animale, 10 Avenue de la Ballastière, 33500 Libourne, France
[4]VITROME, UMR Aix-Marseille University, IRD, SSA, APHM, IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France

ARTICLE DE RECHERCHE

Publié dans : Parasites and Vectors, 2020, 13(1), 319

(doi.10.1186/s13071-020-04185-0)

Parasites & Vectors

RESEARCH Open Access

Development of a multiplex qPCR-based approach for the diagnosis of *Dirofilaria immitis*, *D. repens* and *Acanthocheilonema reconditum*

Younes Laidoudi[1,2], Bernard Davoust[1,2], Marie Varloud[3], El Hadji Amadou Niang[1,2], Florence Fenollar[2,4] and Oleg Mediannikov[1,2*]

Abstract

Background: *Dirofilaria immitis*, *D. repens* and *Acanthocheilonema reconditum* are the main causative agents of zoonotic canine filariosis.

Methods: We developed a combined multiplex approach for filaria and *Wolbachia* detection using the *28S*-based pan-filarial and *16S*-based pan-*Wolbachia* qPCRs, respectively, involving a fast typing method of positive samples using triplex qPCR targeting *A. reconditum*, *D. immitis* and *D. repens*, and a duplex qPCR targeting *Wolbachia* of *D. immitis* and *D. repens*. The approach was complemented by a duplex qPCR for the differential diagnosis of heartworms (*D. immitis* and *Angiostrongylus vasorum*) and pan-filarial *cox*1 and pan-*Wolbachia ftsZ* PCRs to identify other filarial parasites and their *Wolbachia*, respectively. A total of 168 canine blood and sera samples were used to validate the approach. Spearman's correlation was used to assess the association between filarial species and the strain of *Wolbachia*. Positive samples for both the heartworm antigen-test after heating sera and at least one DNA-positive for *D. immitis* and its *Wolbachia* were considered true positive for heartworm infection. Indeed, the presence of *D. repens* DNA or that of its *Wolbachia* as well as *A. reconditum* DNA indicates true positive infections.

Results: The detection limit for *Wolbachia* and filariae qPCRs ranged from 5×10^{-1} to 1.5×10^{-4} mf/ml of blood. When tested on clinical samples, 29.2% (49/168) tested positive for filariae or *Wolbachia* DNA. Filarial species and *Wolbachia* genotypes were identified by the combined multiplex approach from all positive samples. Each species of *Dirofilaria* was significantly associated with a specific genotype of *Wolbachia*. Compared to the true positives, the approach showed excellent agreement ($k = 0.98–1$). Unlike *D. immitis* DNA, no *A. vasorum* DNA was detected by the duplex qPCR. The immunochromatographic test for heartworm antigen showed a substantial ($k = 0.6$) and a weak ($k = 0.15$) agreements before and after thermal pre-treatment of sera, respectively.

Conclusions: The proposed approach is a reliable tool for the exploration and diagnosis of occult and non-occult canine filariosis. The current diagnosis of heartworm disease based on antigen detection should always be confirmed by qPCR essays. Sera heat pre-treatment is not effective and strongly discouraged.

Keywords: Canine filariosis, Multiplex qPCRs, Differential diagnosis, Heartworm disease, *Dirofilaria immitis*

Background

Canine filariosis includes diseases caused by parasitic nematodes called filariae, belonging to the order Spirurida. There are several species of veterinary and human importance. Dogs seem to be the natural hosts for several species, such as *Dirofilaria immitis*, *D. repens*,

*Correspondence: olegusss1@gmail.com
[1] Microbes, Evolution, Phylogeny and Infection (MEPHI), UMR Aix-Marseille University, IRD, APHM, IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France
Full list of author information is available at the end of the article

Acanthocheilonema reconditum, *A. dracunculoides*, *Cercopithifilaria grassii*, *Brugia ceylonensis*, *B. patei*, *B. malayi*, *B. pahangi*, *Onchocerca lupi* and *Thelazia callipaeda* [1–4]. These arthropod-borne filarioids produce blood, cutaneous or mucous microfilariae, where they are available to arthropod vectors [5]. The most common and medically important species affecting dogs are *D. immitis*, *D. repens* and *A. reconditum* [6]. In addition to their veterinary importance, they can also affect human health. *Dirofilaria immitis* causes pulmonary and cardiopulmonary dirofilariosis in humans and dogs, respectively. Cardiopulmonary dirofilariosis usually called heartworm disease has recently been considered as an emerging disease in Europe. In the USA, *D. immitis* is the most important life-threatening parasitic infection in dogs [7]. Elsewhere in the world, particularly in eastern European countries, *D. repens* is the most endemic parasitic nematode causing subcutaneous infection, which is less virulent but more zoonotic than that caused by *D. immitis* [8]. *Acanthocheilonema reconditum* is an occasional zoonotic agent that affects the subcutaneous tissue and the perirenal fat [9, 10] causing a common but clinically less important infection in dogs [11].

Once mature, these filarioids can produce microfilariae circulating in the bloodstream. This larval stage (L1) is also a target for the diagnosis by microscopic detection of the larvae or by detection of their DNA in the host blood [10]. *Dirofilaria immitis*, the agent of heartworm disease, is distributed worldwide and is responsible for heart failure in dogs after colonization of pulmonary arteries and the right ventricle, where it can be fatal if untreated. Due to the gravity of the disease, it remains the most commonly diagnosed filariosis in dogs due to the detection of antigen circulating in the blood [11–13]. Several problems with the current diagnostic methods have been raised, such as morphological confusion between the microfilariae of *D. immitis*, *A. reconditum* and *D. repens.* Commercially available diagnostic kits for the detection of *D. immitis* antigens may also cross-react with filarial and non-filarial nematodes, such as *D. repens*, *A. reconditum* and *Onchocerca* spp. [14], *Spirocerca lupi* and *Angiostrongylus vasorum*, especially the latter which can cause a cross-reaction without prior heat pre-treatment of the sera [15–17]. *Angiostrongylus vasorum*, the agent of French heartworm disease, should also be taken into account in the differential diagnosis of pulmonary disease [17]. This so-called occult heartworm is characterized by the absence of microfilaremia or an amicrofilaremia. This may result from the host's immune response, low parasite load and infertility or, incidentally, the microfilaricidal effect observed in dogs receiving macrocyclic lactone prevention [14]. When the occult heartworm occurs in a co-infection with another filariosis, the diagnosis is even more challenging. The association of the heartworm with *D. repens* infection may result in an unexplained suppressive effect on the production of microfilariae of *D. immitis* [18, 19]. In such cases, the cross-reactivity between *D. immitis* and *D. repens* may result in misdiagnosis. Therefore, there is an urgent need for a more sensitive diagnostic method to detect occult as well as non-occult canine filariosis and to identify the pathogen. Detection of *D. immitis* has gained more and more attention; many trials have been performed for improving the quality of heartworm diagnostic tools, such as the detection of a specific antigen released by these worms [20], or the use of a recombined antigen of *D. immitis* for specific antibody detection [21].

The endosymbiotic intracellular bacteria of the genus *Wolbachia* are associated with some filarial species of two subfamilies of the Onchocercidae: Onchocercinae and Dirofilariinae [22]. These bacteria are host-specific, and each species of filarial worm is associated with a specific bacterial genotype. *Wolbachia* spp. have been targeted for the indirect diagnosis of *D. immitis* infection in dead-end hosts such as humans and cats, whereby the strong reaction of the host against the parasite prevents them to achieving their maturation, and, therefore, the production of microfilariae may not be achieved [23]. In such cases, the detection of filaria-specific *Wolbachia* may indicate a filarial infection and can serve as an alternative diagnostic tool in endemic areas [23, 24]. In around 40–60% of canine heartworm cases, both *Wolbachia* and parasite DNA may be detected using conventional PCR [25–27]. Indeed, the combined detection of *Wolbachia* and *Dirofilaria* DNA was suggested to improve heartworm detection [27].

In the present study, we developed a multiplex real-time PCR-based approach allowing a specific, rapid and simultaneous detection of *D. immitis*, *D. repens* and *A. reconditum* as well as the occult *Dirofilaria* spp. infections in dogs. In addition, it was completed by a duplex real-time PCR-based assay for the simultaneous detection of *D. immitis* and *A. vasorum* as a differential diagnostic for canine heartworms. The approach can be used in routinely in a diagnostic laboratory. We also evaluated the effectiveness of a novel molecular approach to conventional serological diagnosis and assessed the importance of serum heating.

Methods

Probes, primers design and PCR amplification protocol

Custom protocol and in silico validation

First, for each PCR assay, the target gene was chosen to meet the objective of each system. Fasta files were constructed from the sequences of the representative members of the family Onchocercidae or *Wolbachia* genotypes

available in the GenBank database. The sequences were aligned using BioEdit v 7.0.5.3 software [28] to reveal the highly conserved inter- and intra-species regions as target regions for primers and probes. This region was submitted to Primer3 online software v. 0.4.0 (http://primer3.ut.ee), in order to determine valuable candidate primers and probes; the selection was based on the criteria for primer and probe design.

Physicochemical characteristics, annealing temperature and the possibility for hairpin, self- and hetero-dimers were tested using free online software Oligo-Analyzer 3.1 [29]. Primer sets and probes were also checked within DNA databases of metazoans (taxid:33208), vertebrates (taxid:7742), bacteria (taxid:2), Canidae (taxid:9608), Felidae (taxid:9682) and humans (taxid:9605) using primer-BLAST [30]. This was completed for all possible forward-reverse and probe-reverse combinations of each PCR system. Primers were synthesized by Eurogentec (Liège, Belgium) and the hydrolysis probe was synthesized by Applied Biosystems™ (Foster City, CA, USA).

TaqMan simplex qPCR targeting filarial nematodes

The choice of the large subunit rRNA (*LSU*) gene, also called *28S* gene, was based on several criteria such as: the tandem repetition of about 150 times in the filarial nematode genome, which improves the PCR detectability [31]; availability on GenBank for representatives of all nematode families; and sharing a highly conserved region within the Onchocercidae. The primers qFil-28S-F, qFil-28S-R and a TaqMan® hydrolysis probe (qFil-28S-P) were designed to amplify *28S* gene for most filarial species (Table 1).

TaqMan triplex qPCR targeting D. immitis, D. repens and A. reconditum

The gene encoding for the cytochrome *c* oxidase subunit 1 gene (*cox*1) was selected for the development of the triplex TaqMan qPCR system targeting *D. immitis*, *D. repens* and *A. reconditum* (Table 1). This choice was based on the availability of *cox*1 for the three species on GenBank. Indeed, the *cox*1 gene is recognized for its high sensitivity (a high copy number relative to the nuclear gene in each cell) [32]. The *cox*1 gene has been described as a "barcode gene" for filarial nematodes [33]. The primers Fil.COI.749 and dg.Fil.COI.914 (Table1) were designed to amplify a 166 bp-long *cox*1 fragment for most members of the Onchocercidae. The system's specificity was confined to the TaqMan probes, namely P.imm.COI.777 specific to *D. immitis* and P.rep.COI.871 specific to both *D. repens* and "*Candidatus* Dirofilaria (Nochtiella) honkongensis" affecting dogs and humans in Japan [34]. Finally, the probe P.rec.COI.866 is specific to *A. reconditum*. In the triplex TaqMan system, three different dyes were used for specific detection: FAM and VIC with a non-fluorescent quencher-TAMRA confined to *D. immitis* and *D. repens* probes, respectively; Cyanine 5 (Cy5) with a non-fluorescent quencher-BHQ-3 for the *A. reconditum* probe (Table 1).

TaqMan duplex qPCR targeting D. immitis and A. vasorum

The duplex *cox*1-based qPCR was designed (Table 1) with primers Hw.COI.723-F and Hw.COI.950-R to amplify partial *cox*1 gene (227 bp) of both filarial and non-filarial nematodes, including *D. immitis* and *A. vasorum*. The primers were chosen to flank the probe P.imm.COI.777, previously designed for *D. immitis*. In addition, we designed a new probe named A.vas.COI.813-P specific to *A. vasorum*. The TaqMan probes were labelled with FAM and VIC, respectively, with a non-fluorescent quencher TAMRA.

TaqMan simplex qPCR targeting Wolbachia

The *16S* rDNA gene has been reported as the most commonly used gene for *Wolbachia* phylogeny [35]. The simplex-qPCR was developed and validated *in silico* for the conserved region of the first third of the *16S* rDNA gene. The qPCR system (Table 1) is composed of primers Wol.16S.301f and Wol.16S.478r with the probe Wol.16S.347p targeting all *Wolbachia* lineages.

TaqMan duplex qPCR targeting filarial Wolbachia

Wolbachia ftsZ gene, the homologue of the eukaryotic protein tubulin, provides sufficient discrimination between *Wolbachia* spp. of supergroups C and D found in filarial nematodes, and those of supergroups A and B found in arthropods with a higher divergence between filarial *Wolbachia* of supergroups C and D [36]. The *ftsZ*-based duplex-qPCR was designed with primers WDiro.ftsZ.490f and wDiro.ftsZ.600r targeting filarial *Wolbachia* belonging to supergroup C, which includes those found in *Dirofilaria* sp. However, the specificity of the duplex-qPCR was confined to probes wDimm.ftsZ.523p and wDrep.ftsZ.525p specific to *Wolbachia* sp. of *D. immitis* and that of *D. repens*, respectively (Table 1).

Run protocols

The simplex, duplex and triplex qPCR reactions were carried out in a final volume of 20 µl, containing 5 µl of DNA template, 10 µl (2×) of Master Mix Roche (Eurogentec, Liège, Belgium). Volume of each primer per reaction was 0.5 µl (50 µM) for the simplex qPCR and 0.75 µl (50 µM) for both the duplex and triplex qPCR, with 0.5 µl of both UDG (1 U/µl) and each probe (20 µM). The final volume was made up to 20 µl

Table 1 Primers and probes developed in this study

System name	Target gene	Primer and probe name	Sequence (5′-3′)	Assay specificity
Pan-fil *28S* qPCR-based system	*28S* rRNA	qFil-28S-F	TTGTTTGAGATTGCAGCCCA	Filariae
		qFil-28S-P	6FAM-CAAGTACCGTGAGGGAAAGT-TAMRA	
		qFil-28S-R	GTTTCCATCTCAGCGGTTTC	
All-Wol *16S* qPCR-based system	*16S* rRNA	all.Wol.16S.301-F	TGGAACTGAGATACGGTCCAG	*Wolbachia*
		all.Wol.16S.347-P	6FAM-AATATTGGACAATGGGCGAA-TAMRA	
		all.Wol.16S.478-R	GCACGGAGTTAGCCAGGACT	
Triplex TaqMan *cox*1 qPCR-based system	*cox*1	Fil.COI.749-F	CATCCTGAGGTTTATGTTATTATTTT	*D. immitis*, *D. repens* and *A.reconditum*
		D.imm.COI.777-P	6FAM-CGGTGTTTGGGATTGTTAGTG-TAMRA	
		D.rep.COI.871-P	6VIC-TGCTGTTTTAGGTACTTCTGTTTGAG-TAMRA	
		A.rec.COI.866-P	Cy5-TGAATTGCTGTACTGGGAACT-BHQ3	
		Fil.COI.914-R	CWGTATACATATGATGRCCYCA	
Duplex Wol-Diro ftsZ qPCR-based system	*ftsZ*	WDiro.ftsZ.490-F	AAGCCATTTRGCTTYGAAGGTG	Endosymbiotic *Wolbachia* of *D. immitis* and *D. repens*
		WDimm.ftsZ.523-P	6FAM-CGTATTGCAGAGCTCGGATTA-TAMRA	
		WDrep.ftsZ.525-P	6VIC-CATTGCAGAACTGGGACTGG-TAMRA	
		WDiro.ftsZ.600-R	AAACAAGTTTTGRTTTGGAATAACAAT	
Duplex HWs *cox*1 qPCR-based system	*cox*1	Hw.COI.723-F	TCAGCATTTGTTTTGGTTTTT	*D. immitis* and *A. vasorum*
		D.imm.COI.777-P	6FAM-CGGTGTTTGGGATTGTTAGTG-TAMRA	
		A.vas.COI.813-P	6VIC-TGACTGGGAAGAAGGAGGTG-TAMRA	
		Hw.COI.950-R	GCASTAAAATAAGYACGAGWATC	

using DNAse-RNAse free UltraPure water (Eurogentec, Liège, Belgium). The TaqMan cycling conditions included two hold steps at 50 °C for 2 min followed by 15 min at 95 °C, and 39 cycles of two steps at 95 °C for 30 s and 60 °C for 30 s. These reactions were performed in a CFX96 Touch Real-time PCR Detection System (Bio-Rad, Marnes-la-Coquette, France) after activating the appropriate absorption channels for the dyes used in each qPCR system.

The accumulation of the relative fluorescence units (RFUs) was recorded during the extension step of each qPCR and was used to set-up the cut-off value for each TaqMan system, according to the formula described [37]. The tolerance value was fixed at 5% for all systems. The qPCR reaction was considered positive only if the RFU value was higher than the cut-off value. qPCR data analysis was performed using the CFX Manager Software Version 3 [37].

Design of conventional PCR primers, amplification and sequencing protocols

In order to complete the molecular identification of filariae and their *Wolbachia* spp., we designed two sets of degenerate primers (Table 2): (i) Fspec.COI.957f and Fspec.COI.1465r targeting a 509-bp fragment of the *cox*1 gene of filarioids; and (ii) Wol.ftsZ.363.f and Wol.ftsZ.958.r targeting a 595-bp fragment of the *ftsZ* gene of *Wolbachia* lineages that may be associated with filarioids. All PCR reactions were carried out in a total volume of 50 µl, consisting of 25 µl (2×) of AmpliTaq Gold master mix, 18 µl of DNAse-RNAse free UltraPure water (Eurogentec, Liège, Belgium), 1 µl of each primer (20 µM) and 5 µl of DNA template (except no-template controls). The thermal cycling conditions were: incubation step at 95 °C for 15 min, followed by 40 cycles at 95 °C for 1 min, 30 s at the annealing temperature (with a different melting temperature for each PCR assay, see Table 2), 72 °C for 45 s for elongation, followed by a final extension step at 72 °C for 5 min (Table 1). PCR amplification was performed in a Peltier PTC-200 thermal cycler (MJ Research Inc., Watertown, MA, USA). Amplicons were purified using the filter plate Millipore NucleoFast 96 PCR kit following the manufacturer's recommendations (Macherey Nagel, Düren, Germany). Purified DNA was sequenced using the BigDye® terminator v3.3 cycle sequencing kit DNA in line with the manufacturer's instructions (Applied Biosystems). Sequencing was performed using 3500xL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and a capillary electrophoresis fragment analyzer (Applied Biosystems). The nucleotide sequences were assembled and edited using ChromasPro 2.0.0 and were then checked using the Basic Local Alignment Search Tool (BLAST) [38].

Specificity, sensitivity and system validation

DNA samples summarized in Additional file 1: Table S1 were used for the *in vitro* validation of all PCR systems as follows. Nineteen samples of genomic DNA from filarial parasites were used to validate the pan-filarial *28S* qPCR. DNA from eight strains of *Wolbachia* endosymbionts of *Aedes albopictus*, *Anopheles gambiae*, *Cimex lectularius*, *C. hemipterus* (PL13 strain), *D. immitis* microfilariae, *D. repens*, *Onchocerca lupi*, *Wuchereria bancrofti* and *Brugia* sp. were used for the *Wolbachia 16S*-based qPCR system. *Dirofilaria immitis*, *D. repens* and *A. reconditum* DNA were used to validate the triplex qPCR. *Dirofilaria immitis* and *A. vasorum* DNA were used to validate the duplex qPCR targeting heartworms. Finally, DNA of *Wolbachia* endosymbiont of *D. immitis* and that of *D. repens* were used for the duplex *ftsZ*-based qPCR system.

All PCR systems were tested for their specificity using several nematodes, arthropods, laboratory-maintained colonies as well as human, monkey, donkey, horse, cattle, mouse and dog DNA. DNA samples used to test the sensitivity and specificity of PCR systems are summarized in Additional file 1: Table S1 and Additional file 2: Table S2.

The analytical sensitivity was assessed using a 10-fold dilution of DNA templates, then standard curves and derived parameters (PCR efficiency, slope, Y-intercept and correlation coefficient) were generated using CFX Manager Software Version 3 [37]. The triplex and pan-filarial qPCR systems were challenged in detecting the related numbers of microfilariae of *D. immitis*, *D. repens* and *A. reconditum*. The DNA of each species was obtained from naturally infected canine blood, *D. immitis* (Corsica, 2018), *D. repens* (France, 2018) and *A. reconditum* (Côte d'Ivoire, 2018). First, 1 ml of each blood sample was examined by the modified Knott's test [9] to identify the microfilariae species and their number (Fig. 1). Then, the microfilariae concentration was adjusted to 1500 mf/ml by adding Hank's balanced salt solution (HBSS; Gibco-BRL, Life Technologies, Eragny, France). Thereafter, two extractions were performed from 200 µl: (i) from each separately calibrated sample; and (ii) after mixing an equal volume of each of them to generate a concentration of 500 mf/ml per species. These were used to evaluate the pan-filarial and triplex qPCRs, respectively. Finally, a serial 10-fold dilution of DNA extracted from microfilaremic blood (Corsica, 2018) containing 4033 microfilariae of *D. immitis* was used to assess the analytical sensitivity of both the triplex and duplex (Wol-Diro *ftsZ*) qPCRs in the direct and the indirect detection of single infection with *D. immitis.*

PCR tools validation by sample screening and identification of filarial infection on biological samples

A pre-existing collection of canine blood and serum samples was used in this study. This included: (i) 8 samples composed of nematode-free laboratory Beagles from the biobank of the Veterinary Research Center of the IHU Méditerranée Infection were used as a negative control group; and (ii) 136 dogs enrolled in March 2017 from Corsica where heartworms are endemic; 7 military working dogs from France recruited on October 2018; and 17 dogs enrolled in April 2018 from Côte d'Ivoire in which blood microfilariae were recorded. Canine blood samples were collected by a veterinarian using cephalic venipuncture into a citrate and serum separator tube. The serum collected and citrate blood were then stored at −20 °C. These 168 samples were subsequently processed for molecular and serological analysis. Genomic DNA was extracted from the blood and the microfilaria-containing tissues using the Qiagen DNA tissue extraction kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. The extracted DNA was eluted in a total volume of 100 µl and stored at −30 °C.

First, all DNA samples were screened for both filarial and *Wolbachia* DNA using the pan-filarial and pan-*Wolbachia* qPCRs, respectively. Then, partial *cox*1 and *ftsZ* genes were amplified and sequenced according to the previous protocol from all positive samples for filarioids and *Wolbachia*, respectively. Secondly, the fast typing method based on the direct identification of filarial and *Wolbachia* genotypes used the approach combining the triplex *cox*1 and duplex Wol-Diro *ftsZ* qPCR-based systems. Finally, all samples were screened for heartworms using the duplex *cox*1-based qPCR in order to differentiate between *D. immitis* and *A. vasorum* DNA.

The serological analysis was performed on all sera using the DiroCHEK® heartworm antigen test kit

Table 2 PCR/sequencing primers developed in this study, their characteristics and conditions

System name	Target gene	Primer name	Sequence (5′-3′)	Amplicon size (bp)	Tm (°C)	Specificity
Pan-fil *cox*1 PCR	*cox*1	Fwd.957	ATRGTTTATCAGTCTTTTTTTATTGG	509	52.0	Filariae
		Rwd.1465	GCAATYCAAATAGAAGCAAAAGT			
Wol ftsZ PCR	*ftsZ*	Wol.ftsZ.363.f	GGRATGGGTGGTGGYACTGG	560	59.5	*Wolbachia*
		Wol.ftsZ.958.r	GCATCAACCTCAAAYARAGTCAT			

Abbreviation: Tm, melting temperature

(Zoetis, Lyon, France). The test consisting of an enzyme-linked immunosorbent assay sandwich ELISA, targeting the antigen secreted by adult female heartworms [14]. Each serum sample was tested using two different protocols: (i) 200 µl of serum was heated at 104 °C for 10 min followed by centrifugation at 16,000×*g*; (ii) the second protocol was performed without heat-treatment of the sera following the recommendations by Beall et al. [39] regarding the immune complex dissociation to detect any heartworm antigen if present.

In order to evaluate the performance of molecular and serological assays in the absence of the gold standard test (necropsy), we developed the following approach to determine true positive samples. The sample was considered a true positive for heartworm if it was positive for: (i) at least one of the molecular markers of heartworm (DNA of *D. immitis* or its *Wolbachia*); and (ii) a positive antigen test after immune complex dissociation by heating sera. This approach eliminates false-positive serological results that may be obtained by increasing the detection threshold (sensitivity) after heat-treatment of the sera before use, which is subsequently confirmed by the molecular markers specific to *D. immitis*. Once the DNA of *A. reconditum* was identified, the sample was considered a true positive. Finally, samples positive for at least one DNA marker of *D. repens* or DNA of its *Wolbachia* were considered to be true positives.

Statistical analysis

Results generated through laboratory analysis were recorded in Microsoft Excel (Microsoft Corp., Redmont, USA). In order to assess how *Wolbachia* sp. strains correlated with filarial species, Spearman's correlation coefficient was calculated. In order to evaluate the relevance of each diagnostic approach, the prevalence, correct classification, misclassification, sensitivity, specificity, false positive rate, false negative rate, positive and negative predictive value and Cohen's Kappa (*k*) measure agreement was calculated. According to the scale of Landis & Koch [40], the agreement quality of Kappa values was interpreted as follows: <0, no agreement; 0–0.2, slight agreement; 0.2–0.4, fair agreement, 0.4–0.6, moderate agreement; 0.6–0.8, substantial agreement; 0.8–1, almost perfect agreement. Statistical analyses were performed using Addinsoft 2018 (XLSTAT 2018: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France).

Results

Validation of the PCR systems

The *in silico* validation revealed that the pan-filarial systems (*28S* qPCR and *cox*1 PCR) were specific for filarial

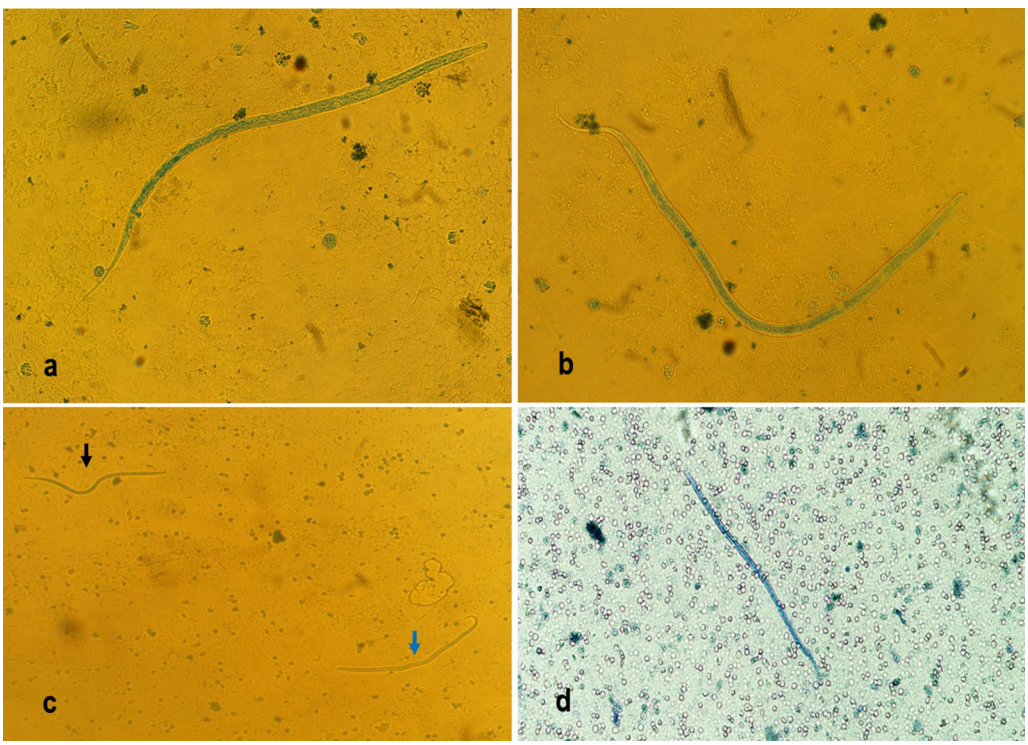


Fig. 1 Microfilariae in canine blood by modified Knott's test. **a** *Dirofilaria immitis* microfilaria. **b** *Dirofilaria repens* microfilaria. **c** *Dirofilaria immitis* (black arrow) and *D. repens* (blue arrow) co-infection. **d** *Acanthocheilonema reconditum* microfilaria

parasites belonging to the subfamilies Dirofilariinae, Onchocercinae, Setariinae, Oswaldofilariinae, Icosiellinae and Waltonellinae. The *16S* qPCR targeting *Wolbachia* strains was specific for all the lineages known so far. However, the *ftsZ* PCR showed specificity for *Wolbachia* strains belonging to supergroups C, D, F and J, that may be associated with filarioids. Likewise, the multiplex qPCRs were also specific for the target species without failure. For each qPCR system, primer melting temperatures were closely identical and were lower than that of the probe. Indeed, the absence of primer-dimer formation and hairpin structures was also confirmed. Furthermore, the specificity was confirmed again by an *in vitro* validation, as shown in Additional file 1: Table S1, where positive reaction was obtained only from the target DNA and no negative control was amplified. Despite using single-species or pooled DNAs, the specific fluorescence signals generated through the multiplex qPCR systems were successfully related to the target DNA (Additional file 3: Table S3, Additional file 4: Figure S1).

Determining assay performance characteristics

The assay characteristics were assessed for the pan-filarial, the triplex and the duplex qPCR targeting *Wolbachia* sp. endosymbiont of *Dirofilaria* spp. The analytical sensitivity of the pan-filarial qPCR was confirmed three times using *D. immitis*, *D. repens* and *A. reconditum* DNA sharing the same microfilariae concentration. This assay was able to detect up to 1.5×10^{-4} microfilariae per ml (mf/ml) (corresponding to 0.75×10^{-6} mf/5 µl). Efficiency ranged from 99.1 to 100.7%, with a slope from −3.34 to −3.30, Y-intercept values from 21.71 to 21.72 and an R^2 from 0.996 to 0.999 for microfilariae of all species tested (Additional file 5: Table S4, Additional file 6: Figure S2). However, the analytical sensitivity of the triplex qPCR, using pooled DNA of three species, was confirmed by the detection of up to 5×10^{-1} mf/ml (corresponding to 2.5×10^{-3} mf/5 µl) of each species simultaneously (Additional file 7: Table S5, Additional file 8: Figure S3). qPCR efficiency ranged from 100.4 to 103.7%, with a slope from −3.30 to −3.24, Y-intercept values from 32.89 to 33.19 and an R^2 from 0.993 to 0.999. Finally, the analytical sensitivity was confirmed for both the *cox*1-triplex and the *ftsZ* duplex qPCRs in detecting the infection by *D. immitis* (Additional file 9: Table S6).The detection limit was up to 4.03×10^{-2} and 4.03×10^{-1} mf/ml, respectively (corresponding to 2.01×10^{-3} and 2.01×10^{-2}, respectively) mf/5 µl, qPCR efficiency was 104.8 and 100.5%, respectively, slopes were −3.212 and −3.309, respectively. Y-intercept values were 31.17 and 35.98, respectively, and R^2 was above 0.995 for both systems (Additional file 9: Table S6, Additional file 10: Figure S4).

Molecular diagnostic approaches

Results of molecular screening followed by the sequence typing approach are detailed in Fig. 2a, b and Additional file 11: Table S7. Of the 168 samples tested, 49 (29.17%) were positive for DNA of at least one filaria species or its *Wolbachia* genotype. All positive results were grouped in: (i) 19 blood samples positive only for filarioid DNA; (ii) 9 samples positive only for *Wolbachia* DNA; and (iii) 21 samples positive for both filarial and *Wolbachia* DNA. Although partial *cox*1 and *ftsZ* amplicons were amplified from all positive samples for filariae and *Wolbachia*, *cox*1 gene sequence-based identification allowed the identification of the causative agent of filariosis in 35 (87.5%) out of 40 samples amplified by PCR (Fig. 2a). We here report 12 (30%) cases of *D. immitis*, 7 (17.5%) cases of *D. repens*, 15 (37.5%) cases of *A. reconditum* and one case of both *D. repens* and *A. reconditum* DNA. Noteworthy, the amplicon sequences were obtained separately from this latter case after serial dilution of blood before DNA extraction. However, the *ftsZ* gene sequence-based identification allowed the identification of *Wolbachia* sp. genotype in 25 (83.33%) out of 30 samples amplified by PCR (Fig. 2b). Twenty-two (73.33%) of the *Wolbachia* sequences were closely related to the strain identified in *D. immitis* (GenBank: AJ010272, 99.58%) and 3 (10%) were similar to the strain identified in *D. repens* (GenBank: AJ010273, 99.80%). However, sequence-based identification failed to yield sequences from amplified DNA in 5 cases for each system, which corresponds to 12.5% and 16.7% of filaria and *Wolbachia* DNA samples, respectively. The combined multiplex approach based on the triplex *cox*1 qPCR targeting filariae and the duplex qPCR targeting *Wolbachia*, allowed the detection of 49 samples previously considered positive based on filariae markers (Fig. 3a and Additional file 12: Table S8). The triplex *cox*1 qPCR identified the corresponding species from all the positive samples for filariae ($n = 40$, 100%). Of these, 34 (34.85%) samples had DNA from a single filarial species. *Dirofilaria immitis* was identified in 12 (30%), *D. repens* in 7 (17.5%) and *A. reconditum* in 15 (37.5%) of these samples. Five samples (12.5%) were positive for DNA of two filarial species, of which 4 were positive for *D. immitis* and *D. repens* and one was positive for *D. repens* and *A. reconditum*. Positivity for three filarial species was detected for only one sample (2.5%). The duplex Wol-Diro-*ftsZ* qPCR allowed the identification of *Wolbachia* genotype from all samples positive for *Wolbachia* DNA. Twenty-one samples (21.70%) were positive for *Wolbachia* sp. endosymbiont of *D. immitis*, three samples were positive for *Wolbachia* sp. endosymbiont of *D. repens* and five

samples (16.67%) were positive for both strains. Among the 168 samples screened by duplex qPCR for heartworms (*D. immitis* and *A. vasorum*), 17 (10.12%) were positive for *D. immitis* DNA and no *A. vasorum* DNA was detected.

Link between *Wolbachia* genotype and filarial species within the infected host

The results of the distribution of filarioid markers obtained by multiplex qPCRs are shown in Fig. 3a and Additional file 12: Table S8. Interestingly, most samples which were positive for filarioid DNA were also positive for *Wolbachia* (21/40, 52.5%). Indeed, *Wolbachia* DNA was associated with at least one *Dirofilaria* spp. in 80% (20/25) of the samples having dirofilarial DNA. Analysis of the correlation between *Wolbachia* strains and *Dirofilaria* species are shown in Table 3. Seventy-five percent (9/12) of the samples positive for *D. immitis* DNA alone were also positive for *Wolbachia* genotype known to be associated with this filarioid, which corresponds to a significant correlation ($r=0.509$, $P<0.0001$). In addition, there was a significant correlation ($r=0.181$, $P<0.019$) between the presence of *D. repens* DNA alone and that of *Wolbachia* strain commonly associated with this filarioid. In general, the presence of *D. repens* DNA was correlated with the presence of DNA of both *Wolbachia* strains ($r=0.454$, $P<0.0001$). The presence of both *D. immitis* and *D. repens* DNA was associated with the presence *Wolbachia* endosymbiont of *D. immitis* ($r=0.244$, $P<0.002$) and also with the presence of both *Wolbachia* strains together ($r=0.175$, $P=0.023$). On the other hand, 29.63% (8/27) of the samples harboring the *Wolbachia* endosymbiont of *D. immitis* were free of filarioid DNA (Fig. 3a) and 25% (2/8) of the samples positive for *Wolbachia* endosymbiont of *D. repens* DNA were also free for filarioid DNA. No correlation was observed between *A. reconditum* and *Wolbachia* strains.

Heartworm antigen detection and infection status

Of the 168 dog sera tested for heartworm antigen, 16.67% (28) were positive before pre-treatment of the sera and were grouped into three groups (Fig. 3b): (i) 9 (5.36%) were mono-infected by heartworm and were positive for both *D. immitis* and its *Wolbachia* sp. DNA except one, which was positive for *D. immitis* DNA only; (ii) 10 (5.95%) were samples co-infected with at least one other filarioid detected by PCR, comprising 8 (4.76%) positive for *D. repens* and *D. immitis* and 2 (1.19%) positive for *D. immitis*, *D. repens* and *A. reconditum*; and (iii) 9 (5.39%) were positive for filariae other than *D. immitis*, with 7 (4.17%) samples positive for *A. reconditum* DNA only, 1 (0.6%) positive for DNA of *Wolbachia* endosymbiont of *D. repens* and 1 (0.6%) positive for DNA of both *A. reconditum* and *Wolbachia* endosymbiont of *D. repens*.

Once the heat pre-treatment of sera was performed, the rate of positive samples increased up to 71.43% ($n=120$). Of these, 39.17% ($n=47$) harbored at least one DNA marker of filarial parasites or their *Wolbachia*. However,

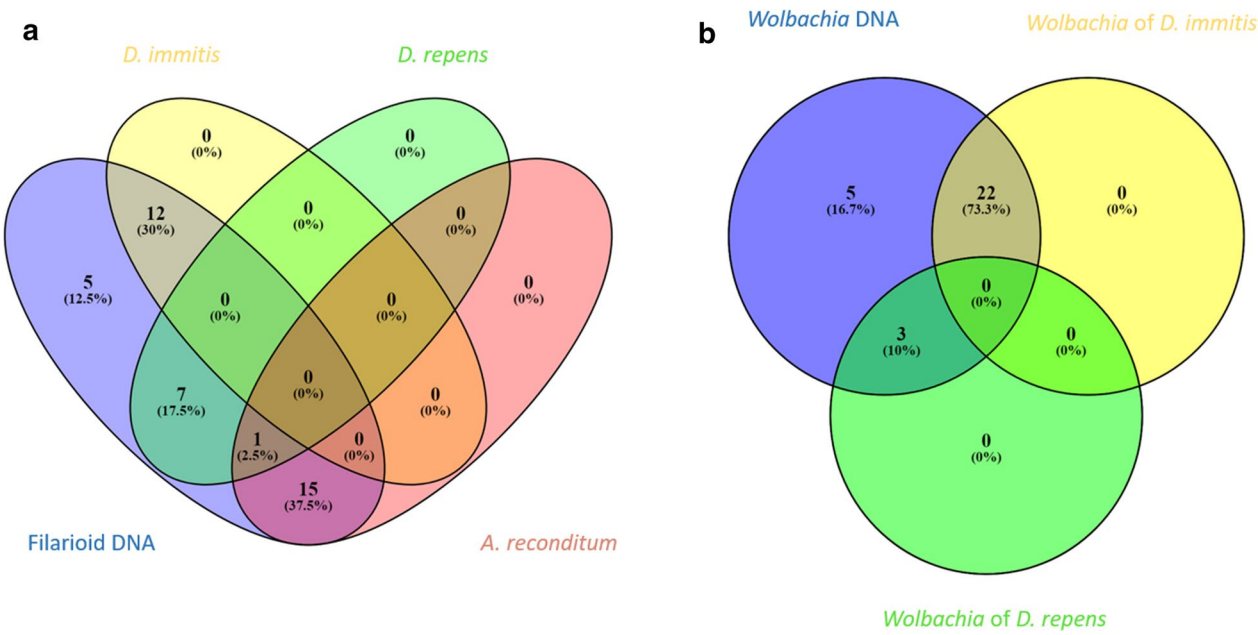


Fig. 2 Venn diagram depicting the distribution of positive samples for filariae and *Wolbachia* DNA. **a** Sequence typing method based on the *cox*1 gene of filariae. **b** *Wolbachia* genotyping based on the *ftsZ* gene

two samples (1.19%), one positive for *Wolbachia* endosymbiont of *D. immitis* by qPCR and the other positive for *D. repens* by qPCR, remained serologically negative. For 73 samples (43.46%) no filarioid marker was detected; these were considered positive for unknown antigens (Fig. 3b and Additional file 13: Table S9). No positive results in the negative control group for both serological and molecular assays were obtained.

Performance characteristics comparison of the diagnostic tools

Once the true positive samples for each filariosis were determined, the diagnostic value was evaluated for each test in the specific detection of filariosis. The sequence typing approach combining the identification of the filariae and *Wolbachia* allowed the diagnosis of heartworm infection in 86.21% (25/29) of cases, which corresponds to a specificity of 82.8% and a sensitivity of 99.3%, thus resulting in an almost perfect agreement with the true positive rate ($k = 0.87$). Compared to the gold standard, the approach combining the multiplex qPCR systems detected one more positive sample for *Wolbachia* endosymbiont of *D. immitis* (Fig. 3b). This approach showed a sensitivity of 100% and a specificity of 99.3%, with an almost perfect agreement ($k = 0.98$) (Additional file 14: Table S10), whereas the detection of heartworm antigen prior to heat pre-treatment of sera showed a sensitivity of 65.5% and a specificity of 93.3%, corresponding to moderate agreement ($k = 0.6$). Additionally, the heat pre-treatment of sera allowed the detection of 71.4% ($n = 120$) including 24.17% ($n = 29$) positive for *D. immitis* infection, 15% ($n = 18$) positive for filariae other than *D. immitis* and 60.83% ($n = 73$) without molecular markers of filariae. The performance characteristics of this

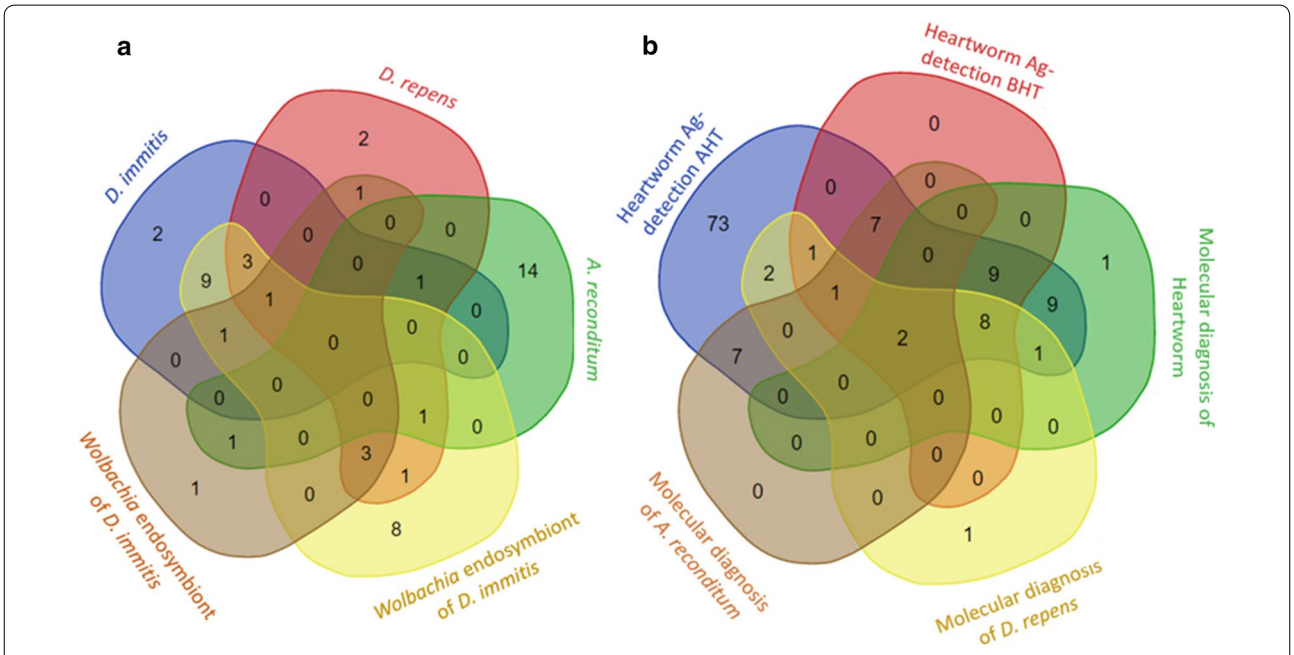


Fig. 3 Venn diagram depicting the distribution of positive samples detected by molecular and serological assays. **a** Molecular identification of filariae and associated *Wolbachia* using the multiplex approach. **b** Comparison between molecular diagnosis and heartworm antigen detection before and after heat pre-treatment of sera

Table 3 Spearman correlation matrix depicting the strength of association between filaria and *Wolbachia* species within infected dogs

Groups of *Wolbachia* DNA	Filarial DNA	*D. immitis* and *D. repens* DNA	Single-species DNA of *D. immitis*	Single-species DNA of *D. repens*	*A. reconditum* DNA
Wolbachia DNA	0.506 (< 0.0001)	0.284 (0.0002)	0.565 (< 0.0001)	0.447 (< 0.0001)	− 0.053 (0.490)
Both *Wolbachia* of *D. immitis* and *D. repens* DNA	0.313 (< 0.0001)	0.175 (0.023)	0.087 (0. 259)	0.454 (< 0.0001)	− 0.059 (0.449)
Single-species DNA of *Wolbachia* ex *D. immitis*	0.478 (< 0.0001)	0.244 (< 0.002)	0.509 (< 0.0001)	0.079 (0.381)	− 0.072 (0.335)
Single-species DNA of *Wolbachia* ex *D. repens*	0.334 (< 0.0001)	− 0.024 (0.614)	− 0.037 (0.630)	0.181 (0.019)	0.104 (0.180)

Note: The first number represents the correlation coefficient. Values close to zero reflect the absence of correlation. The associated *P*-values are in parentheses

tool in detecting heartworm infection when the sera were heated were 100% sensitivity and 34.5% specificity with a slight agreement ($k=0.15$) (Additional file 14: Table S10). Taking the combined multiplex approach as the gold standard, the sequence typing method had a specificity of 100% and a sensitivity of 62.6% and 94.1% for the detection of *D. repens* and *A. reconditum*, respectively. A substantial ($k=0.75$) and an almost perfect ($k=0.97$) agreement with the gold standard test was observed for the detection of *D. repens* and *A. reconditum*, respectively (Additional file 15: Table S11).

Discussion

qPCR system validation and assay performance characteristics

The newly developed PCR assay systems have shown specific detection of the target DNA for which they were designed. The pan-filarial *28S* qPCR system aims the detection of filarial DNA from biological samples. It has been adapted for the detection of the filarial parasites known to date, such as members of the subfamilies Dirofilariinae and Onchocercinae parasitizing mammals, reptiles and birds, and those of the subfamily Setariinae, confined to large mammals, and Oswaldofilariinae parasites of reptiles, and amphibian parasites of the subfamilies Icosiellinae and Waltonellinae [5]. The *LSU* rRNA (*28S*) gene targeted by this system is known for its conserved regions between the filarial species [41]. The second qPCR system was customized for the detection of *Wolbachia* DNA irrespective of their lineages. It targets the first part of the *16S* gene which is highly conserved between *Wolbachia* lineages [35]. Another qPCR system for *Wolbachia* targeting the *16S* gene has been proposed as a complementary diagnosis from human blood of the lymphatic filariosis caused by *Wuchereria bancrofti* [42].

In addition to being specific, the multiplex qPCRs were discriminatory towards targeted DNA without failure (Additional file 3: Table S3). These features are directly related to the choice of the target genes, which offer sufficient discrimination between species, as is the case with the *cox*1 gene representing a nematode barcode [34, 43] used for the development of the triplex qPCR for *D. immitis*, *D. repens* and *A. reconditum* and the duplex qPCR targeting *D. immitis* and *A. vasorum* agents of heartworm diseases. *Wolbachia ftsZ* gene, mainly used for the characterization of *Wolbachia* supergroups [36], was used for the development of the duplex qPCR for both *Wolbachia* genotypes associated with *D. immitis* and *D. repens*. A real time PCR for *Wolbachia* endosymbiont of *Brugia pahangi* targeting the same gene has been described [44].

It is worth noting that molecular diagnosis combining the detection of filarial and *Wolbachia* DNA is an improvement and a tool for evaluating treatment protocols targeting filariae and *Wolbachia* [44]. In the present study, the analytical sensitivity of the new qPCR assays ranged from 99.3% to 107.6%, with the slope value of the standard curves ranging from −3.34 to −3.15 and coefficients of determination (R^2) higher than 0.99. These characteristics are directly derived from the design protocol, where the formation of heterodimers and hairpins inside and between primers and probes was avoided. Primer sets share a similar melting temperature which is lower than that of the probes, offering a better sensitivity of the qPCR reaction.

The sensitivity of the pan-filarial *28S* qPCR system was much higher than the triplex qPCR for the detection of *D. immitis*, *D. repens* and *A. reconditum* DNA, where the detection limit was 1.5×10^{-4} and 5×10^{-2} mf/ml, respectively. Indeed, the reference baseline for mitochondrial DNA retrieved from the EZ1 DNA-tissue kit was at 41.4 copies per nuclear genome [45]. Estimated genomic rRNA copy number of 150 in *B. malayi* [32] suggests that the *28S* rRNA gene enables a high amplification efficiency rather than the mitochondrial *cox*1 gene of filarial nematodes. However, the sensitivity of the triplex qPCR in detecting single-species DNA of *D. immitis* was much higher than that of the duplex *ftsZ* qPCR in detecting single-species DNA of *Wolbachia* endosymbiont of *D. immitis*, where the detection limit was 4.03×10^{-3} and 4.03×10^{-1} mf/ml, respectively. Rao et al. [42], reported that filarial DNA is more frequently detected than *Wolbachia* DNA from *W. bancrofti* microfilaremic blood using qPCR assays. The difference of sensitivity could be explained by the weaker infection density by *Wolbachia* at this parasite larval stage [42].

Molecular diagnostic approaches

Here, we developed and assessed two molecular approaches in detecting and identifying canine filariosis. The first combined the screening and sequence typing of both filarial and *Wolbachia* DNA. The genomic DNA was identified with an almost perfect specificity ranging from 99.3 to 100%. However, the sensitivity ranged from moderate (62.5%) to perfect (94.1%) regarding the presence or absence of co-infection. Overlapping peaks corresponding to different nucleotides on electropherograms of the sequenced samples suggest co-infection [46]. The second approach combines two multiplex qPCR systems targeting *A. reconditum*, *D. immitis*, *D. repens* and the *Wolbachia* genotypes associated with the latter two species. All samples were positive for at least one molecular marker which were detected and identified with an almost perfect sensitivity and specificity using this approach. This method is fast, simple to use, sensitive and highly specific in detecting occult and non-occult

filariosis within the infected hosts. The present results reinforce the utility of multiplex qPCR in detecting co-infections, confirm the resolution limits of the sequence typing method in the identification of co-infections [47], and avoiding the sequencing procedure needed using PCR with filaria generic primers [48].

Linkage between *Wolbachia* strains and filarial species within the infected host

As expected, *Wolbachia* DNA was significantly associated with *Dirofilaria* species in 80% (20/25) of the samples positive for at least one *Dirofilaria* spp. DNA, reinforcing the idea that this endosymbiosis relationship is present in *Dirofilaria* spp. and not in *A. reconditum* [27]. Of the samples positive for *D. immitis* DNA, 75% (9/12) were also found to be positive for the *Wolbachia* genotype known to be associated with this filarioid, resulting in a significant correlation (Table 3). As previously reported, *Wolbachia* DNA was detected in 64.0% of the samples positive for *D. immitis* [27], and in 81.6% of the samples positive for *D. repens* [49]. In the present study, we investigated the link between *Wolbachia* genotype and *D. repens* infection. The samples positive for a single-species DNA of *D. repens* had a significant correlation with the *Wolbachia* genotype known to be associated with this filarioid. This result corroborates the data by Vytautas et al. [49]. Interestingly, the presence of the single-species DNA of *D. repens* was also strongly correlated with the presence of both *Wolbachia* strains associated with *Dirofilaria* spp. This association could be explained, either by the presence of an occult co-infection with *D. immitis*, or by an exchange of *Wolbachia* strains between *Dirofilaria* spp. The first suggestion is supported by the fact that co-infection of *D. repens* and *D. immitis* is often associated with an occult form. This phenomenon results from a competitive suppression between microfilariae species [19]. On the other hand, *Wolbachia* sp. of *D. immitis* was detected in 29.63% (8/27) of the samples in which *D. immitis* DNA was not detected and, in the same samples, an antigen was detected after heat pre-treatment of sera. This result confirms the possibility to detect *Wolbachia* DNA in occult infections. The utility of *Wolbachia* as a diagnosis target for the occult heartworm disease has been demonstrated in the dead-end host, such as humans and cats, where the parasite cannot achieve its maturation and the infection might be amicrofilariaemic [23]. However, the second suggestion related to the exchange (horizontal transfer) of *Wolbachia* strains between *Dirofilaria* species is in contrast with the published data. *Wolbachia* transmission principally occurs *via* eggs of female worms (vertical transfer) [36, 50]. The vertical transfer of *Wolbachia* leads to the specialization of the host-symbiotic relationship [51]. Taylor et al. [52] have indicated that experimental crosses between *B. pahangi* and *B. malayi* have demonstrated *Wolbachia* transmission through female worms only [52]. Theoretically, exchange of *Wolbachia* between *D. immitis* and *D. repens* is hardly possible in natural conditions, because these filariae do not share the same site and the adult worms will not have contact inside the host organism [53]. In addition, it has been reported that each genotype of *Wolbachia* has a specific filarial host [36], and that live worms can release their *Wolbachia* endosymbionts into host tissues [52]. We believe that the presence of a specific genotype of *Wolbachia* is a reliable marker for the presence of its filarial host.

Performance characteristics of the heartworm antigen detection tests

In the present study, all diagnostic approaches did not react with samples from the negative control group. We assessed the diagnostic value of LISA (DiroCHEK®) in detecting heartworms. The direct exploration of heartworm antigen from sera without heating the sera showed a moderate performance, with sensitivity and specificity values of 65.52% and 93.3%, respectively. Positive antigen tests were obtained from 19 out of 29 (65.52%) of the samples determined as true positives for heartworm and these often harbored both *D. immitis* and its *Wolbachia* sp. DNA. However, 10 samples (34.38%) of which 8 (80%) harbored only DNA of the *Wolbachia* endosymbiont of *D. immitis*, remained undetected by serology. The lack of sensitivity of this assay was unexpected. This may be due in part to the presence of juvenile parasites, which do not produce detectable antigens [54]. Nevertheless, similar discordances have recently been reported, where 41 (38.7%) positive PCR-confirmed microfilaremic samples were negative for heartworm antigen [55]. Therefore, filarial *Wolbachia* interact with the host by activating the Th1 type protective-immune response [56], which could be implemented in the clearance of heartworm antigens.

Finally, nine out of 28 (32.14%) samples were positive for *A. reconditum* and/or *D. repens*; these filariae are known to generate a cross-reactivity in heartworm antigen tests even in the absence of heat treatment of the sera [14]. However, 29 (96.67%) of the samples positive for at least one molecular heartworm marker tested positive for heartworm antigen after heating the sera; this step has recently been added to improve the sensitivity of this test under certain conditions [15, 41]. In contrast, the heat pre-treatment of sera strongly altered the specificity of the heartworm antigen test. Cross-reactivity was observed overall in the samples positive for any filarial parasite, as well as in samples positive for unknown antigens. Filarial (*D. repens* and *A. reconditum*) and non-filarial nematodes (*A. vasorum* and *S. lupi*), are known to

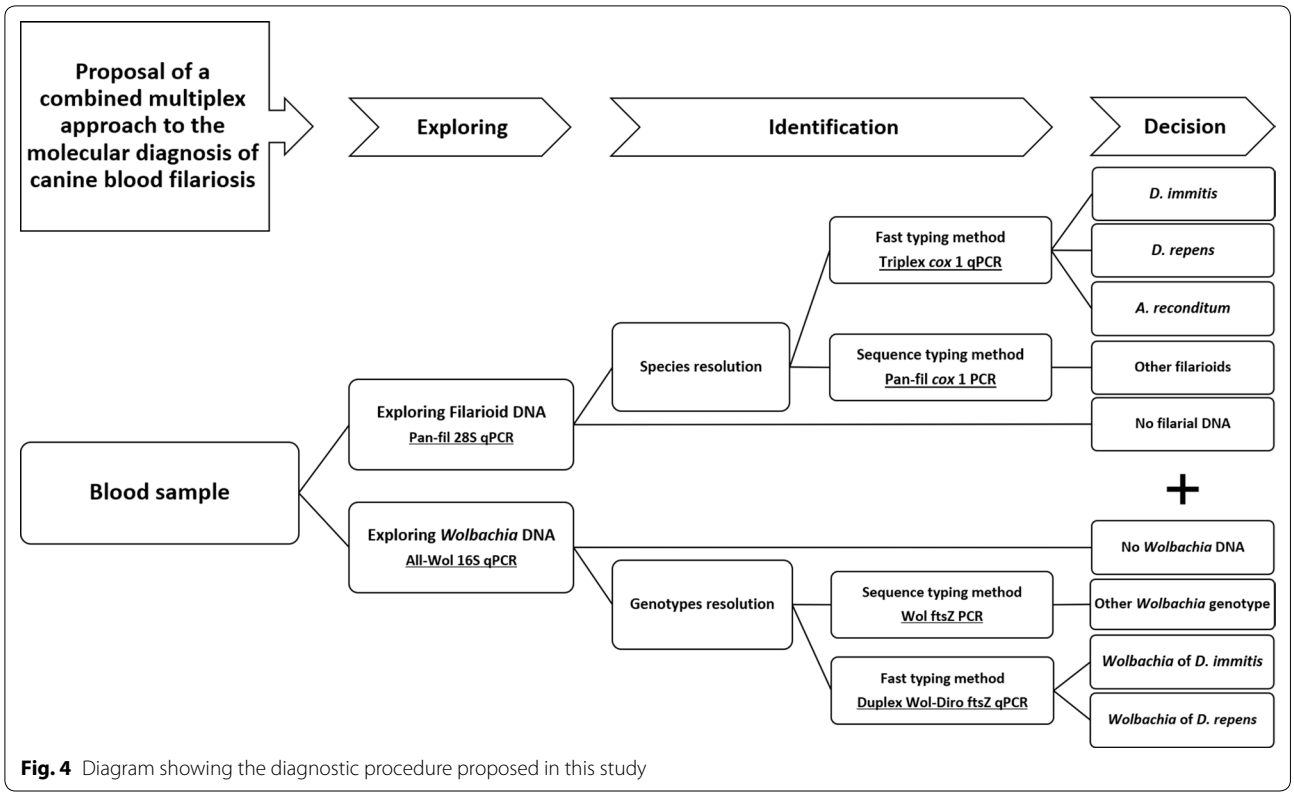


Fig. 4 Diagram showing the diagnostic procedure proposed in this study

cross-react with heartworm antigen tests [15, 57]. Therefore, molecular and serological diagnosis of *A. vasorum* from canine blood showed a close efficiency [58]. Regarding the life-cycle of *A. vasorum* [59], the absence of its DNA from all blood samples tested in the present study is not sufficient enough to rule out infection. However, the qPCR targeting *A. vasorum* developed in this study can be used as a simplex for detecting parasite larvae from other biological samples, such as faeces, pharyngeal swabs as well as in the intermediate hosts. In the absence of circulating microfilaria, the antigen detection can neither confirm nor exclude the occult heartworm in area endemic for the other species that cross-react with the test, as is the case of *D. repens* (Fig. 2). *Dirofilaria repens* microfilariae induce a suppressive effect on those of *D. immitis* [18] which induces the occult form of the latter. The European Society of Dirofilariosis and Angiostrongylosis (ESDA) does not recommend routine heat pre-treatment of sera in an area endemic for these parasites; this is recommended for use only to resolve the discrepancy between other tests, especially when a dog is positive for the microfilaria test and negative for serology, or to confirm a suspicion of clinical disease suggestive of microfilaremia [54].

Conclusions

The molecular approach developed herein represents an improvement in the diagnosis of canine filariosis. It relies principally on TaqMan multiplex qPCR technologies. We encourage researchers to follow the molecular procedure summarized in Fig. 4. The approach allows the detection of filarial parasites as well as their *Wolbachia* endosymbionts at the family level from canine blood. Furthermore, we have implemented a highly sensitive and specific triplex qPCR assay for the simultaneous detection of *D. immitis*, *D. repens* and *A. reconditum*, the most frequent agents of canine filariosis. A duplex qPCR is presented for the simultaneous identification of *Wolbachia* genotypes from *D. immitis* and *D. repens* as a complementary diagnostic of canine dirofilariosis and their occult forms. Two primer sets are proposed for PCR/sequencing of filariae and *Wolbachia* DNAs. Finally, the approach is complemented by a duplex qPCR for *D. immitis* and *A. vasorum*, agents of canine heartworm disease. Moreover, this approach is useful in epidemiological surveillance, in diagnosis and therapeutic follow-up of both filarial parasites and their *Wolbachia* endosymbionts. The specific detection of *Wolbachia* genotypes could be used for the diagnosis of filariosis and the assessment of related pathogeny within dead-end hosts, as is the case of *D. immitis* infection in humans and cats. Heartworm

antigen testing without heat treatment of the sera is not reliable in an endemic area with other filarial species, including *A. reconditum* and *D. repens*. We discourage the use of heat pre-treatment of sera, which significantly alters the specificity of the assay due to the cross-reactivity between many filarial and non-filarial nematodes and the possibility of false-positive results that may induce unnecessary heavy treatment of heartworm disease.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04185-0.

Additional file 1: Table S1. DNA used as a positive control in this study.

Additional file 2: Table S2. DNA used as a negative control in this study.

Additional file 3: Table S3. *In vitro* validation of the triplex *cox*1-based qPCR.

Additional file 4: Figure S1. Assessment of the specificity of the triplex *cox*1-based qPCR in detecting the target DNA.

Additional file 5: Table S4. Analytical sensitivity of the pan-filarial *28S*-based qPCR in detecting single-species DNA.

Additional file 6: Figure S2. a, **b**, **c**-1. Efficiency of pan-filarial *28S*-based qPCR using single-species DNA of *D. immitis*, *D. repens* and *A. reconditum*. **a**, **b**, **c**-2. Standard curves generated from serial 10-fold dilution of each DNA.

Additional file 7: Table S5. Analytical sensitivity of the triplex *cox*1-based qPCR using pooled DNA.

Additional file 8: Figure S3. a Efficiency of the triplex *cox*1-based qPCR using pooled DNA. **b** Standard curves generated from serial 10-fold dilution of DNA.

Additional file 9: Table S6. Analytical sensitivity of the triplex *cox*1 and the duplex *ftsZ*-based qPCRs in detecting single-species DNA of *D. immitis* and its *Wolbachia*.

Additional file 10: Figure S4. a, b Efficiency of the triplex *cox*1 and the duplex *ftsZ*-based qPCRs using single-species DNA of *D. immitis* and its *Wolbachia*. **c**, **d** Standard curves generated from serial 10-fold dilution of DNA.

Additional file 11: Table S7. Samples distribution according to filariae and *Wolbachia* DNA detected by the sequence typing approach.

Additional file 12: Table S8. Samples distribution according to filariae and *Wolbachia* DNA detected by the multiplex approach.

Additional file 13: Table S9. Samples tested positive by at least by one diagnostic approach.

Additional file 14: Table S10. Performance of molecular and serological assays in detecting *D. immitis* infection.

Additional file 15: Table S11. Performance of molecular approaches in detecting *D. repens* and *A. reconditum*.

Abbreviations

FAM: 6-FAM (6-carboxyfluorescein); VIC: VIC (2′-chloro-phenyl-1,4- dichloro-6-carboxyfluorescein); Cy5: cyanine 5; TAMRA: tetra-methyl-rhodamine; BHQ: black hole quencher.

Acknowledgements

We gratefully thank Sebastien Cortaredona for assistance with statistical analysis. We also thank Professor Domenico Otranto who provided us with positive control DNA. We express our gratitude to Zoetis who provided us with the DiroCHEK® tests.

Authors' contributions

YL, BD, MV and OM designed the study. YL, BD, OM designed, and YL carried out the data analysis. YL, MV, EAN and OM drafted the manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by the Institut-Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI. Funding sources had no role in the design or conduct of the study; collection, management, analysis or interpretation of the data; or preparation, review or approval of the manuscript.

Availability of data and materials

The data supporting the conclusions of this article are included within the article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Microbes, Evolution, Phylogeny and Infection (MEPHI), UMR Aix-Marseille University, IRD, APHM, IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France. [2] IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France. [3] Ceva Santé Animale, 10 Avenue de la Ballastière, 33500 Libourne, France. [4] VITROME, UMR Aix-Marseille University, IRD, SSA, APHM, IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France.

Received: 15 October 2019 Accepted: 13 June 2020
Published online: 22 June 2020

References

1. Ravindran R, Varghese S, Nair SN, Balan VM, Lakshmanan B, Ashruf RM, et al. Canine filarial infections in a human *Brugia malayi* endemic area of India. Biomed Res Int. 2014;2014:630160.
2. Latrofa MS, Annoscia G, Colella V, Cavalera MA, Maia C, Martin C, et al. A real-time PCR tool for the surveillance of zoonotic *Onchocerca lupi* in dogs, cats and potential vectors. PLoS Negl Trop Dis. 2018;12:e0006402.
3. Seixas F, Travassos P, Coutinho T, Lopes AP, Latrofa MS, dos Pires MA, et al. The eyeworm *Thelazia callipaeda* in Portugal: current status of infection in pets and wild mammals and case report in a beech marten (*Martes foina*). Vet Parasitol. 2018;252:163–6.
4. Otranto D, Dantas-Torres F, Brianti E, Traversa D, Petrić D, Genchi C, et al. Vector-borne helminths of dogs and humans in Europe. Parasit Vectors. 2013;6:16.
5. Adamson RC. Nematode parasites of vertebrates. Their development and transmission. Wallingford: CAB International; 2000.
6. Ilie MS, Imre K, Hotea I, Dărăbuș G. Survey of canine dirofilariosis from south-western Romania—preliminary results. In: 3rd European Dirofilaria Days, 21–22 June 2012, Parma, Italy; 2012. p. 68.
7. Simón F, González-Miguel J, Diosdado A, Gómez PJ, Morchón R, Kartashev V. The complexity of zoonotic filariasis epidemiology and its consequences: a multidisciplinary view. Biomed Res Int. 2017;2017:6436130.
8. Genchi C, Kramer L. Subcutaneous dirofilariosis (*Dirofilaria repens*): an infection spreading throughout the old world. Parasit Vectors. 2017;10:517.
9. Magnis J, Lorentz S, Guardone L, Grimm F, Magi M, Naucke TJ, et al. Morphometric analyses of canine blood microfilariae isolated by the Knott's test enables *Dirofilaria immitis* and *D. repens* species-specific and *Acanthocheilonema* (*syn. Dipetalonema*) genus-specific diagnosis. Parasit Vectors. 2013;6:48.

10. Nutman BT. Filarial infections. In: Jong EC, Sanford CA, editors. The travel and tropical medicine manual. 4th ed. Philadelphia: Saunders/Elsevier; 2008. p. 611–25.
11. Luca F, Luigi V. Filarial infections. In: Day MJ, editor. Arthropod-borne infectious diseases of the dog and cat. 2nd ed. Boca Raton: CRC Press; 2016.
12. Simsek S, Ciftci AT. Serological and molecular detection of *Dirofilaria* species in stray dogs and investigation of *Wolbachia* DNA by PCR in Turkey. J Arthropod Borne Dis. 2016;10:445–53.
13. Bowman DD, Atkins CE. Heartworm biology, treatment, and control. Vet Clin North Am Small Anim Pract. 2009;39:1127–58.
14. Little S, Saleh M, Wohltjen M, Nagamori Y. Prime detection of *Dirofilaria immitis*: understanding the influence of blocked antigen on heartworm test performance. Parasit Vectors. 2018;11:186.
15. Aroch I, Rojas A, Slon P, Lavy E, Segev G, Baneth G. Serological cross-reactivity of three commercial in-house immunoassays for detection of *Dirofilaria immitis* antigens with *Spirocerca lupi* in dogs with benign esophageal spirocercosis. Vet Parasitol. 2015;211:303–5.
16. Venco L, Manzocchi S, Genchi M, Kramer LH. Heat treatment and false-positive heartworm antigen testing in *ex vivo* parasites and dogs naturally infected by *Dirofilaria repens* and *Angiostrongylus vasorum*. Parasit Vectors. 2017;10:476.
17. Rinaldi L, Cortese L, Meomartino L, Pagano TB, Pepe P, Cringoli G, et al. *Angiostrongylus vasorum*: epidemiological, clinical and histopathological insights. BMC Vet Res. 2014;10:236.
18. Monica A, Matei IA, Amico GD, Bel LV, Dumitrache MO, Modrý D, et al. *Dirofilaria immitis* and *D. repens* show circadian co-periodicity in naturally co-infected dogs. Parasit Vectors. 2017;10:116.
19. Mircean M, Ionică AM, Mircean V, Györke A, Codea AR, Tăbăran FA, et al. Clinical and pathological effects of *Dirofilaria repens* and *Dirofilaria immitis* in a dog with a natural co-infection. Parasitol Int. 2017;66:331–4.
20. Sassi AJ, Geary JF, Leroux LP, Moorhead AR, Satti M, Mackenzie CD, et al. Identification of *Dirofilaria immitis* proteins recognized by antibodies from infected dogs. J Parasitol. 2014;100:364–7.
21. Solgi R, Sadjjadi SM, Mohebali M, Zarei Z, Golkar M, Raz A. Development of new recombinant DgK antigen for diagnosis of *Dirofilaria immitis* infections in dogs using ELISA technique and its comparison to molecular methods. Iran Biomed J. 2018;22:283–9.
22. Martin C, Gavotte L. The bacteria *Wolbachia* in filariae, a biological Russian dolls'system: new trends in antifilarial treatments. Parasite. 2010;17:79–89.
23. Turb ME, Zambon E, Zannoni A, Russo S, Gentilini F. Detection of *Wolbachia* DNA in blood for diagnosing filaria-associated syndromes in cats. J Clin Microbiol. 2012;50:2624–30.
24. Dingman P, Levy JK, Kramer LH, Johnson CM, Lappin MR, Greiner EC, et al. Association of *Wolbachia* with heartworm disease in cats and dogs. Vet Parasitol. 2010;170:50–60.
25. Landum M, Ferreira CC, Calado M, Alho AM, Maurício IL, Meireles JS, et al. Detection of *Wolbachia* in *Dirofilaria* infected dogs in Portugal. Vet Parasitol. 2014;204:407–10.
26. Rossi MID, Aguiar-Alves F, Santos S, Paiva J, Bendas A, Fernandes O, et al. Detection of *Wolbachia* DNA in blood from dogs infected with *Dirofilaria immitis*. Exp Parasitol. 2010;126:270–2.
27. Maia C, Altet L, Serrano L, Cristóvão JM, Tabar MD, Francino O, et al. Molecular detection of *Leishmania infantum*, filariae and *Wolbachia* spp. in dogs from southern Portugal. Parasit Vectors. 2016;9:170.
28. Hall T, Biosciences I, Carlsbad C. BioEdit: an important software for molecular biology. GERF Bull Biosci. 2011;2:60–1.
29. Owczarzy R, Tataurov AV, Wu Y, Manthey JA, McQuisten KA, Almabrazi HG, et al. IDT SciTools: a suite for analysis and design of nucleic acid oligomers. Nucleic Acids Res. 2008;36:163–9.
30. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinform. 2012;13:134.
31. Bik HM, Fournier D, Sung W, Bergeron RD, Thomas WK. Intra-genomic variation in the ribosomal repeats of nematodes. PLoS ONE. 2013;8:e78230.
32. Lagatie O, Merino M, Debrah LB, Debrah AY, Stuyver LJ. An isothermal DNA amplification method for detection of *Onchocerca volvulus* infection in skin biopsies. Parasit Vectors. 2016;9:624.
33. Ferri E, Barbuto M, Bain O, Galimberti A, Uni S, Guerrero R, et al. Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). Front Zool. 2009;6:1.
34. Yilmaz E, Fritzenwanker M, Pantchev N, Lendner M, Wongkamchai S, Otranto D, et al. The mitochondrial genomes of the zoonotic canine filarial parasites *Dirofilaria* (*Nochtiella*) *repens* and *Candidatus* Dirofilaria (Nochtiella) honkongensis provide evidence for presence of cryptic species. PLoS Negl Trop Dis. 2016;10:e0005028.
35. Willems A, Watteyne S, Mertens J, Gillis M, Vandekerckhove TTM, Swing JG. Phylogenetic analysis of the *16S* rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. Fems Microbiol Lett. 1999;180:279–86.
36. Bandi C, Anderson TJC, Genchi C, Blaxter ML. Phylogeny of *Wolbachia* in filarial nematodes. Proc R Soc B Biol Sci. 1998;265:2407–13.
37. Bio-Rad, L. Real-time PCR applications guide. Hercules: Bio-Rad Laboratories Inc; 2006. p. 41.
38. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10.
39. Beall MJ, Arguello-Marin A, Drexel J, Liu J, Chandrashekar R, Alleman AR. Validation of immune complex dissociation methods for use with heartworm antigen tests. Parasit Vectors. 2017;10:481.
40. Landis JR, Koch GG. An application of hierarchical kappa-type statistics in the assessment of majority agreement among multiple observers. Biometrics. 1977;33:363–74.
41. Chilton NB, Huby-Chilton F, Gasser RB. First complete large subunit ribosomal RNA sequence and secondary structure for a parasitic nematode: phylogenetic and diagnostic implications. Mol Cell Probes. 2003;17:33–9.
42. Rao RU, Atkinson LJ, Ramzy RM, Helmy H, Farid HA, Bockarie MJ, et al. A real-time PCR-based assay for detection of *Wuchereria bancrofti* DNA in blood and mosquitoes. Am J Trop Med Hyg. 2006;74:826–32.
43. Prosser SWJ, Velarde-Aguilar MG, León-Règagnon V, Hebert PDN. Advancing nematode barcoding: a primer cocktail for the cytochrome *c* oxidase subunit I gene from vertebrate parasitic nematodes. Mol Ecol Resour. 2013;13:1108–15.
44. Simoncini L, Casiraghi M, Bazzocchi C, Sacchi L, Bandi C, Genchi C. Real-time PCR for quantification of the bacterial endosymbionts (*Wolbachia*) of filarial nematodes. Parassitologia. 2001;43:173–8.
45. Fazzini F, Schöpf B, Blatzer M, Coassin S, Hicks AA, Kronenberg F, et al. Plasmid-normalized quantification of relative mitochondrial DNA copy number. Sci Rep. 2018;8:15347.
46. Kommedal Ø, Karlsen B, Sæbø Ø. Analysis of mixed sequencing chromatograms and its application in direct *16S* rRNA gene sequencing of polymicrobial samples. J Clin Microbiol. 2008;46:3766–71.
47. Irshad M, Gupta P, Mankotia DS, Ansari MA. Multiplex qPCR for serodetection and serotyping of hepatitis viruses: a brief review. World J Gastroenterol. 2016;22:4824–34.
48. Rishniw M, Barr SC, Simpson KW, Frongillo MF, Franz M, Dominguez Alpizar JL. Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet Parasitol. 2006;135:303–14.
49. Sabūnas V, Radzijevskaja J, Sakalauskas P, Petkevičius S, Karvelienė B, Žiliukienė J, et al. *Dirofilaria repens* in dogs and humans in Lithuania. Parasit Vectors. 2019;12:177.
50. Rao RU. Endosymbiotic *Wolbachia* of parasitic filarial nematodes as drug targets. Indian J Med Res. 2005;122:199–204.
51. Taylor MJ, Bandi C, Hoerauf A. *Wolbachia* bacterial endosymbionts of filarial nematodes. Adv Parasitol. 2005;60:245–84.
52. Taylor MJ, Hoerauf A. *Wolbachia* bacteria of filarial nematodes. Parasitol Today. 1999;15:437–42.
53. McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L. Heartworm disease in animals and humans. Adv Parasitol. 2008;66:193–285.
54. Nelson CT, McCall JW, Jones S, Moorhead A. Current canine guidelines for the prevention, diagnosis, and management of heartworm (*Dirofilaria immitis*) infection in dogs. American Heartworm Society; 2018. https://www.heartwormsociety.org/veterinary-resources/american-heartworm-society-guidelines. Accesed 25 Dec 2019.
55. Alho AM, Landum M, Ferreira C, Meireles J, Gonçalves L, de Carvalho LM, et al. Prevalence and seasonal variations of canine dirofilariosis in Portugal. Vet Parasitol. 2014;206:99–105.

56. Tabar MD, Altet L, Martínez V, Roura X, Veterinario H, Vicente S, et al. *Wolbachia*, filariae and *Leishmania* coinfection in dogs from a Mediterranean area. J Small Anim Pract. 2013;54:174–8.
57. Schnyder M, Deplazes P. Cross-reactions of sera from dogs infected with *Angiostrongylus vasorum* in commercially available *Dirofilaria immitis* test kits. Parasit Vectors. 2012;5:258.
58. Jefferies R, Morgan ER, Helm J, Robinson M, Shaw SE. Improved detection of canine *Angiostrongylus vasorum* infection using real-time PCR and indirect ELISA. Parasitol Res. 2011;109:1577–83.
59. Ferdushy T, Hasan MT. *Angiostrongylus vasorum*: the "French heartworm". Parasitol Res. 2010;107:765–71.

Publisher's Note

Publication N°3

Molecular Approach for the Diagnosis of Blood and Skin Canine Filariids

Younes Laidoudi[1,2,3], Samia Bedjaoui[4], Hacène Medkour[1,2,3], Stefania Latrofa[5], Idir Bitam[6,7], Bernard Davoust[1,2], Domenico Otranto[5] and Oleg Mediannikov[1,2*]

[1]Aix Marseille Univ, IRD, AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France
[2]IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France
[3] PADESCA Laboratory, Veterinary Science Institute, University Constantine-1, 25100 El Khroub, Algeria
[4]Laboratory of Food Hygiene and Quality Insurance System (HASAQ), Higher National veterinary school, Issad Abbes, Oued Smar, 16000 Algiers, Algeria
[5]Department of Veterinary Medicine, University of Bari, Valenzano, Italy
[6]Aix-Marseille University, IRD, AP-HM, SSA, VITROME, 13385 Marseille CEDEX 05, France
[7]Superior School of Food Sciences and Food Industries, Algiers 16004, Algeria

ARTICLE DE RECHERCHE

Soumis à : Microorganisms

microorganisms

MDPI

Article

Molecular Approach for the Diagnosis of Blood and Skin canine Filarioids

Younes Laidoudi1,2,3, Samia Bedjaoui4, Hacène Medkour1,2,3, Maria Stefania Latrofa5, Abdeslam Mekroud3, Idir Bitam6,7, Bernard Davoust1,2, Domenico Otranto5,8 and Oleg Mediannikov1,2*

[1] Aix Marseille Univ, IRD, AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France ; younes.laidoudi@yahoo.com (Y.L.) ; hacenevet1990@yahoo.fr (H.M.) ; bernard.davoust@gmail.com (B.D.) ; olegusss1@gmail.com (O.M.)
[2] IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France
[3] PADESCA Laboratory, Veterinary Science Institute, University Constantine-1, 25100 El Khroub, Algeria; mekroudabdeslam@gmail.com (A. M.)
[4] Laboratory of Food Hygiene and Quality Insurance System (HASAQ), Higher National veterinary school, Issad Abbes, Oued Smar, 16000 Algiers, Algeria; sbedjaoui@hotmail.com (S.B.)
[5] Department of Veterinary Medicine, University of Bari, Valenzano, Italy ; maria.latrofa@uniba.it (M.S.L.); domenico.otranto@uniba.it (D.O.)
[6] Aix-Marseille Univ, IRD, AP-HM, SSA, VITROME, 13385 Marseille, France; idirbitam@gmail.com (I.B.)
[7] Superior School of Food Sciences and Food Industries, Algiers 16004, Algeria
[8] Department of Pathobiology, Faculty of Veterinary Science, Bu-Ali Sina University, Felestin Sq, Hamedan, Iran
* Correspondence: Oleg Mediannikov olegusss1@gmail.com

Received: date; Accepted: date; Published: date

Abstract: The zoonotic *Onchocerca lupi* and tick-transmitted filarioids of the genus *Cercopithifilaria* remain less well known also due to the difficulties in accessing to skin samples as target tissues. Here, we proposed a molecular approach reliying on multiplex qPCR assays that allow the rapid identification of filarioids from canine blood, skin and tick samples. This includes two newly developed duplex qPCR tests, the first one targeting filarial and *C. grassii* DNA (**CanFil-*C. grassii***). and the second qPCR assay designed for the detection of *Cercopithifilaria bainae* and *Cercopithifilaria* sp. II DNAs (***C. bainae*-C.spII**). The third one is a triplex TaqMan *cox-1* assay targeting DNA of blood microfilariae (e.g. *D. immitis, D. repens* and *A. reconditum*). The novel duplex qPCRs developed were validated in *silico* and by screening of known DNA collection. The qPCR assays were also used for screening the blood and tick samples of 72 dogs from Algeria. This allowed the identification of canine filariasis infection with 100% of specificity and 89.47% and 100% of sensitivity from naturally infected blood and tick samples, respectively. The prevalences of 26.39% for *D. immitis* and 5.56% for both *D. repens* and *A. reconditum* were reported in blood and tick samples. *Cercopithifilaria* DNAs were detected only in tick samples, with a prevalence of 4.17% and 5.56% for *C. bainae* and *Cercopithifilaria* sp. II, respectively. Co-infections were diagnosed in 6.94% and 13.89% of blood and tick samples, respectively. Whereas no *C. grassii* DNA was detected in any sample. The use of engorged ticks instead of blood and skin samples could be an easier option for the surveillance of all canine filarioids herein investigated. The multiplex qPCR assays herein validated showed to be useful in the detection of filarial co-infections by overcoming sequencing of positive samples.

Keywords: Canine filarioses; *Dirofilaria immitis*; *Dirofilaria repens*; *Cercopithifilaria bainae*; *Cercopithifilaria grassii; Cercopithifilaria* sp. II ; *Onchocerca lupi*; Skin; Ticks; multiplex qPCR

1. Introduction

Canine filarioses are a group of diseases caused by arthropod-borne filarioids (Spirurida: Onchocercidae) belonging to the genera *Dirofilaria*, *Acanthocheilonema, Cercopithifilaria, Brugia*, and *Onchocerca* [1–3]. In addition to their veterinary importance, many of them are zoonotic. The adult filarioids live in different districts from the hearth (*Dirofilaria immitis*) to the ocular cavities (*Onchocerca lupi*) and, many of them, in the subcutaneous tissues (i.e., *Cercopithifilaria grassii, Cercopithifilaria* sp. I *and Cercopithifilaria* sp. II, *Dirofilaria repens* and *Acanthocheilonema reconditum*). Once mature, the viviparous nematodes produce blood or cutaneous microfilariae (mfs), which are available to an arthropod vectors for their cycle to complete to the infective third stage larvae [1]. The availability of the microfilariae in different animal tissues and anatomical regions is related to their detection [4] and, therefore, to their diagnosis. For example, microfilariae in the subcutaneous tissues such as *Cercopithifilaria* spp. and the zoonotic *O. lupi* are less diagnosed or completely non-diagnosed in comparison to those circulating in blood such as *D. immitis, D. repens, A. reconditum* and *Acanthocheilonema dracunculoides,* where they are routinely diagnosed by several assays such as morphological identification, molecular and serological tests [5]. Recently, great importance has been given to cutaneous filariases caused by *O. lupi* and *Cercopithifilaria* spp. [6–9]. These latter are transmitted by hard ticks of the family Ixodidae [10], whilst for the first one pathogen the vector is still unknown. *Onchocerca lupi* was firstly detected from a Caucasian wolf (Canis lupus) in Georgia [11] and subsequently diagnosed in domestic animals (i.e., dogs and cats) from European countries (i.e., Hungary, Greece, Germany and Portugal) and USA [12–19]. At present, the diagnosis of *Cercopithifilaria* spp. and *O. lupi* is based mainly on microscopic examination of dog cutaneous sediments. However, this method is quite invasive since it requires a skin biopsy, therefore representing a major limitation to this diagnosis technique in the clinical routine [20]. Molecular techniques have recently been standardized for the detection of *O. lupi* DNA [21].

After the first description of *Cercopithifilaria grassii* in 1907 by Noè in dogs in Italy, this filariasis remained mysterious until 1982, when larvae of a *Cercopithifilaria* spp. were observed in ixodid ticks in Switzerland [22], then in the brown tick of the dog *Rhipicephalus sanguineus* in northern Italy [23]. In 1984, Almeida and Vicente managed to identify another cutaneous canine filarial species, *Cercopithifilaria bainae*, these latter have been identified again in a Sicilian dog by Otranto et *al.* (2011) and named as *Cercopithifilaria* sp. I and subsequently redescribed as *Cercopithifilaria bainae* by Otranto *et al.* (2013) [10]. A third species of *Cercopithifilaria* sp. mfs have been identified as *Cercopithifilaria* sp. II as a formal description was not carried out since adult specimens have never been detected [10,24]. In 2012, Otranto et *al.* have realized a morphological and molecular characterization of *C. grassii* and *Cercopithifilaria* sp. II mfs from samples derived from European dogs. This extensive study has shown that dogs can be parasitized by three dermal species namely, *C. grassii*, *C. bainae* and *Cercopithifilaria* sp. II [7,25]. In 2014, Solinas *et al.* conducted a study whose objective was to determine the genetic constitution of *C. bainae* and *Cercopithifilaria* sp. II. [26].

In order to improve the molecular diagnosis of canine filariasis and to better understand the interactions of the filaroids among them, we propose in this study a novel multiplex qPCR approach. It consists primarily on a two duplex and one triplex TaqMan *cox*-1-based qPCR assays for the simultaneous detection and differentiation of *D. immitis, D. repens, A. reconditum, C. grassii, C. bainae* and *Cercopithifilaria* sp. II DNA. The approach was completed by PCR/sequencing assay to detect the other canine filarioids having blood and skin mfs. Secondly, the approach was standardized on ticks infesting dogs as a suitable sample to molecularly explore all etiological agents of canine filariasis.

2. Materiel and methods

2.1. Design protocol and specificity-Based Principles of the Duplex Real time qPCRs

The mitochondrial gene encoding for the cytochrome *c* oxidase subunit 1 (*cox* 1 gene) was targeted for its presence in several copies by cell and described as a "barcode gene" for filarial nematodes [27]. PCR design was performed according to the criteria for primers and probes

protocol [28]. Briefly, primers and probes of two duplex qPCR assays (Table 1) targeting filarial nematodes and *C. grassii* DNA (i.e., **CanFil-*C.grassii*)** and those of *C. bainae* and *Cercopithifilaria* sp. II (i.e., ***C. bainae*-C.spII**), were designed by alignment of sequences from representative members of *Onchocercidae* family available from GenBank database, using primer3 software v. 0.4.0 (http://primer3.ut.ee). Subsequently, all possible combinations of forward-reverse and probe-reverse of each qPCR system were checked within the DNA databases of metazoans (taxid:33208), vertebrates (taxid:7742), bacteria (taxid:2), *Canidae* (taxid:9608), *Felidae* (taxid:9682) and humans (taxid:9605) using primer-BLAST [29]. Primers and hydrolysis probes were synthetized by Eurogentec (Liège, Belgium) and Applied Biosystems™ respectively.

Table 1. Primers and probes designed for duplex qPCR assays accordingly to species examined.

Assay name	Sequences names	Sequences	Specificity
CanFil- *C. grassii*	C.Fil.354f	GATCGTAATTTTARTACYTCTTTTATGA	Filaria/*C. grassii*
	Can-fil.411p	6VIC- TATCAGCATTTGTTTTGGTTTTT-TAMRA	
	C.grassii .433p	6FAM- GGAAGGGTGGTAATCCTCTTCTTT-TAMRA	
	C.Fil.564r	CAGCAATCCAAATAGAAGCAAA	
***C. bainae*-C.spII**	T-Fil-62f	TTGTCTTTTTGGTTTACTTTTGTGG	*C. bainae/Cercopithifilaria* sp.II
	C.bai.121p	6FAM- AGGGGGTGCTGGTAGCAGG-TAMRA	
	C.spII.116p	6VIC- GTTGGTAGAGGCCCTGGGAGT-TAMRA	
	T-Fil-337r	GAAGTCAAATAAGAAGTRCAAACAAACA	

2.2. Run protocols

Both duplex qPCR reactions were carried out in a total volume of 20 µl. Reaction mixture containing 10 µl of Master Mix Roche (Eurogentec), 2µl of ultra-purified water DNAse-RNAse free, 0.5 µl of each primer (20 µM of concentration), 0.5 µl for both UDG and each probe (5 µM of concentration). Finally, 5µl of DNA template was added to the mixture. The TaqMan cycling protocol included two hold steps at 50°C for 2 minutes followed by 15 minutes at 95°C, and 39 cycles of two steps each (95°C for 30 seconds and 60°C for 30 seconds). These reactions were performed in a thermal cycler CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France).

2.3. Assays validation

2.3.1. Specificity validation

Duplex-qPCR assays were challenged against two DNA collection: (i) A total of 46 genomic DNA, consisting of 36 samples from adult or larval filarioids from 14 species (i.e., *Cercopithifilaria* sp. II, *C. grassii, C. bainae, Onchocerca lupi, Dirofilaria immitis, D. repens, Acanthocheilonema reconditum, Setaria digitata, Mansonella sp., M. perstans, Wuchereria bancrofiti, Loa loa, Brugia sp., B. malayi*), and 10 filarial free samples (5 skin samples and 5 ticks) and (ii) 57 DNA samples (Table S1) consist of non-filarial nematodes, arthropods, laboratory-maintained colonies as well as human, monkey, donkey, horse, cattle, mouse and dog DNAs.

2.3.2. Analytical Sensitivity and Efficiency Assessment

The analytical sensitivity of the newly developed qPCRs was assessed using a serial 10-fold dilutions of both single-species and spiked DNAs. The DNA of *O. lupi* and *C. grassii* as well as the spiked DNA from both species were used for the duplex qPCR **CanFil- *C.grassii*** while the DNA from *C. bainae* and *Cercopithifilaria* sp. II and the spiked DNA samples were used for the duplex qPCR ***C. bainae* - *C.*spII**. The sensitivity of each assay was assessed by generating the standard curves and by analyses of the derived parameters (i.e., efficiency, Slope, Y-intercept and correlation coefficient) within CFX Manager Software Version 3 (Bio-Rad, 2006).

132 2.3.3. Microfilariae Quantification protocol

133 The quantification protocol has been performed for the duplex qPCR filaria-*C. grassii* to
134 evaluate the detection limit in term of mfs concentration from biological samples. A serial 10-fold
135 dilutions of *D. immitis* DNA extracted from 200 µL of infected canine blood [31] containing 470 mfs
136 per ml (i. e. 94 mf/200µL of eluted DNA and 2.35 mfs/5µL of qPCR reaction) were tested. In
137 addition to the standard curves, the relative fluorescence units (RFUs) from the dye (VIC, Table 1)
138 were used to evaluate the qPCR efficiency in detecting the related mfs DNA as previously
139 described [5]. The cut-of value was determined with a tolerance coefficient of 5% according to the
140 formula described [30].

141 *2.4. Set Up of a Molecular Approach for the Diagnosis of Blood and Skin Filarioids*

142 2.4.1. Panel of biological samples

143 During an expedition to the Northern Algeria canine samples (blood and ectoparasites) have
144 been collected [32]. The study area was known to be endemic for ticks and tick-borne pathogens
145 such as [32] *Rickettsia massiliae, Rickettsia conorii and Ehrlichia canis* [33]. A total of 567 ticks were
146 collected from 72 (32%) dogs out of 227 animals sampled [32]. Ticks from each dog were kept in
147 tubes containing 70° of ethanol and were conducted to our laboratory for further analysis. One
148 engorged tick and blood samples of each infested dog (n=72) were selected and analyzed.
149 Genomic DNA was extracted individually from all tick body and blood samples. In order to
150 minimize PCR inhibitors from tick samples, we followed the extraction protocol described by
151 HALOS et al., 2004 [34]. Briefly, a bead-based physical disruption of the tick body within the
152 Tissue-Lyser apparatus (QIAGEN, Germany), and 24-hour of enzymatic digestion at 56° C using
153 buffer G2 supplemented with 25% of proteinase K were performed prior DNA extraction. While,
154 blood samples were subjected only to the enzymatic digestion. The extraction was performed
155 using the EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France), in line with the manufacturer's
156 instructions. DNA was eluted in a final volume of 200 µL and stored at -20 C until analysis.

157 2.4.2. Diagnosis Approach standardization on biological samples

158 To gain further insight into the diagnosis value of filarial infection from canine samples, we
159 assessed a multiplex qPCRs approach based on two duplexes qPCRs [CanFil-C.grassii and Cerco
160 spI-spII] herein developed and another multiplex qPCR system [Triplex TaqMan cox1] [35]. The
161 approach was proposed to explore the presence of filarial DNA followed by species-level
162 identification of *C. grassii, C. bainae, Cercopithifilaria* sp. II, *D. immitis, D. repens* and *A. reconditum*.
163 The amplification and sequence typing approach using filaria generic PCR primers and probes
164 [Pan-fil cox1 PCR] [35] targeting the partial (509 bp) *cox1* gene of filarial nematodes have been used.
165 To achieve the identification at the species level, PCR positive products were resolved in 0.5x
166 GelRed stained (Biotium, CA, USA) agarose gels (2%), then purified using NucleoFast® 96 PCR
167 DNA purification plate then run on the BigDye™ Terminator v3.1 Cycle Sequencing Kit [Applied
168 Biosystems, Foster City, CA]. The BigDye products were purified on the Sephadex G-50 Superfine
169 gel filtration resin prior the sequencing on the ABI Prism 3130XL. Finally, *cox* 1 nucleotide
170 sequences were edited using ChromasPro 2.0.0, aligned against the closely related species using
171 MAFFT [36]. Best fit model and maximum likelihood phylogeny were performed on MEGA 6 [37].
172 Phylogram was edited using iTOL v4 software [38].
173 Standardization of the assays was performed on two different panels of canine samples (i.e.
174 blood and tick samples). However, because the gold standard tests (dog necropsy, blood
175 concentration test and tick dissection) were absent, filarial true-positive dogs were considered if at
176 least one DNA sequence was obtained from at least one sample (i.e., tick or blood) of each dog.
177 However, samples that had yielded unreadable DNA sequences (overlapping peaks in the
178 electropherograms) from both tick and blood samples of the same dog were removed from the
179 analysis. Finally, Prevalence, correct classifcation, misclassifcation, sensitivity, specifcity, false

positive rate, false negative rate, positive and negative predictive value and Youden index were calculated for each approach [39–41].

3. Results

The *in-silico* and *in-vitro* validations revealed that, the newly designed duplex-qPCRs were specific for the target species. The duplex COI-based qPCR for *C. grassii* and canine filarioids amplified all filarial species (n=15) assessed from different biological sources discriminating *C. grassii* DNA (Table 2). The duplex COI-based qPCR for *Cercopithifilaria* spp. was able to detect and discriminate between *C. bainae* and *Cercopithifilaria* sp. II. No DNA amplification was obtained from both free-filarial tick and skin samples as well as from the panel of negative controls summarized in Table S1.

Table 2. *In-vitro* validation of the newly customized duplex qPCRs trough the DNA panel of filarioids and negative hosts (dogs and ticks).

Panels of tested DNA from filarioids			CanFil- *C.grasii*		*C. bainae* - *C.*spII	
Species name	**Specimens**	**Tested samples (n)**	**Filarial DNA**	***C. grassii***	***C. bainae***	***C. sp. II***
C. bainae	Adult worms	1	1	Negative	1	Negative
	Larva F1 "Microfilaria"	2	2	Negative	2	Negative
	Infected ticks	5	5	Negative	5	Negative
***Cercopithifilaria* sp. II**	Larva F1 "Microfilaria"	1	1	Negative	Negative	1
***C. bainae* + *C.* sp. II**	Mixed DNA	3	3	Negative	3	3
C. grassii	Larva F1 "Microfilaria"	1	1	1	Negative	Negative
C. grassii* + *C. bainae	Mixed DNA	3	3	3	3	Negative
***C. grassii* + *C.* sp. II**	Mixed DNA	2	2	2	Negative	2
O. lupi	Larva F1 "Microfilaria"	1	1	Negative	Negative	Negative
	Infected skin	6	6	Negative	Negative	Negative
D. immitis	Adult worms	2	2	Negative	Negative	Negative
D. repens	Adult worms	1	1	Negative	Negative	Negative
A. reconditum	Blood microfilaria	1	1	Negative	Negative	Negative
T. callipaeda	Adult worms	2	2	Negative	Negative	Negative
S. digitata	Adult worms	2	2	Negative	Negative	Negative
Mansonella sp.	Blood microfilaria	1	1	Negative	Negative	Negative
M. perstens	Blood microfilaria	1	1	Negative	Negative	Negative
W. bancrofiti	Blood microfilaria	2	2	Negative	Negative	Negative
Loa loa	Blood microfilaria	1	1	Negative	Negative	Negative
***Brugia* sp.**	Infected mosquitoes	3	3	Negative	Negative	Negative
B. malayi.	Infected mosquitoes	3	3	Negative	Negative	Negative
Filarial free samples	Ticks	5	Negative	Negative	Negative	Negative
	Dog skin	5	Negative	Negative	Negative	Negative

Analytical sensitivity and assay performance characteristics

The analytical sensitivity of each duplex qPCR was confirmed by qPCR efficiencies values ranging from 99.3% to 104.9%, with slope from -3.34 to -3.21, Y-intercept values from 40.541 to 45.792 with an $R^2 \geq 0.99$ for all qPCR reactions (Table 3, Figure S1 and S2).

Table 3. Analytical sensitivities and performance characteristics of the duplex qPCRs in detecting single species and pooled DNAs.

Assays	DNA target		Efficiency (%)	Coefficient of determination (R^2)	Slope	Y-intercept
CanFil-C.grasii	Single-species DNA	*Onchocerca lupi*	103.2	0.994	-3.247	40.541
		Cercopithifilaria grassii	99.3	0.999	-3.38	41.018
	Pooled DNAs	*O. lupi*	103.8	0.993	-3.235	43.74
		C. grassii	99.3	0.996	-3.34	45.107
C. bainae - C.sp.II	Single-species DNA	*Cercopithifilaria bainae*	100.3	0.996	-3.314	43.902
		Cercopithifilaria sp. II.	100.3	0.997	-3.315	45.792
	Pooled DNAs	*C. bainae*	99.5	0.995	-3.334	44.918
		Cercopithifilaria sp. II.	104.9	0.994	-3.21	43.694

Results of the detection limit of the duplex qPCR targeting canine filarioids and *C. grassii* are detailed in Table 4. The assay was able to detect up to 4.7 $\times 10^{-2}$ mfs/ml (i.e., corresponding to 2.35 x 10^{-4} mfs/5µl) with an efficiency of 99.8% and a slope of -3.327 and with a perfect adjustment (R^2=0.999) (Figure S3).

Table 4. Sensitivity and assay performance characteristics of the duplex COI-based system in detecting *D. immitis* microfilariae

SQ mfs/ml	SQ per qPCR reaction from mfs/5µl	Ct Mean	E-RFU	SCRS
$4.7 \times 10^{+2}$	2.35×10^{0}	24.26	1554	[E= 96.9%] [S=-3.398] [Y.int=33.2] [R^2=0.998]
$4.7 \times 10^{+1}$	2.35×10^{-1}	27.35	1363	
4.7×10^{0}	2.35×10^{-2}	31.03	1046	
4.7×10^{-1}	2.35×10^{-3}	34.02	596	
4.7×10^{-2}	2.35×10^{-4}	37.92	175	
4.7×10^{-3}	2.35×10^{-5}	N/A	N/A	
4.7×10^{-4}	2.35×10^{-6}	N/A	N/A	
4.7×10^{-5}	2.35×10^{-7}	N/A	N/A	
4.7×10^{-6}	2.35×10^{-8}	N/A	N/A	
4.7×10^{-7}	2.35×10^{-9}	N/A	N/A	
Cut Off Value		38.0	161	
Negative Control		N/A	6.09	

SQ: Starting Quantity, **mfs**: microfilaria, **Cq**: cycle quantification value; **N/A**: No amplification, **E-RFU**: End of relative fluorescence unit, **SCRS**: Standard Curve Results Spreadsheet, **E**: Efficiency, **S**: Slope, **Y.int:** Y-intercept.

The detailed results of molecular identification of filarial DNA from blood and tick samples are shown in Figure 1. Overall, the multiplex qPCR approach allowed the identification of 22 (30.55%) filarial-positive samples. In particular, 14 (19.44%), two (2.78%) and one (1.39%) blood samples were positive for *D. immitis*, *D. repens* and *A. reconditum*, respectively and five (6.94%) were coinfected, two (2.78%) with *D. immitis* and *D. repens* and three (4.17%) with *D. immitis* and *A. reconditum*. Any blood samples scored positive for *Cercopithifilaria* spp. Accordingly, all filarial species identified in the blood of dogs were also detected in their ticks. Whilst, *Cercopithifilaria* spp. DNA was found in six ticks, four (5.56%) *D. immitis* positive samples and one (1.39%) *D. immitis-D. repens* coinfected sample

were also positive for *C. bainae*. *Cercopithifilaria* sp. II was detected in 3 (4.14%) ticks, one among them was also positive for *D. immitis*.

Accordingly, all filaria-positive samples by the qPCR were also amplified by filaria generic PCR [Pan-fil cox1 PCR]. High quality DNA sequences were obtained from 17 (23.61%) blood samples, including 14 (19.44%) *D. immitis*, two (2.78%) *D. repens* and one (1.39%) *A. reconditum* and 14 (19.44%) tick samples, including nine (12.5%) *D. immitis*, two (2.78%) *D. repens*, one(1.39%) *A. reconditum* and two (2.78%) *Cercopithifilaria* sp. II. Whilst, five (6.94%) and 10 (13.89%) samples have yielded unreadable DNA sequences (overlapping peaks in the electropherograms) from blood and ticks respectively. Phylogenetic analysis confirmed the molecular identification of each filarial detected from blood and/or tick samples by clustering the representative sequences within the clades of the same reference species (Figure 2). DNA sequences generated during the present study were deposited in GenBank under the accession number xx.

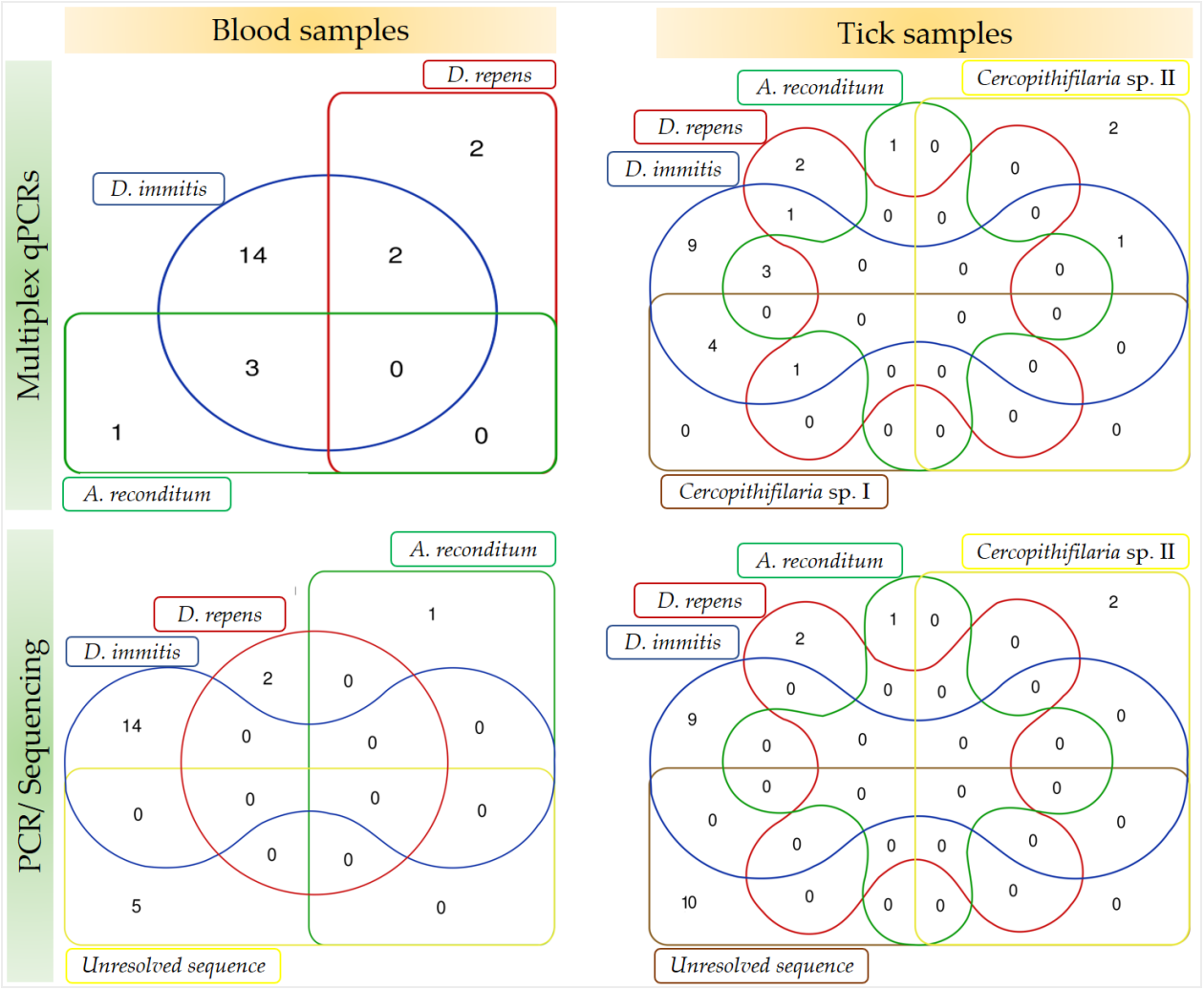


Figure 1. Venn diagrams depicting the distribution of positive samples detected by each molecular assay (i.e. multiplex qPCRs and PCR/sequencing) from canine blood and tick samples.

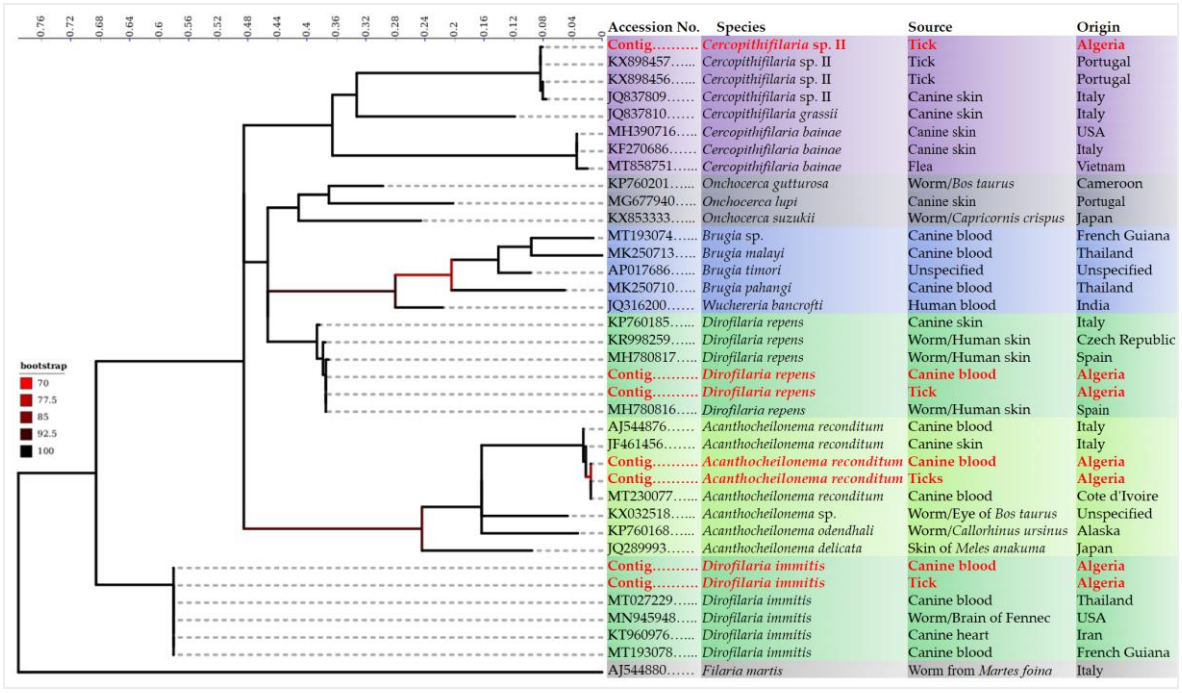


Figure 2. Phylogenetic tree showing the clusterization of filarial genotypes identified in the present study with the other filarioids. The tree was inferred using the Maximum Likelihood method based on 1000 bootstraps and the Tamura-Nei model. The analysis involved 36 partials (453 bp) *cox* 1 sequences of filarioids. Outgroup taxons *Filaria martis* (AJ544880) is drawn at root. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5779)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 49.5935% sites). Log likelihood was -2738.5083. The axis showed the global distance observed throughout the trees. The identity of each taxa is colour-coded according to the genus. Branches are colour-coded according to the bootstraps percent's.

Despite the successful species resolution of the multiplex approach from tick and blood samples of all filarial positive dogs (n=24), specificity and sensitivity analysis involved only 19 among them (Table S2). These latter were considered as filarial true positives dogs, from which at least one DNA sequence was obtained from their blood and / or tick samples (Table S2). A total of 48 dogs were negative for filarial DNA from both blood and tick samples. Five dogs were excluded from the analysis because they were coinfected and have yielded unreadable DNA sequences (overlapping peaks in the electropherograms) from their blood and tick samples.

Compared to the gold standard (Table 5), the multiplex qPCRs approach combining the identification of *D. immitis, D. repens, A. reconditum, C. grassii, C. bainae* and *Cercopithifilaria* sp. II allowed the diagnosis of canine filarioids with 100% of specificity in 89.47% and 100% of cases from their blood and ticks respectively (Youden index of 0.86 and 1, respectively). While the sequence typing approach allowed the diagnosis of canine filariasis with 100% of specificity in 89.47% and 73.68% of cases from their blood and ticks respectively (Youden index of 0.86 and 0.74, respectively).

Table 5. Performance characteristics of molecular assays in identifying filarial DNA from canine blood and ticks

Performances (in % unless specified)	Multiplex qPCRs approach		Sequence typing approach	
	Ticks	Blood	Ticks	Blood
True positive (n=19)	19	17	14	17
True negative (n=48)	48	48	48	48
False positive (n)	0	0	0	0
False negative (n)	0	2	5	2
Total	67	67	67	67
Sensitivity	100	89.47	73.68	89.47
Specificity	100	100	100	100
Predictive positive value (PPV)	100	100	100	100
Predictive negative value (PNV)	100	96	90.57	96
False positive rate	0	0	0	0
False negative rate	0	4	9.43	4
Correct classifcation	28.36	28.36	28.36	28.36
Prevalence	28.36	25.37	20.9	25.37
Youden index	1	0.86	0.74	0.86

4. Discussion

In this study we assessed a molecular approach relaying on multiplex qPCR assays that allow the rapid identification of filarioids from canine blood, skin and tick samples. Canine filarial agents such as the zoonotic *O. lupi* and tick-transmitted filarioids of the genus *Cercopithifilaria* speread in many areas of Europe, medetarnean bassin and several part of the world [42–44]. Furthermore, their life cycle, vectors as well as the parasite itself are less or completely unkown for some of them [3,7]. Depending on monitoring progress and vector surveillance, their detection contributes in avoiding the introduction and/or spread of these vector-borne helminths causing diseases [45]. Therefore, considering the zoonotic role for some of these parasites with an increasing of the public health implications, the diagnosis and or the monitoring assays must provide species-level identification to properly assist in decisions for medical and preventive treatments. The identification of filarial agent provides a better understanding on the distribution and prevalence of the disease. In addition to the poorly developed veterinary diagnostic services [45], diagnosing skin filarial agents remains labour and difficult because of the limited acces to skin samples and parasite material. As a consequence, the approaches for managing these health treathering parasites might be incomplete and need more deveopement.

To the best of our knowledge, molecular detection of the majority of canine filariasis of the genus *Cercopithifilaria* relies heavily on sequence typing method. This method is based on the use of filaria generic primers for DNA amplification and sequencing analysis [8,46], since filaria generic primers can theoretically amplify any filarial DNA [35]. However, as we demonstrated here and elsewhere [31,35,47], the sequence typing method may not allow species identification when the samples are coinfected with two or more specimens. Our findings showed that, the new duplex-qPCRs (CanFil-C. grassii and C. bainae-CspII) were specific to the target species without failure. A high analytical sensitivity was provided by each duplex qPCR in detecting single-species and/or pooled DNA with an efficiency ranged from 99.3% to 104.9% and a coefficient of determination (R^2) greater than 0.99. Furthermore, the duplex CanFil-C. grassii also explore the presence of filarial DNA, which assist in decision for further investigations and allowed rapidly information about the presence/absence of filarial DNA, an important step when the diagnosis approach relies on several species-specific assays. Although when used together, the novel duplex qPCRs and the triplex TaqMan *cox-1* assays allowed the identification of canine filariasis caused by *C. bainae*, *Cercopithifilaria* sp. II, *D. immitis*, *D. repens* and *A. reconditum* with 100% of specificity and 89.47% and 100% of sensitivity from naturally infected blood and tick samples, respectively. These

features were higher than those of the sequence typing approach, which consolidates the usefulness of multiplex qPCR in the detection of filarial co-infections and reinforces the previous studies [35,48].

Another limitation of either molecular or parasitological diagnosis of canine filariasis is the choice of samples, since both methods are often targeting mfs specimens. However, these larval stages have different locations in the host. Indeed some of them such as *D. immitis*, *D. repens* and *A. reconditum* are located in the bloodstream with a different density, while *Cercopithifilaria* spp. and other skin mfs are distributed unevenly in superficial dermal tissues [21,49]. The only two reports of the blood *C. bainae* DNA remain inconclusive and could just be an accidental detection of micro-fragments from dead microfilaria [47,50]. These features indicating that the exhaustive diagnosis of canine filariasis should rely on both blood and skin samples.

Data haerein presented demonstrated that, tick samples are more suitable for exploring both blood and skin microfilaria when the assay is able to discriminate at the species-level the coinfections. In addition to their role as vector for *Cercopithifilaria* spp. [26], tick are co-evolved with these filarioids and shown the same predilection sites on their hosts (dogs) [49], which indicates the close contact of *Cercopithifilaria* microfilaria and ticks within infected dogs. The recent study of Lineberry et al., (2020), reported that ticks infesting positive dogs for *C. bainae* are also positive for the DNA of this latter [46]. Furthermore, several studies reported the presence of filarial DNA from ticks infesting animals [51,52], indicating that ticks could be considered as equivalent to blood sample in detecting filarioids. The use of the hematophagous arthropod as an alternative blood sampling method was demonstrated for Triatomine bugs. This sampling method was advantages in obtaining blood samples without anaesthesia from animals where veins are inaccessible [53]. Though the absence of skin from sample panels herein tested, which may represent a limitation of the multiplex qPCR approach, the use of ticks infesting dogs provides an alternative to the complicated sampling methods requiring both blood and skin samples from the same dogs, thereby, exploring filarial DNA from engorged ticks offers the possibility to detect both skin and blood mfs and reduces the sampling and analyzing steps.

In this study, the prevalence of 33% and 29% of filarial infections had been observed in ticks and blood samples from Algerian dogs, respectively, which are almost identical to those previously observed in dog blood samples from India (26.5%) [54] and from Italy (23%) [55,56]. In addition to *D. immitis* already described, for the first time, we report the presence of *D. repens, A. reconditum, C. bainae* and *Cercopithifilaria* sp. II in Algeria. Here the prevalence observed for *D. immitis* was 23.61%, which is close to that reported from the same study area (Northern Algeria) by Ben-Mahdi and Madani (YEAR). In 2009, where 24.46% of dogs were *D. immitis*-antigens positive [57], but it was higher than that reported by Tahir et al. in 2015, who reported a prevalence of 1.4% for *D. immitis* in dog blood samples by molecular tests [58]. A very high prevalence of canine microfilaraemia of 42.68% was observed in Cherthala in the state of Kerala, a southern area of India [2]. In Northern Virginia, 0.74% *Amblyomma americanum* ticks carried filarial nematode DNA [59]. In Southern Connecticut, infection rate of *Acanthocheilonema* filarial nematode in *Ixodes* ticks is relatively high with 22% and 30% in nymph and adult Ixodes ticks, respectively [51]. The overall prevalence of *Cercopithifilaria* sp. in the sampled animal populations was 13.9% and 10.5% by microscopy of skin sediments and by PCR on skin samples, respectively. The higher prevalence rate of infested animals was recorded in Spain either by microscopical examination of skin sediments (21.6%) or by molecular detection on skin samples (45.5%) whereas the lower positivity rate was in Greece (4.3%). In Italy, according to the sites and to the diagnostic tests employed, the prevalence of *Cercopithifilaria* spp. infestation in dogs varied from 5.3% up to 19.5% [10]. Differences in reported prevalence levels among studies may due to diagnosis tool performances, the different tissues sampled, the number of animals tested, but also due to the geographical distribution of tick vectors transmitting pathogens.

5. Conclusion

The diagnosis approach combining species-specific multiplex qPCR assays allowed the identification of *D. immitis, D. repens, A. reconditum, C. grassii, C. bainae* and *Cercopithifilaria* sp. II despite the presence of coinfection. The use of ticks infesting dogs instead of blood and skin samples could be an easier way that contribute to disease progress monitoring and to the surveillance of canine filariasis. This would be particularly relevant, since most of them are pathogenic for dogs and constitutes an emergent zoonosis. Although the specific detection of *Cercopithifilaria* species may ultimately assist in the quest to identify the elusive adult *Cercopithifilaria* sp. II. We demonstrated how intense the challenge was for dogs in Algeria. We draw general attention to public health risks, since dogs are sentinels for human infections. An urgent need for the implementation of preventive strategies against canine vector-borne diseases in general and filariasis in particular. Finally, we encourage researchers to follow the molecular procedure summarized in Figure 3 to explore, diagnose, and monitoring canine filariasis from ticks infesting dogs unless combining blood and skin samples.

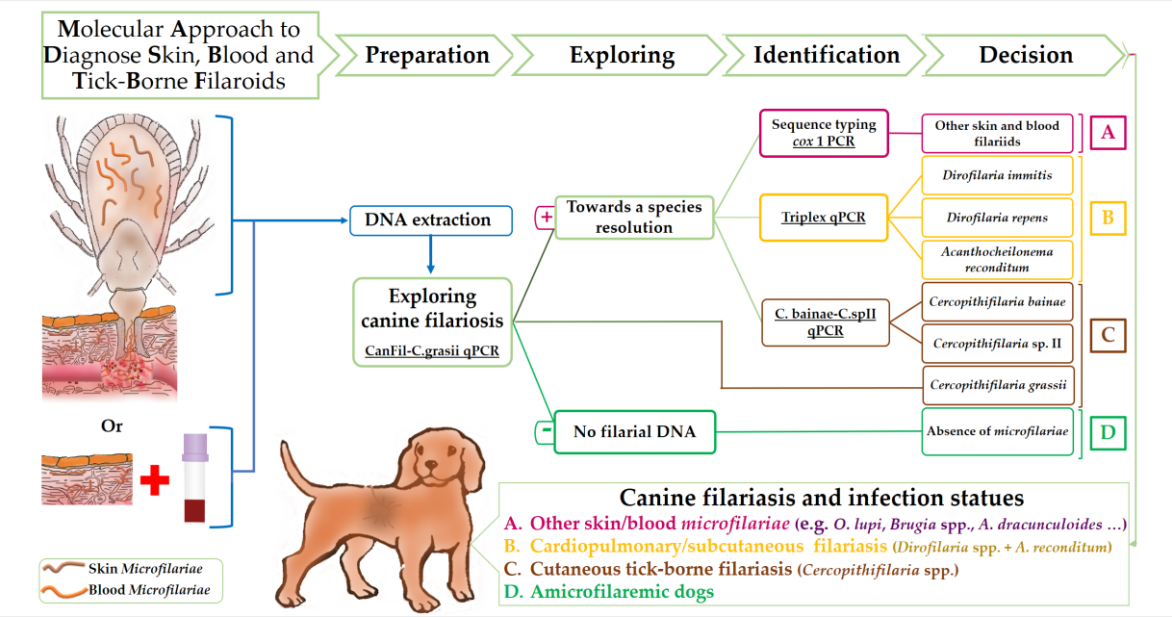


Figure 3. Flow Diagram showing the specificity-based principles of the proposed molecular approach in detecting canine filariasis from ticks infesting dogs

Supplementary Materials: Table S1: Negative control DNA used for specificity determination of designed qPCR systems; **Table S2**: Molecular identification of filarial DNA from positive samples (tick/blood). **Figure S1**: Analytical sensitivity of the CanFil- C.grassii assay in detecting (a) filarial DNA (i. e. *Onchocerca lupi*), (b) *Cercopithifilaria grassii* and (c) pooled DNA of *O. lupi* and *C. grassii*. **Figure S2**: Analytical sensitivity of the C. bainae - C.sp.II assay in detecting (a) *Cercopithifilaria* bainae, (b) *Cercopithifilaria* sp. II and (c) pooled DNA of *C. bainae* and *Cercopithifilaria* sp. II. **Figure S2**: Analytical sensitivity and detection limit of the CanFil-C.grassii assay using a serial 10-fold dilution of single-species-DNA of *Dirofilaria immitis microfilariae*.

Ethics approval and consent to participate: Dogs were examined by veterinarians with the assistance and acceptance of their owners. Ethical aspects related to dog sampling were treated in accordance with Algerian legislation guidelines. Risk assessment was submitted to and approved by the ethics committee and decision board of the veterinary practitioners from the wilayas of the North of Algeria. These institutions are affiliated with the Algerian Ministry of Agriculture and Rural Development (Directions des Services Vétérinaires). Protocol of the study was also approved by the scientific college (Procès-Verbal du CSI N°6, 2018) at the Veterinary Science Institute, University Constantine 1, Algeria. To facilitate field work, collaborations were established with veterinary doctors and their assistants working in these establishments.

Author Contributions: Y.L. and O.M. designed the study; Y.L. performed the lab work and data analysis; YL., HM., IB., and AM. field investigations; YL., S.B., HM., MSL., DO., and OM., writing—review and editing the article. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Institut Hospitalo-Universitaire Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: We thank veterinarians and dog owners for their valuable help with the field work.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Anderson, R.C. Nematode parasites of vertebrates: their development and transmission.; 2000; ISBN 9780851994215.
2. Ravindran, R.; Varghese, S.; Nair, S.N.; Balan, V.M.; Nair, A.S.; Malayil, A.; Chandrasekhar, L.; Juliet, S.; Kopparambil, D.; Ramachandran, R.; et al. Canine Filarial Infections in a Human Brugia malayi Endemic Area of India Canine Filarial Infections in a Human Brugia malayi Endemic Area of India. **2014**, doi:10.1155/2014/630160.
3. Latrofa, M.S.; Annoscia, G.; Colella, V.; Cavalera, M.A.; Maia, C.; Martin, C.; Šlapeta, J.; Otranto, D. A real-time PCR tool for the surveillance of zoonotic Onchocerca lupi in dogs, cats and potential vectors. *PLoS Negl. Trop. Dis.* **2018**, *12*, 1–11, doi:10.1371/journal.pntd.0006402.
4. Otranto, D.; Brianti, E.; Abramo, F.; Gaglio, G.; Napoli, E.; Latrofa, M.S.; Ramos, R.A.; Dantas-Torres, F.; Bain, O. Cutaneous distribution and localization of Cercopithifilaria sp. microfilariae in dogs. *Vet. Parasitol.* **2012**, doi:10.1016/j.vetpar.2012.05.016.
5. Younes Laidoudi, Bernard Davoust, Marie Varloud, El Hadji Amadou Niang, Florence Fenollar, O.M. Development of a multiplex qPCR-based approach for the diagnosis of Dirofilaria immitis, D. repens and Acanthocheilonema reconditum. *Parasit. Vectors* **2020**, *13*, doi:Parasites & Vectors.
6. Diseases, W.; Directorate, V.D.; Office, C.A. Onchocercosis : A newly recognized disease in dogs. **2008**, *151*, 1–13, doi:10.1016/j.vetpar.2007.09.008.
7. Brianti, E. Species diversity of dermal microfilariae of the genus Cercopithifilaria infesting dogs in the Mediterranean region. **2012**, doi:10.1017/S0031182012001357.
8. Cortes, H.C.E.; Cardoso, L.; Giannelli, A.; Latrofa, M.S.; Dantas-Torres, F.; Otranto, D. Diversity of Cercopithifilaria species in dogs from Portugal. *Parasit. Vectors* **2014**, *7*, 1–4.
9. Cantey, P.T.; Weeks, J.; Edwards, M.; Rao, S.; Ostovar, G.A.; Dehority, W.; Alzona, M.; Swoboda, S.; Christiaens, B.; Ballan, W. The emergence of zoonotic Onchocerca lupi infection in the United States–a case-series. *Clin. Infect. Dis.* **2016**, *62*, 778–783.
10. Otranto, D.; Brianti, E.; Latrofa, M.; Annoscia, G.; Weigl, S.; Lia, R.; Gaglio, G.; Napoli, E.; Giannetto, S.; Papadopoulos, E.; et al. On a Cercopithifilaria sp. transmitted by Rhipicephalus sanguineus: A neglected, but widespread filarioid of dogs. *Parasites and Vectors* **2012**, doi:10.1186/1756-3305-5-1.
11. Rodonaja, T.E. A new species of Nematode, Onchocerca lupi n. sp., from Canis lupus cubanensis.; 1967;
12. Otranto, D.; Giannelli, A.; Trumble, N.S.; Chavkin, M.; Kennard, G.; Latrofa, M.S.; Bowman, D.D.; Dantas-Torres, F.; Eberhard, M.L. Clinical case presentation and a review of the literature of canine onchocercosis by Onchocerca lupi in the United States. *Parasites and Vectors* **2015**, *8*, doi:10.1186/s13071-015-0699-3.
13. Hermosilla, C.; Hetzel, U.; Bausch, M.; Grübl, J.; Bauer, C. First autochthonous case of canine ocular onchocercosis in Germany. *Vet. Rec.* **2005**, *156*, doi:10.1136/vr.156.14.450.
14. Széll, Z.; Erdélyi, I.; Sréter, T.; Albert, M.; Varga, I. Canine ocular onchocercosis in Hungary. *Vet. Parasitol.* **2001**, *97*, doi:10.1016/s0304-4017(01)00397-1.
15. Labelle, A.L.; Daniels, J.B.; Dix, M.; Labelle, P. Onchocerca lupi causing ocular disease in two cats. *Vet. Ophthalmol.* **2011**, *14*, doi:10.1111/j.1463-5224.2011.00911.x.
16. Labelle, A.L.; Maddox, C.W.; Daniels, J.B.; Lanka, S.; Eggett, T.E.; Dubielzig, R.R.; Labelle, P. Canine ocular onchocercosis in the United States is associated with Onchocerca lupi. *Vet. Parasitol.* **2013**, *193*, doi:10.1016/j.vetpar.2012.12.002.
17. Maia, C.; Annoscia, G.; Latrofa, M.S.; Pereira, A.; Giannelli, A.; Pedroso, L.; Otranto, D. Onchocerca lupi nematode in cat, Portugal. *Emerg. Infect. Dis.* 2015, *21*.
18. Otranto, D.; Dantas-Torres, F.; Giannelli, A.; Latrofa, M.S.; Papadopoulos, E.; Cardoso, L.; Cortes, H. Zoonotic Onchocerca lupi infection in dogs, Greece and Portugal, 2011-2012. *Emerg. Infect. Dis.* **2013**, *19*, 2000–2003, doi:10.3201/eid1912.130264.
19. Miró, G.; Montoya, A.; Checa, R.; Gálvez, R.; Mínguez, J.J.; Marino, V.; Otranto, D. First detection of Onchocerca lupi infection in dogs in southern Spain. *Parasites and Vectors* **2016**, *9*, doi:10.1186/s13071-016-1587-1.
20. Otranto, D.; Dantas-Torres, F.; Giannelli, A.; Abramo, F.; Ignjatović Ćupina, A.; Petrić, D.; Cardoso, L.; Mutafchiev, Y.; Cortes, H. Cutaneous Distribution and Circadian Rhythm of Onchocerca lupi Microfilariae in Dogs. *PLoS Negl. Trop. Dis.* **2013**, doi:10.1371/journal.pntd.0002585.

21. Latrofa, M.S.; Annoscia, G.; Colella, V.; Cavalera, M.A.; Maia, C.; Martin, C.; Šlapeta, J.; Otranto, D. A real-time PCR tool for the surveillance of zoonotic Onchocerca lupi in dogs, cats and potential vectors. *PLoS Negl. Trop. Dis.* **2018**, doi:10.1371/journal.pntd.0006402.
22. Bain, O.; Aeschlimann, A.; Chatelanat, P. Présence, chez des tiques de la région de Genève, de larves infestantes qui pourraient se rapporter à la filaire de chien Dipetalonema grassii . *Ann. Parasitol. Hum. Comparée* **1982**, doi:10.1051/parasite/1982576643.
23. S Pampiglione, G Canestri Trotti, S.M.-P. Ritrovamento di Diptalonema grassii (Noè, 1907) in Rhipicephalus sanguineus su cane in Italia e descrizione di alcuni suoi stadi larvali. *Parassitologia* **1983**, *25*.
24. Otranto, D.; Varcasia, A.; Solinas, C.; Scala, A.; Brianti, E.; Dantas-Torres, F.; Annoscia, G.; Martin, C.; Mutafchiev, Y.; Bain, O. Redescription of Cercopithifilaria bainae Almeida & Vicente, 1984 (Spirurida, Onchocercidae) from a dog in Sardinia, Italy. *Parasites and Vectors* **2013**, doi:10.1186/1756-3305-6-132.
25. Otranto, D.; Latrofa, M.S.; Brianti, E.; Annoscia, G.; Parisi, A.; Dantas-Torres, F.; Bain, O.; Gasser, R.B. An assessment of genetic variability in the mitochondrial cytochrome c oxidase subunit 1 gene of Cercopithifilaria sp. (Spirurida, Onchocercidae) from dog and Rhipicephalus sanguineus populations. *Mol. Cell. Probes* **2012**, doi:10.1016/j.mcp.2011.12.002.
26. Solinas, C.; Varcasia, A.; Brianti, E.; Giannetto, S.; Pipia, A.P.; Columbano, N.; Tosciri, G.; Dantas-Torres, F.; Garippa, G.; Otranto, D.; et al. Cercopithifilaria spp. in dogs in Sardinia Island (Italy). *Parasitol. Res.* **2014**, doi:10.1007/s00436-013-3695-6.
27. Ferri, E.; Barbuto, M.; Bain, O.; Galimberti, A.; Uni, S.; Guerrero, R.; Ferté, H.; Bandi, C.; Martin, C.; Casiraghi, M. Integrated taxonomy: Traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Front. Zool.* **2009**, *6*, 1–12, doi:10.1186/1742-9994-6-1.
28. SantaLucia, J. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, doi:10.1073/pnas.95.4.1460.
29. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer- BLAST: A tool to design target- specific primers for polymerase chain reaction. **2012**, doi:10.1186/1471-2105-13-134.
30. QtvPcr, B. Real-Time PCR Applications Guide. *Methods* **2006**, 2–4.
31. Laidoudi, Y.; Ringot, D.; Watier-Grillot, S.; Davoust, B.; Mediannikov, O. A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France. *Parasite* **2019**, *26*, 72, doi:10.1051/parasite/2019073.
32. Medkour, H.; Laidoudi, Y.; Lafri, I.; Davoust, B.; Mekroud, A.; Bitam, I.; Mediannikov, O. Canine vector-borne protozoa: Molecular and serological investigation for Leishmania spp., Trypanosoma spp., Babesia spp., and Hepatozoon spp. in dogs from Northern Algeria. *Vet. Parasitol. Reg. Stud. Reports* **2020**, doi:10.1016/j.vprsr.2019.100353.
33. Bessas, A.; Leulmi, H.; Bitam, I.; Zaidi, S.; Ait-Oudhia, K.; Raoult, D.; Parola, P. Molecular evidence of vector-borne pathogens in dogs and cats and their ectoparasites in Algiers, Algeria. *Comp. Immunol. Microbiol. Infect. Dis.* **2016**, doi:10.1016/j.cimid.2016.01.002.
34. Halos, L.; Jamal, T.; Vial, L.; Maillard, R.; Suau, A.; Le Menach, A.; Boulouis, H.J.; Vayssier-Taussat, M. Determination of an efficient and reliable method for DNA extraction from ticks. *Vet. Res.* **2004**, *35*, doi:10.1051/vetres:2004038.
35. Laidoudi, Y.; Davoust, B.; Varloud, M.; Niang, E.H.A.; Fenollar, F.; Mediannikov, O. Development of a multiplex qPCR-based approach for the diagnosis of Dirofilaria immitis, D. repens and Acanthocheilonema reconditum. *Parasit. Vectors* **2020**, *13*, 319, doi:10.1186/s13071-020-04185-0.
36. Katoh, K.; Misawa, K.; Kuma, K.I.; Miyata, T. MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* **2002**, *30*, doi:10.1093/nar/gkf436.
37. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, doi:10.1093/molbev/mst197.
38. Letunic, I.; Bork, P. Interactive Tree of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Res.* **2019**, *47*, doi:10.1093/nar/gkz239.
39. GU Yule On the methods of measuring association between two attributes. *J. R. Stat. Soc.* **1912**, *75*, 579–652.
40. Bonett, D.G.; Price, R.M. Statistical inference for generalized Yule coefficients in 2× 2 contingency tables. *Sociol. Methods Res.* **2007**, *35*, 429–446.
41. Warrens, M.J. On association coefficients for 2× 2 tables and properties that do not depend on the marginal distributions. *Psychometrika* **2008**, *73*, 777.

42. Otranto, D.; Dantas-Torres, F.; Brianti, E.; Traversa, D.; Petrić, D.; Genchi, C.; Capelli, G. Vector-borne helminths of dogs and humans in Europe. *Parasites and Vectors* 2013.
43. Brianti, E.; Otranto, D.; Dantas-torres, F.; Brianti, E.; Traversa, D.; Petri, D. Europe Vector-borne helminths of dogs and humans in Europe. **2017**, doi:10.1186/1756-3305-6-16.
44. Dantas-Torres, F.; Otranto, D. Overview on Dirofilaria immitis in the Americas, with notes on other filarial worms infecting dogs. *Vet. Parasitol.* **2020**, *282*, doi:10.1016/j.vetpar.2020.109113.
45. Otranto, D.; Dantas-Torres, F.; Breitschwerdt, E.B. Managing canine vector-borne diseases of zoonotic concern: part one. *Trends Parasitol.* 2009.
46. Lineberry, M.W.; Sundstrom, K.D.; Little, S.E.; Stayton, E.M.; Allen, K.E. Detection of Cercopithifilaria bainae infection in shelter dogs and ticks in Oklahoma, USA. *Parasites and Vectors* **2020**, *13*, doi:10.1186/s13071-020-04089-z.
47. Laidoudi, Y.; Mari, J.; Tahir, D.; Watier-grillot, S. Detection of Canine Vector-Borne Filariasis and Their Wolbachia Endosymbionts in French Guiana. **2020**.
48. de Almeida, M.; Nascimento, F.S.; Mathison, B.A.; Bishop, H.; Bradbury, R.S.; Cama, V.A.; da Silva, A.J. Duplex Real-Time PCR Assay for Clinical Differentiation of Onchocerca lupi and Onchocerca volvulus. *Am. J. Trop. Med. Hyg.* **2020**, doi:10.4269/ajtmh.20-0113.
49. Otranto, D.; Brianti, E.; Abramo, F.; Gaglio, G.; Napoli, E.; Latrofa, M.S.; Ramos, R.A.; Dantas-Torres, F.; Bain, O. Cutaneous distribution and localization of Cercopithifilaria sp. microfilariae in dogs. *Vet. Parasitol.* **2012**, *190*, 143–50, doi:10.1016/j.vetpar.2012.05.016.
50. Rojas, A.; Rojas, D.; Montenegro, V.M.; Baneth, G.; Rica, C. Detection of Dirofilaria immitis and other arthropod-borne filarioids by an HRM real-time qPCR , blood-concentrating techniques and a serological assay in dogs from Costa Rica. **2015**, 1–10, doi:10.1186/s13071-015-0783-8.
51. Namrata, P.; Miller, J.M.; Shilpa, M.; Reddy, P.R.; Bandoski, C.; Rossi, M.J.; Sapi, E. Filarial nematode infection in Ixodes scapularis ticks collected from Southern Connecticut. *Vet. Sci.* **2014**, *1*, doi:10.3390/vetsci1010005.
52. Beaver, P.C.; Burgdorfer, W. A microfilaria of exceptional size from the ixodid tick, Ixodes dammini, from Shelter Island, New York. *J. Parasitol.* **1984**, *70*, doi:10.2307/3281647.
53. Voigt, C.C.; Peschel, U.; Wibbelt, G.; Frölich, K. An alternative, less invasive blood sample collection technique for serologic studies utilizing Triatomine bugs (Heteroptera; Insecta). *J. Wildl. Dis.* **2006**, *42*, doi:10.7589/0090-3558-42.2.466.
54. Azaziah, P.; Abd, M.; Irwin, P.J.; Gatne, M.; Coleman, G.T.; Mcinnes, L.M.; Traub, R.J. A survey of canine filarial diseases of veterinary and public health significance in India. **2010**, 11–13.
55. Magi, M.; Guardone, L.; Prati, M.C.; Tozzini, G.; Torracca, B.; Monni, G.; Macchioni, F. Canine filarial infections in Tuscany, central Italy. *J. Helminthol.* **2012**, *86*, 113.
56. Latrofa, M.S.; Dantas-Torres, F.; Giannelli, A.; Otranto, D. Molecular detection of tick-borne pathogens in Rhipicephalus sanguineus group ticks. *Ticks Tick. Borne. Dis.* **2014**, *5*, doi:10.1016/j.ttbdis.2014.07.014.
57. Meriem-Hind, B.-M.; Mohamed, M. Prevalence of canine Dirofilaria immitis infection in the city of Algiers, Algeria. *African J. Agric. Res.* **2009**, *4*, 1097–1100.
58. Tahir, D.; Damene, H.; Davoust, B.; Parola, P. First molecular detection of Dirofilaria immitis (Spirurida: Onchocercidae) infection in dogs from Northern Algeria. *Comp. Immunol. Microbiol. Infect. Dis.* **2017**, *51*, doi:10.1016/j.cimid.2017.04.001.
59. Henning, T.C.; Orr, J.M.; Smith, J.D.; Arias, J.R.; Rasgon, J.L.; Norris, D.E. Discovery of filarial nematode DNA in Amblyomma americanum in Northern Virginia. *Ticks Tick. Borne. Dis.* **2016**, *7*, doi:10.1016/j.ttbdis.2015.11.007.

Chapitre 2 :

Diagnostic et caractérisation des nématodoses vectorielles

2.2 : Caractérisation de nématodes à transmission vectorielle chez des primates non-humains

Préambule

Il est connu que les maladies parasitaires en général et les nématodoses en particulier sont des maladies fréquentes chez les primates non-humains (PNHs) (Strait et al. 2012), aussi bien, en captivité qu'en liberté. De nombreux genres de protozoaires et de métazoaires ont été décrits comme source de parasitisme chez les principaux groupes de PNHs. Bien que certains d'entre eux soient considérés comme non pathogènes (ou du moins leurs effets néfastes sur l'hôte n'ont pas encore été élucidés), un grand nombre peuvent entraîner chez l'hôte, des troubles physiologiques, une perte nutritionnelle, et même des lésions qui entraînent une débilitation grave, donnant souvent lieu à des complications de surinfections bactériennes, qui peuvent être mortelles. Les infestations les plus marquantes demeurent celles dues aux nématodes. Ces parasites affectent les PNHs de la bouche à l'anus, du cœur au poumon, de la peau aux viscères, de l'œil au cerveau, mettant parfois en péril la vie des sujets infestés. La diversité de localisation est, en fait, la résultante d'une grande variété des espèces impliquées. On dénombre, aujourd'hui, plus de 167 espèces de nématodes identifiés chez les PNHs, appartenant à 17 superfamilles, à savoir : Rhabditoidea, Ancylostomatoidea, Strongyloidea, Trichostrongyloidea, Metastrongyloidea, Oxyruridea, Ascaridoidea, Subuluroidea, Habronematoidea, Spiruroidea, Thelazioidea, Rictularioidea, Physalopteroidea, Dracunculoidea, Filarioidea, Trichuroidea (Strait et al. 2012). Il semble que ces parasitoses n'épargnent aucun groupe de primates, selon la littérature, au moins 26 espèces de nématodes ont été identifiés chez les prosimiens (*Prosimia* ou *Prosimii*), 39 espèces chez les singes du Nouveau Monde, 49 espèces chez les singes de l'Ancien Monde et environ 25 espèces chez les grands singes (Strait et al. 2012). Par conséquent, ces parasitoses qui sont souvent massives, constituent un risque menaçant la survie des PNHs qui pour certains sont en voie de disparition. On doit aussi considérer le risque zoonotique car les contacts directs et indirects avec l'homme sont possibles. A cet égard, leur implication, en tant que réservoir pour les

nématodes à transmission vectorielle, doit être mieux connue. Par exemple, toutes les filaires humaines ont été identifiées chez les PNHs des zones endémiques (Strait et al. 2012). Il est donc clair que l'éradication de ces parasitoses humaines sera difficile en l'absence de leur surveillance chez ces primates. On note, également, qu'en dépit de l'abondance de la littérature, les études détaillées sur la pathogénie de ces infestations restent rares, et les données manquent. De plus, les diagnostics sont limités par l'usage de techniques classiques, telle que la coprologie. L'identification a souvent été limitée au genre du parasite. En me basant sur l'approche inductive, j'ai tenté d'apporter des réponses à des questions se posant à la suite d'observations.

Ainsi, il a été rapporté, par des chercheurs de l'IHU, la présence de deux vers ayant l'aspect d'ascaris dans des fèces de chimpanzés du Sénégal. J'ai ensuite mené une recherche parasitologique. L'identification morphologique de ces deux spécimens a révélé leur appartenance à l'espèce zoonotique *Abbreviata caucasica*, une espèce de nématode gastrointestinal qui avait été oubliée, depuis longtemps. Cette espèce a, initialement, été identifiée chez l'homme Caucasien par Linstow en 1902, puis en Afrique chez l'homme et les primates (Fain et Vandepitte 1964). Nonobstant, l'espèce *Abbreviata caucasica,* rencontrée en Afrique, présentait les mêmes critères taxo-morphologiques que celle observée au Caucase, mais avec une taille presque deux fois plus grande. Disposant de ces parasites, j'ai alors cherché à mettre au point une technique moléculaire pour leur génotypage, afin de pouvoir améliorer les connaissances liées à la taxonomie, mais aussi au diagnostic et à la surveillance épidémiologique. Au départ, j'ai conçu quatre jeux d'amorces, ciblant les gènes *ITS2*, *12S*, *16S* et *cox1* des nématodes. Ensuite, à partir des séquences obtenues, j'ai conçu une qPCR ciblant spécifiquement le 12S d'*Abbreviata caucasica*. Elle est capable de détecter jusqu'à $1{,}13 \times 10^{-3}$ œufs/gramme de fèces. Il est également important de noter qu'environ 52% (n=48) des chimpanzés du Sénégal, suivis par l'IHU, sont infestés par ce nématode. Parallèlement,

nous avons émis une hypothèse pour identifier l'hôte intermédiaire de ce nématode, en ciblant les termites, qui font partie de l'alimentation des chimpanzés (**Publication N°4**).

L'exploration coprologique des fèces de chimpanzés, nous a révélé l'existence d'œufs de nématodes appartenant à une dizaine d'espèces. Ceci nous a conduit à élargir nos recherches, à la fois, en termes d'échantillonnage et d'agents pathogènes recherchés, en incluant, des métazoaires et des protozoaires. En plus des échantillons de fèces de chimpanzés du Sénégal, nous avons étudié un panel d'échantillons de fèces provenant de babouins du Sénégal (n=7) et de Djibouti (n=6) et de macaques d'Algérie (n=69). Par ailleurs, en République du Congo, afin d'explorer le rôle des PNHs en tant que réservoirs épidémiologiques pour les parasites zoonotiques, nous avons eu accès à des échantillons fécaux de 39 gorilles et à 38 prélèvements de selles des hommes (éco-gardes) vivant au voisinage de ces grands singes. Le niveau du parasitisme détecté est élevé, notamment, chez les chimpanzés avec 79,6%, suivi par 46,6% pour les gorilles et 31,6% chez les hommes. Environ 7% des macaques et des babouins ont été trouvés parasités. La caractérisation moléculaire de ces parasites révèle au moins 11 espèces différentes de nématodes chez les PNHs à savoir, des filaroides, *Mansonella* spp., *Mansonella perstens, Abbreviata caucasica, Necator americanus, Ascaris lumbricoides, Enterobius vermicularis, Strongyloides stercoralis, Oesophagostomum muntiacum*, et d'autres nématodes non identifiés. En outre, les gorilles et les hommes de la République du Congo partagent au moins six génotypes identiques de nématodes à savoir : des filaroides, *Necator americanus, Ascaris lumbricoides, Strongyloides stercoralis,* et d'autres nématodes non identifiés. A cela, s'ajoute la présence du même génotype de *Giardia lamblia* chez les deux espèces (**Publication N°5**).

En Amérique du Sud, dans le département français de la Guyane, nous avons eu accès à des échantillons de sang prélevés chez quelques alouates, ou singes-hurleurs, (*Alouatta*

macconnelli), primates chassés par la population autochtone amérindienne. Les prélèvements ont été effectués directement sur des animaux morts, puis adressés aux laboratoires de l'IHU, selon une procédure réglementaire. Notre objectif était de faire le diagnostic moléculaire des filarioses. En effet, chez l'homme, ces helminthoses sévissent en Amérique Latine. Dans cette région, le programme international de traitement de masse préventif (praziquantel, ivermectine et albendazole) a donné de très bons résultats, principalement, contre les mansonelloses humaines à *Mansonella ozzardi* et *M. perstens* et la filariose lymphatique à *Wuchereria bancrofti*. Bien que les PNHs de l'Ancien Monde soient réputés comme étant des hôtes susceptibles, aussi bien, expérimentalement que naturellement, pour *Wuchereria bancrofti,* les *Mansonella* spp., les *Brugia* spp. et les autres filarioses humaines, il n'existe cependant pas, à notre connaissance, d'étude rapportant des cas de filarioses humaines chez ces PNHs américains. Cependant, les études précédentes ont rapporté par identification morphologique, l'existence, d'au moins 14 espèces de *Mansonella* (appartenant toutes au sous-genre *Tetrapetalonema*) et 7 espèces du genre *Dipetalonema*, exclusifs de ces hôtes PNHs (Lefoulon et al. 2015). Notre étude a permis, par biologie moléculaire, de détecter la forte prévalence d'une espèce de *Mansonella* sp. sous-genre *Tetrapetalonema*, mais l'identification précise de l'espèce n'a pas été possible, en l'absence de séquences d'ADN dans la base de données GenBank et en l'absence de matériel biologique permettant l'identification morphologique de l'espèce. Parallèlement, j'ai pu identifier une espèce du genre *Brugia* et une espèce non identifiée d'Onchocercidae. A l'issue de cette étude, j'ai proposé deux PCRs spécifiques aux genres *Mansonella* et *Brugia* avec une qPCR pour les parasites du genre *Brugia*, afin de pouvoir étudier (diagnostic, surveillance épidémiologique, identification du vecteur, etc.) ce parasite qui est, potentiellement, zoonotique (**Publication N°6**).

Publication N°4

Zoonotic *Abbreviata caucasica* in Wild Chimpanzees (*Pan troglodytes verus*) from Senegal

Younes Laidoudi[1,2], Hacène Medkour[1,2], Maria Stefania Latrofa[3], Bernard Davoust[1,2], Georges Diatta[2,4,5], Cheikh Sokhna[2,4,5], Amanda Barciela[6], R. Adriana Hernandez-Aguilar[6,7], Didier Raoult[1,2], Domenico Otranto[3] and Oleg Mediannikov[1,2*]

[1] Aix Marseille Univ, IRD, AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France
[2] IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France
[3] Department of Veterinary Medicine, University of Bari, Valenzano, Italy
[4] Aix-Marseille University, IRD, SSA, APHM, VITROME, IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France
[5] VITROME, IRD 257, Campus International UCAD-IRD, Hann, Dakar, Senegal
[6] Jane Goodall Institute Spain and Senegal, Dindefelo Biological Station, Dindefelo, Kedougou, Senegal
[7] Department of Social Psychology and Quantitative Psychology, Faculty of Psychology, University of Barcelona, Spain

ARTICLE DE RECHERCHE

Publié dans : Pathogens, 2020, 9(7), 517

(doi.10.3390/pathogens9070517)

pathogens

MDPI

Article

Zoonotic *Abbreviata caucasica* in Wild Chimpanzees (*Pan troglodytes verus*) from Senegal

Younes Laidoudi [1,2], Hacène Medkour [1,2], Maria Stefania Latrofa [3], Bernard Davoust [1,2], Georges Diatta [2,4,5], Cheikh Sokhna [2,4,5], Amanda Barciela [6], R. Adriana Hernandez-Aguilar [6,7], Didier Raoult [1,2], Domenico Otranto [3] and Oleg Mediannikov [1,2,*]

1 IRD, AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, Aix Marseille Univ, 19-21, Bd Jean Moulin, 13005 Marseille, France; younes.laidoudi@yahoo.com (Y.L.); hacenevet1990@yahoo.fr (H.M.); bernard.davoust@gmail.com (B.D.); didier.raoult@gmail.com (D.R.)
2 IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France; georges.diatta@ird.fr (G.D.); cheikh.sokhna@ird.fr (C.S.)
3 Department of Veterinary Medicine, University of Bari, 70010 Valenzano, Italy; maria.latrofa@uniba.it (M.S.L.); domenico.otranto@uniba.it (D.O.)
4 IRD, SSA, APHM, VITROME, IHU Méditerranée Infection, Aix-Marseille University, 19-21, Bd Jean Moulin, 13005 Marseille, France
5 VITROME, IRD 257, Campus International UCAD-IRD, Hann, Dakar, Senegal
6 Jane Goodall Institute Spain and Senegal, Dindefelo Biological Station, Dindefelo, Kedougou, Senegal; amanda.b@janegoodall.es (A.B.); r.a.hernandez-aguilar@ibv.uio.no (R.A.H.-A.)
7 Department of Social Psychology and Quantitative Psychology, Faculty of Psychology, University of Barcelona, Passeig de la Vall d'Hebron 171, 08035 Barcelona, Spain
* Correspondence: olegusss1@gmail.com; Tel.: +33-041-373-2401

Received: 19 April 2020; Accepted: 23 June 2020; Published: 27 June 2020

Abstract: *Abbreviata caucasica* (syn. *Physaloptera mordens*) has been reported in human and various non-human primates including great apes. The identification of this nematode is seldom performed and relies on egg characterization at the coproscopy, in the absence of any molecular tool. Following the recovery of two adult females of *A. caucasica* from the feces of wild Senegalese chimpanzees, morphometric characteristics were reported and new data on the width of the esophagus (0.268–0.287 mm) and on the cuticle structure (0.70–0.122 mm) were provided. The molecular characterization of a set of mitochondrial (*cox*1, 16S rRNA, 12S rRNA) and nuclear (18S rRNA and ITS2) partial genes was performed. Our phylogenetic analysis indicates for the first time that *A. caucasica* is monophyletic with *Physaloptera* species. A novel molecular tool was developed for the routine diagnosis of *A. caucasica* and the surveillance of Nematoda infestations. An *A. caucasica*-specific qPCR targeting the 12S gene was assessed. The assay was able to detect up to 1.13×10^{-3} eggs/g of fecal matter irrespective of its consistency, with an efficiency of 101.8% and a perfect adjustment ($R^2 = 0.99$). The infection rate by *A. caucasica* in the chimpanzee fecal samples was 52.08%. Only 6.19% of the environmental samples were positive for nematode DNA and any for *A. caucasica*. Our findings indicate the need for further studies to clarify the epidemiology, circulation, life cycle, and possible pathological effects of this infestation using the molecular tool herein developed.

Keywords: *Abbreviata caucasica*; *Physaloptera mordens*; *Pan troglodytes verus*; wild chimpanzees; nematode; zoonosis; Senegal

1. Introduction

Physalopteriasis is caused by parasitic nematodes from the genus *Physaloptera* (Spirurida, Physalopteridae) [1], which has been distributed in Africa and the Middle East (i.e., Iran) since

prehistoric times [2,3]. Following the first formal description of the *Physaloptera* genus (Rudolphi in 1819), *Physaloptera abbreviata* (Rudolphi, 1819) was designated as the type species [4]. Afterward, *Abbreviata* was defined as a distinct genus, based on the number, mode, and origin of the uteri, which constitute the main keys for genus differentiation [4]. Adult stages of this genus are found in the stomach of a variety of animals such as reptiles and mammals including humans [5].

Abbreviata (=*Physaloptera*) *caucasica* (Linstow 1902), is a gastrointestinal nematode of Simiiformes (Anthropoidea) members [6]. After its discovery in a Caucasian man, Linstow (1902) provided an incomplete description with some erroneous morphological details [7]. In 1926, Schulz gave the complete description of *A. caucasica* after re-examining the original specimens, establishing a close relationship of this species with *Physaloptera mordens* (Leiper, 1908) isolated from humans in Central Africa. The unique difference identified among specimens was the presence/absence of a series of small teeth between and exterior to the large teeth on the inner face of the lip of *A. caucasica* and *P. mordens*, respectively [8]. A few months later, Ortlepp re-examined *P. mordens* and confirmed that the small teeth were missing in the previous examinations [9], therefore synonymizing *P. mordens* (Leiper, 1908) and *A. caucasica* (Linstow, 1902).

The *A. caucasica* infection has been reported in New and Old World monkeys [10], rhesus macaques (*Macaca mulatta*), baboons (*Papio* spp.), and great apes including both captive (*Pongo* spp.) and wild (*Pongo abelii*) [1,11]. It has also been reported in chimpanzees (*Pan troglodytes*) from Gombe (Tanzania) and Ngogo (Uganda) [12,13], although no adult specimens were examined. Furthermore, eggs of *Physaloptera* sp. have been reported in wild Senegalese chimpanzees [12,14]. Human and non-human primates probably constitute the natural host for *A. caucasica* [8]. The adult parasite looks like *Ascaris* sp. under the naked eye [15] and occurs in the digestive tract from the esophagus to the small intestine, where it can induce serious disease manifestations such as abdominal pain, anorexia, vomiting, and bloody diarrhea [2]. Records of the clinical signs in infected chimpanzees are lacking [6]. Nowadays, the detection of *A. caucasica* depends on the identification of eggs in the feces or in the detection of adult stages during post-mortem examination of the gastrointestinal tract of infested hosts [1]. The arthropod intermediate and/or paratenic hosts remain unknown though some experimental evidence indicates that *A. caucasica* could develop to the infective stage in *Blatella germanica* and in *Schistocerca gregaria* [5]. For other *Physaloptera* species, the intermediate arthropod host has been assessed such as *Tribolium confusum* [16], ground beetles, *Harpalus* sp. [17] as well as crickets, *Acheta assimilis*, and grasshoppers, *Melanoplus femurrubrum* [18,19].

Under natural conditions, *A. caucasica* may develop in arthropods and infestation probably occurs through ingestion of beetles, crickets, or other arthropods as well as paratenic hosts containing infective larvae [8,15]. However, the potential involvement of up to 28 paratenic and second intermediate hosts is suspected [6]. Anthelminthic drugs have been reported to be effective for the treatment of physalopteriasis in non-human primates [1]. However, their control should be reinforced by a molecular characterization to avoid the misleading conclusions about this parasite, sanitation and control of the potential paratenic or arthropod hosts as well as the surveillance of the infestation from the colon of non-human primates [1,16].

As part of the control of infectious and zoonotic diseases in the current chimpanzee population from the Dindefelo Community Natural Reserve in Senegal, we present here morphometric and phylogenetic findings to support the occurrence of *A. caucasica* in chimpanzees, providing a molecular characterization of a set of target mitochondrial (*cox*1, 16S rRNA, 12S rRNA) and nuclear (18S rRNA and ITS2) genes and morphological identification of adult specimens collected from feces of West African chimpanzees in Senegal. In addition, we developed a molecular test that could be used in a routine diagnostic laboratory instead of the labor-intensive coprological methods. The molecular test provides detection, egg quantification, and genetic characterization of *A. caucasica* from biological samples. We therefore applied this tool to a surveillance process and molecular xenomonitoring of *A. caucasica* from possible intermediate hosts and our current population of West African chimpanzees from Senegal.

2. Results

2.1. Morphological Characteristics of Adult A. caucasica

Comparative measurements of *A. caucasica* adult females from our study with *A. caucasica* (Linstow, 1902) and its synonymous species *P. mordens* (Lipper, 1908) [8] are detailed in Table 1. Two female complete specimens measuring 54.7 mm and 59.6 mm in length and 2.08 mm and 2.13 mm in width, respectively, were examined. The nematodes were characterized by the anterior end with a short buccal cavity (Figure 1(1a)) and by a cuticle reflecting over the lips to form a cephalic collarette (Figure 1(1b)) with two lateral pseudolabia undivided (Figure 1(1c,1d)). Nerve ring at 0.430 mm from the anterior end.

The esophagus consisting of two parts: muscular esophagus 0.75 mm long (Figure 1(2a)) and 0.287 mm wide, 0.79 mm long, and 0.268 mm wide, respectively. The esophagus total length was 5.52 mm and 4.82 mm, respectively in two samples.

In the mid-body, the cuticle was 0.70–0.122 mm thick and finely striated (Figure 1(2b)). The worms showed the presence of two small symmetrical pins in the front third of the body (Figure 1(2c)). Vulva 1.560 mm from anterior end (Figure 1(2d)). Presence of four uteri (Figure 1(3a)). Eggs were small 36–41 µm × 28–32 µm (Figure 1(3b)). Tail length was 1.084 mm (Figure 1(3c)). The caudal end showed the presence of a caudal appendix (Figure 1(3d)).

Table 1. Comparative measurement (in mm unless specified) of adult female of *A. caucasica* from our study with *A. caucasica* (Linstow, 1902) and its synonymous species *P. mordens* (Lipper, 1908) according to Fain and Vandepitte (1964) [7], Linstow (1902) and Schulz (1926) [8].

Measures		This Study		Measurement of Fain and Vandepitte (1964)		Measurement of Linstow, 1902	Measurement of Schulz (1926)			
		A. caucasica		*A. caucasica (Syn. P. mordens)*		*A. caucasica: Type Species*	*A. caucasica: Type Species*		*P. Mordens: Type Species*	
		Chimpanzee (Senegal)		Men (Congo)		Men (caucasia)	Men (caucasia)		Men (Uganda)	
Organ	**Segment**	**CHS 11**	**CHS 31**	**N1**	**N1**	**Adult Female**	**N1**	**N2**	**N1**	**N2**
Body	Length	54.7	59.6	108	117	27	24.75	23.84	41	100
	Width	2.08	2.13	2	3	1.14	1.18	1.12	1.8	2.8
	Index a	26.3	28	54	39	23.68	20.97	21.29	22.78	35.71
Nerve ring	From the anterior end	0.43	-	0.7	0.78	-	0.454	0.454	-	-
Esophagus (e)	Total length	5.52	4.82	9	11	-	3.5	3.72	-	-
	Length muscolar (e)	0.79	0.75	0.6	0.6	-	0.43	0.35	-	-
	Width	0.268	0.287			-	-	-	-	-
	Index b	9.91	12.37	12	10.63	-	7	6.4	6.2	6.2
Cuticle	Width	0.92–0.102	0.70–0.122	-	-	-	-	-	-	-
Vulva	From the anterior end	1.56	-	21	23	-	3.50	4.62	-	-
Eggs (µm)		37–41 × 28–32	36–39 × 28–31	60–65 × 45–55		57 × 39	57–62 × 42–45		45–49 × 32–34	
Tail	Tail	1.084	-	1.3	1.4	0.51	0.578	0.532	-	-
	Index c	50.46	-	80	83	53	43	45	70	90

CHS 11 and CHS31: Code sample of specimens from the present study. N1, N2: Code samples of specimens from the study of Schulz (1926). Index a, b, and c are the ratio of body length to body width, esophagus length and tail length, respectively.

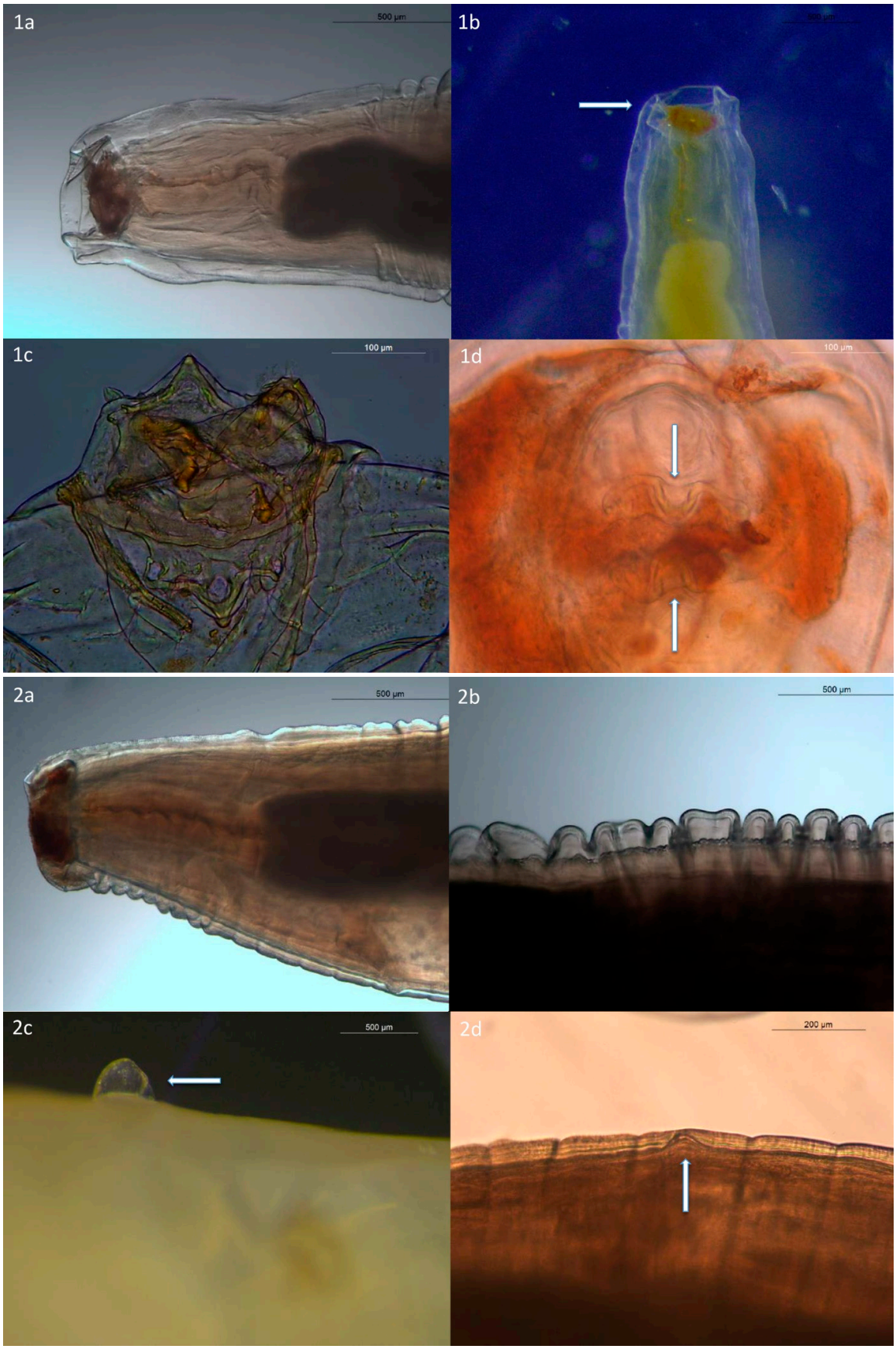


Figure 1. *Cont.*

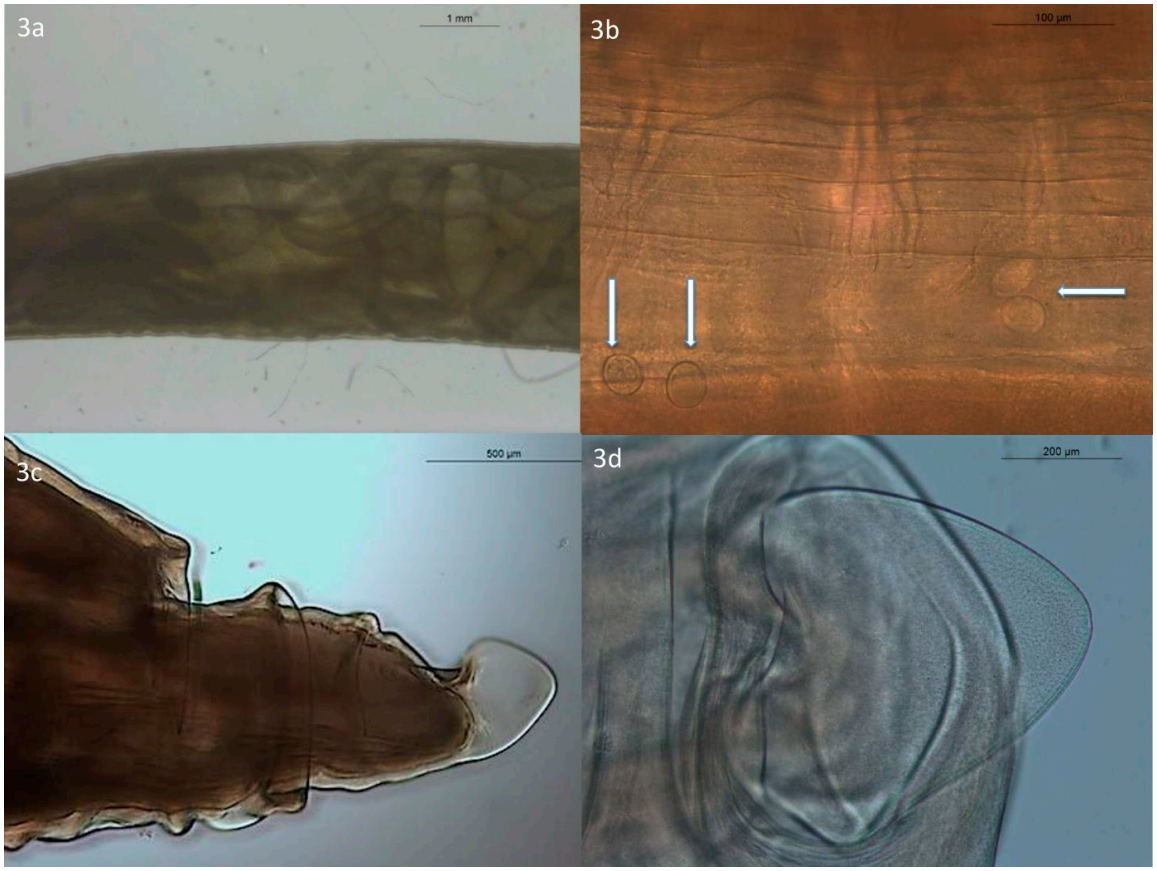


Figure 1. Light microscopy images of *A. caucasica* adult females. (**1a**) Buccal cavity. (**1b**) Cephalic collarette. (**1c**, **1d**) The two lateral pseudolabia. (**2a**) Esophagus. (**2b**) Thick finely striated from the mid-body. (**2c**) Pins. (**2d**) Vulva. (**3a**) Uteri. (**3b**) Eggs. (**3c**) Tail. (**3d**) Caudal appendix.

2.2. *A. caucasica* Eggs from Positive Feces

Eggs of *A. caucasica* were identified morphologically from two fecal samples taken from animals found infested with adult worms. The eggs were apparently identical to the micrograph of *A. caucasica* eggs reported elsewhere [7]. Eggs were embryonated and had a characteristic thick shell and hyaline coat (Figure 2).

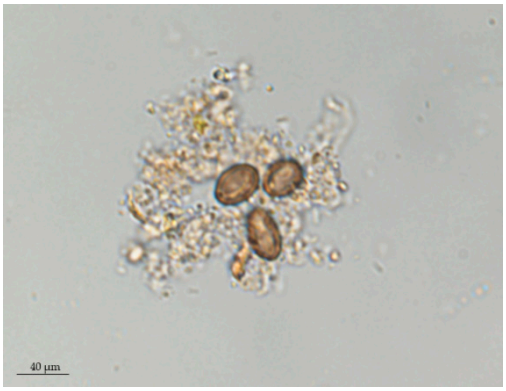


Figure 2. Coproscopy showing the *A. caucasica* eggs found in wild chimpanzee feces (formol-ether method, 100× magnification).

2.3. Molecular Characterization of Adult A. caucasica Worms

First, nearly full-length DNA sequences of the 18S rRNA (AN: MN956824, MN956825), ITS2 (AN: MN956809, MN956810), *cox*1 (AN: MT231294, MT231295), 16S rRNA (AN: MN956826, MN956827), and 12S rRNA (AN: MN956811, MN956812) genes were obtained from adult worms of *A. caucasica.* The sequences of each gene were identical to each other. The BLAST analysis of 1140 bp of the 18S rRNA gene showed the highest query cover (100%) with eight sequences of *Physaloptera* sp. A nucleotide identity of 97.9% (1118/1142) was observed with *Physaloptera apivori* (EU004817) isolated from birds in Germany, followed by 97.89% (1116/1140) for both *Physaloptera* sp. (HM067978) isolated from long-tailed macaques (*Macaca fascicularis*) in China, and *Physaloptera turgida* (DQ503459) isolated from North American opossums (*Didelphis virginiana*) in Louisiana, USA. Finally, an identity ranging from 97.46% to 97.81% was observed within the five other sequences of *Physaloptera* species isolated from reptiles and mammals (EF180069, MG808040, JF934734, AY702703, and EF180065).

In contrast, the BLAST analysis of the partial (759 bp) *cox*1 nucleotide sequence showed the lowest values of query cover and identity with those of *Physaloptera* spp. from the GenBank database, having a greater sequence coverage (i.e., 98 to 100%) with those of Onchocercidae members than that observed for *Physaloptera* species (i.e., 83 to 88%). Among *Physaloptera*, the highest nucleotide identity values observed were 83.7% (529/632) with *Physaloptera* sp. (MH752202) isolated from brown anoles (*Anolis sagrei*) in the USA, 83.5% (530/635) with both *P. turgida* (KT894808) and *Turgida* sp. (KC130680) isolated from opossums (*Didelphis* spp.) in Brazil and Mexico, respectively, and 83.2% (558/671) with *P. amazonica* (MK309356) isolated from Gardner's spiny rat (*Proechimys gardneri*) in Brazil, whilst lower identity values, ranging from 82.1% to 82.9%, were observed for the other five sequences of *Physaloptera* species (MH782844, KT894803, KT894804, KP981418, KT894805).

In contrast, the *Physaloptera cox*1 amino acid sequence appeared first among the top ten sequences of BLASTx [20]. *Abbreviata caucasica* COI sequence (protein id: QIP66136) showed an identity of 88.1% with *P. retusa* (AMX28288) isolated from golden tegu (*Tupinambis teguixin*) in Brazil, 87.7% with *P. mirandai* (AMX28289) isolated from brown four-eye opossums (*Metachirus nudicaudatus*) in Brazil with a coverage of 86% for both, 87.5% of identity and 98% of coverage with *P. rara* (QDF64304) isolated from dogs (*Canis lupus familiaris*), and 87.2% of identity and 86% of coverage with *Physaloptera* sp. (AMX28292), *P. bispiculata* (AMX28291), *P. amazonica* (QDX15779), and *P. hispida* (QCF40948).

BLASTn analyses of 16S rRNA (416 bp) and 12S rRNA (573 bp) sequences identified the first 60 sequences that corresponded to those of *Filarioidea* and *Thelazidae* without any *Physaloptera*. Nucleotide identity of about 75% with a query coverage of more than 99% were observed among these Spiriruds. Finally, the BLASTn analysis of the partial (675 bp) sequence of the ITS2 showed an identity of 93.37% (67/71) and a coverage of 10% with the unique GenBank sequence of *Physaloptera alata* (AY702694) isolated from birds.

The interspecific nucleotide pairwise (INP) distance of the 18S rRNA, *cox*1, 16S rRNA, 12S rRNA, and ITS2 of *A. caucasica* within *Physalopteridae* members are shown in Table S1. All sequences were well resolved in the chromatograms. The partial *cox*1 sequence was correctly aligned against the complete *cox*1 sequence (MH931178) of *P. rara* and no stop codon was observed in the translated amino-acid sequences, suggesting the absence of co-amplified numts. Furthermore, sequence alignment of COI with those of *Physaloptera* species showed nineteen amino-acid changes specific for *A. caucasica* (Figure S1). The interspecific nucleotide pairwise (INP) distance among the 645 bp of *cox*1 corroborated with the IaaP distance, among the corresponding 208 amino acid (Figure 3) and was substantially higher (ten times) between *A. caucasica* and *Physalopteridae* members in comparison with the 18S rRNA sequences.

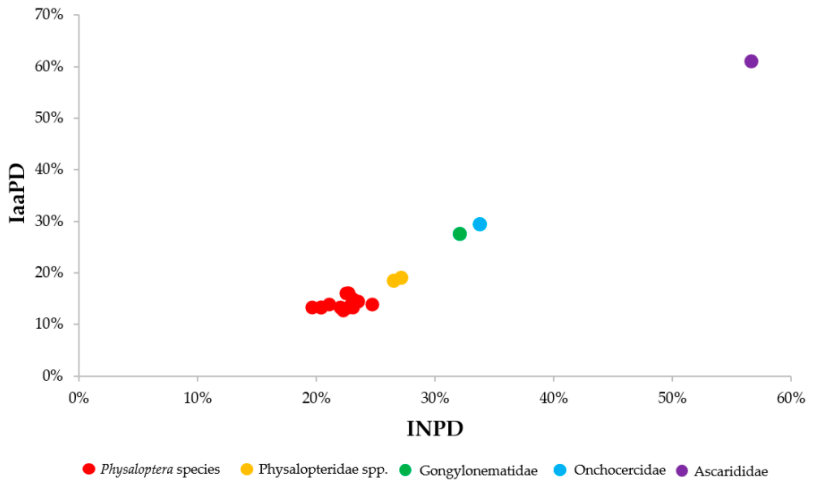


Figure 3. Scatter chart showing the interspecific pairwise distance between the COI sequence of *A. caucasica* and other nematodes based on both IaaPD and INPD. The INP distance was 0.31 (Std Err: 0.03) and 0.21 (Std Err: 0.06) between *A. caucasica* and *Heliconema longissimum* for THE 12S rRNA (GQ332423) and 16S rRNA (GQ332423) sequences, respectively. for the ITS2 sequences, the INP distance observed between *A. caucasica* and Filarioidea (XR 002251420, JQ316671, FM206482, DQ317666, DQ317657, and DQ317652), Spirocercidae (MH038181), Habronematidae (MH038181), and Gongylonematidae (LC026032, LC278392, and LC026029) members ranged from 0.51 (Std Err: 0.06) to 0.54 (Std Err: 0.06). No ITS2 sequences of Physalopteroidea superfamily were available.

The Bayesian trees inferred from *cox*1, nucleotide, and protein sequences, and from 18S rRNA genes are shown in Figure 4A,B and Figure 5, respectively. All phylograms provide evidence that *A. caucasica* is an integral part of the genus *Physaloptera*. In particular, on the *cox*1 tree, *A. caucasica* clustered with *Physaloptera* sp. (MH752202) and *P. retusa* (KT894803) isolated respectively from *Anolis sagrei* in the USA and *Tupinambis teguixin* in Brazil (Figure 4A). Similarly, on the COI tree, *A. caucasica* clustered with *P. rara* (QDF64304), *P. retusa* (AMX28288), *Physaloptera* spp. (QEQ27063, AYA23053), *P. turgida* (AMX28293), and *Turgida* sp. (AFZ99495) (Figure 4B), while on the 18S rRNA tree, *A. caucasica* clustered together with *Physaloptera apivori* (EU004817) and *Physaloptera alata* (AY702703) isolated from birds in Germany (Figure 5).

In addition, all *Physaloptera* and *A. caucasica* haplotypes shared a Euler circuit in the Templeton–Crandall–Sing (TCS) network tree for *cox*1 sequences. *Abbreviata caucasica* was connected by three-step branches to the Euler circuit, while all *Physaloptera* haplotypes were connected to the circuit by one to three-step branches (Figure 6). Hence, the TCS network analysis replicates the same results observed in the Bayesian inferences.

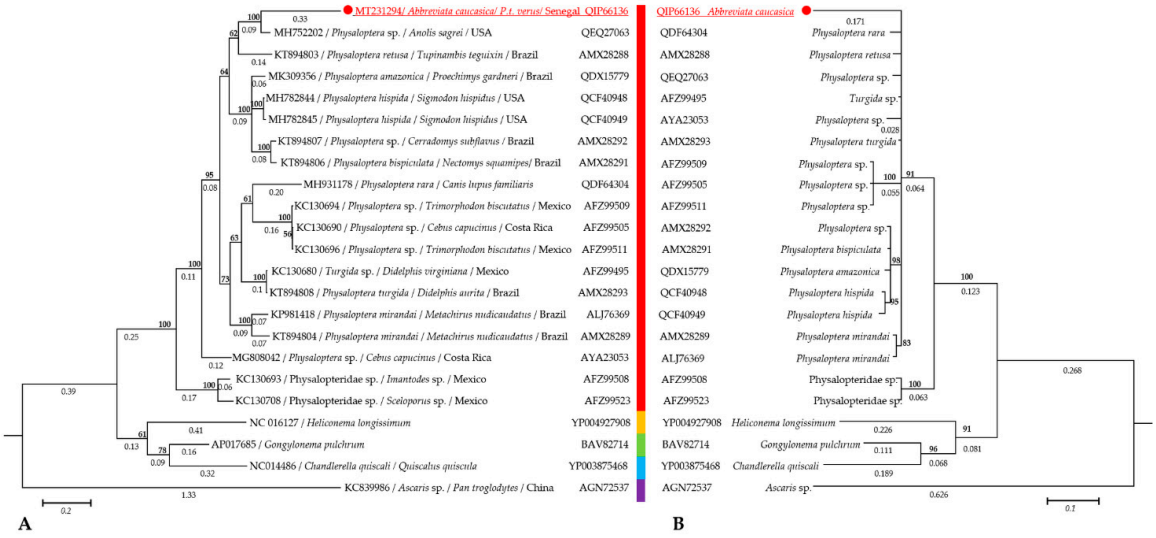


Figure 4. Phylogram generated from Bayesian inference. (**A**) Based on 651 bp from nucleotide sequences of the *cox*1 gene. (**B**) Based on 210 amino-acid from the COI-protein sequences. Numbers above and below branches are the display of nod statistics and branch length, respectively. Host, geographical location (when available), and GenBank accession numbers and protein-id are indicated. The identity of each taxa is color-coded according to the genus. Likelihood was −5448.2 and −1802.86 for nucleotide and protein inferences, respectively.

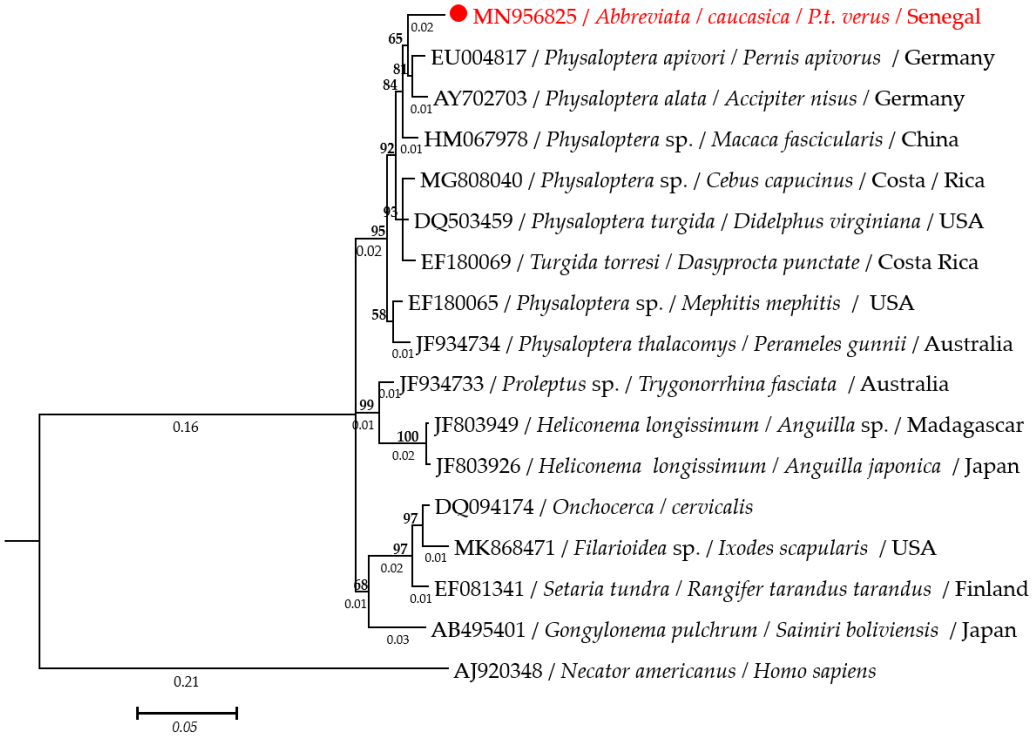


Figure 5. Phylogram generated from Bayesian inference, based on 1209 bp from 18S rRNA sequences. Numbers above and below branches are the display of nod statistics and branches length, respectively. Host, geographical location (when available), and GenBank accession numbers are indicated. Likelihood was −3466.3.

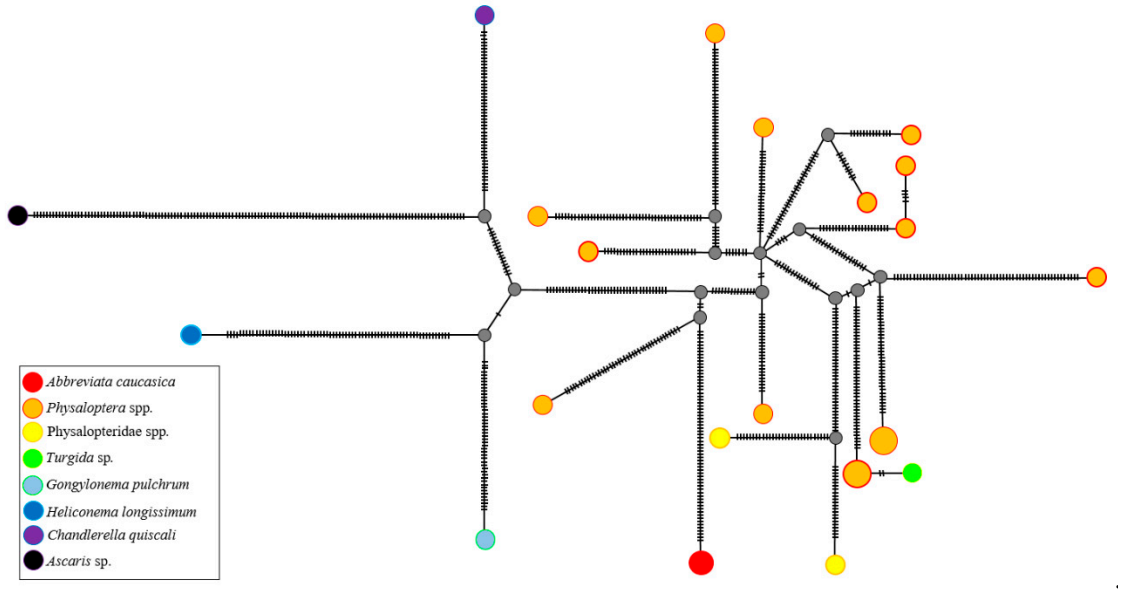


Figure 6. Templeton–Crandall–Sing (TCS) spanning network of the *cox*1 gene (651 bp) fragment. Colored and greyish circles correspond to a species genotype or hypothetical genotype, respectively. Model characteristics were: nucleotide diversity (pi = 1.282), number of segregating sites (361), number of parsimony-informative sites (242), Tajima's D statistic (D = 30.3099), and *p* ("D >= 30.3099" = 0).

2.4. Molecular Investigation of A. caucasica and Nematode Infestation from Biological Samples

The molecular tool developed in the present study was specific for the target DNA without any amplification from the negative controls.

Results of the molecular screening for *A. caucasica* and nematode DNA are detailed in Table 2. Among the 48 fecal samples tested, 52.08% (n = 25) were positive for *A. caucasica*, while all environmental samples tested negative. In addition to the samples that tested positive for *A. caucasica*, the pan-Nematoda qPCR assay allowed for the detection of 29.17% (n = 14) of other fecal samples and 6.2% (n = 7, three soil samples from termite mounds and four termite specimens) of positive environmental samples.

Table 2. *Abbreviata caucasica* and nematode infestations regarding origin and fecal consistency.

	Tested Samples	**Infestation Rate (%) by qPCRs**	
		A. caucasica	**Nematodes**
Localities			
Locality 1	3	0	33.3
Locality 2	6	66.7	100
Locality 3	39	53.8	82.1
Total	48	56.3	81.3
Comparison by localities	Fisher test (p)	0.148	0.052
Fecal consistency			
Fresh	38	40.0	70.0
Degraded	10	55.3	84.2
Fisher test (p)	//	0.49	0.30

Fisher's exact test showed that there were no significant effects of localities and fecal consistency on nematodes and *A. caucasica* prevalences (Table 2).

*Abbreviata caucasica cox*1 species-specific primers successfully amplified a partial sequence (504 bp) from 84% (21/25) of samples identified as positive by the qPCR targeting the 12S rRNA gene. There was no significant difference between both assays according to the McNemar test (p = 0.25). All sequences

were identical to each other and showed 100% similarity to those from adult specimens amplified with pan-Nematoda primers.

All sequences were deposited in the GenBank database under the following accession numbers: MT231296–MT231316.

2.5. The Analytical Sensitivity of A. caucasica 12S rRNA qPCR and Egg Counting

The performance characteristics of the 12S rRNA qPCR are shown in Table S2 and Figure S2. The assay was species specific and was able to detect up to 1.13×10^{-3} eggs/g of positive fecal samples (i.e., corresponding to 1.13×10^{-5} eggs/5 µL of DNA). The qPCR efficiency was 101.8% with −3.28 and 28.68 as a Slope and Y-intercept values, respectively, allowing a perfect adjustment ($R^2 = 0.99$).

Table 3 compares the *A. caucasica* eggs quantified by qPCR in terms of fecal consistency (fresh or degraded samples). Egg concentration in degraded feces ($n = 4$) was low (<1/g), but was higher (mean = 1.4 egg/g) in fresh feces ($n = 21$), while no effect of fecal consistency on egg concentration was observed (ANOVA, R2 = 0.032, Pr > F = 0.403).

Table 3. *Abbreviata caucasica* egg output from the positive samples.

Fecal Consistency	Number of Positive Samples	Quantification (Means Eggs/g) from Positive Samples	
Degraded	4	0.2	
Fresh	21 *	1.4	
Statistics	One-way ANOVA	R2	0.032
		Pr > F	0.403

*: one sample with an abnormal residual was removed before statistical analysis. Degraded: decomposing fecal samples.

3. Discussion

In this study, we report on the presence of *A. caucasica* (adults and eggs) in the feces of western chimpanzees from Senegal. Our data indicate that this population of chimpanzees is exposed to a high nematode infestation (81.3%), particularly *A. caucasica* (52.1%). This corroborates previous data from chimpanzees in southeastern Senegal, in which the reported nematode species-specific prevalence was between 0.78% to 31% where *Physaloptera* sp. was often the most prevalent species (13.26 to 31%) [12,14]. However, it was not specified whether these *Physaloptera* sp. were *A. caucasica* or author *Physaloptera* species. Perhaps the use of molecular assays, which were not applied in these studies, could offer a better species resolution.

The adult worms were designated as *A. caucasica* after careful identification based on the morphological and morphometric features, which was strengthened by previous descriptions by Schulz, (1926), Ortlepp, (1926) and Brede and Burger, (1977) [8,9,21]. In addition to the morphological and morphometric features previously listed, we reported the width of the esophagus (0.268–0.287 mm) and that of the cuticle (0.70–0.122 mm), which may help in the future identification of *A. caucasica*. Morphologically, *Abbreviata* species are closely related to each other [22]. In 1945, Morgan described the utility of uterine morphology (number and mode of origin of the uteri in the female worm) in the taxonomic classification. He classified species from the genus *Abbreviata* ($n = 27$) into more than three classes with two (didelphys), four (tetra-delphys), or more than four (polydelphys) branches. Of those, three were associated with monkeys: *A. caucasica* (Linstow, 1902), *A. poicilomeira* (Sandground, 1936), and *A. multipapillata* (Kreis, 1940) [4]. Based on the uterine morphology, *A. poicilomeira* and *A. multipapillata* are listed in class 5–15 G (5–15 uteri with common trunk), and 9–13 H (9–13 uteri without common trunk), respectively. However, *A. caucasica* can be easily differentiated by the fact that it is in class 4-D (4 uteri with common trunk).

The morphologic-based classification of Physalopteridae members (e.g., *Skrjabinoptera*, *Abbreviata*, and *Physaloptera*) exclude some morphometric measurements from the taxonomic characters such

as the length of the esophagus, vulva position, and egg dimensions. These features seem to variate in the same species and are used only in exceptional cases such as *P. squamatae* (Harwood 1932), *S. chamaeleontis* (Gedoelst 1916), and *S. simplicidens* (Ortlepp 1922) [23]. As expected, our data confirmed the variability of these parameters within the *A. caucasica* (Table 1). This reduced the utility of some commonly used indexes (a, b, and c) in nematode taxonomy [24].

In addition to the important taxonomic characters highlighted by Fain and Vandepitte (1964) (e.g., morphological features of the anterior end posterior ands, the number of uterine branches), the two adult females measured in the present study exhibited morphometric features of body and egg size close to those of *P. mordens* (Lipper, 1908), a species synonymous with *A. caucasica* (Linstow, 1902), where the body size of the female is 41–100 × 1.8–2.8 mm with a small egg of 45–49 × 32–34 µm [7]. Eggs were also similar to those reported by Poinar et al., 1972, where the size is 35–40 × 25–35 µm [5]. However, *A. caucasica* (Linstow, 1902) has been described as having a small body size of 24.75–23.84 × 1.12–1.18 mm and larger eggs of 57–62 × 42–45 µm. In contrast, the measurements from the study of Fain and Vandepitte (1964) showed that the *A. caucasica* (syn. *P. mordens*) adult females had a big body size of 108–117 mm and larger eggs of 60–65 × 45–55 µm (Table 1). Furthermore, the same authors confirmed and described the inconsistency of some measurements within this species [7]. Traditional taxonomic keys are known to be inconclusive for the taxonomic classification of nematodes [25] and should be confirmed by molecular barcoding, which circumvents the limitations of classical morphology-based classification [26]. The question then arises of whether *A. caucasica* (Linstow, 1902) is the same specie as *P. mordens* (Lipper, 1908), as indicated by Ortlepp (1926) and Fain and Vandepitte (1964) using morphologic-based taxonomy [7,9]. To address this question, a molecular comparative characterization of the specimens from the studies of Schulz (1926) and Fain and Vandepitte (1964) [7,8] should be performed to confirm or refute the synonymy of these two species.

In our study, we expanded the genetic data available for *A. caucasica* with sequences of mitochondrial and nuclear DNA (i.e., *cox*1, 12S rRNA, 16S rRNA, 18S rRNA, and ITS2 genes), though the genetic characterization was based on *cox*1 and 18S rRNA genes, due to the limited data on other gene sequences of *Physalopterida* members in the GenBank database.

The molecular analyses carried out in this study such as the phylogenetic comparisons of *cox*1 and 18S rRNA genes, the TCS network analysis of the *cox*1 gene, and the Bayesian inference of both *cox*1 and COI sequences confirmed that *A. caucasica* is monophyletic with *Physaloptera* species (Figures 4A,B and 5). *cox*1 and 18S rRNA genes are widely used as markers for the molecular barcoding of nematodes [27] with *cox*1 sequences of relevance in resolving taxonomic relationships among nematode species [27,28]. This gene is described by an interspecific nucleotide pairwise distance (INPD) of 16% to 27.8% between nematodes species [29].

The description of new species from the genus *Physaloptera* as well as the recording of new hosts has quickly evolved over the last decade [30–37]. However, there is a lack of additional data on the epidemiology, life cycle, clinical signs, and description of larval stages in intermediate hosts, which impedes progress in the understanding of these parasites. This is also related to the limited diagnostic and monitoring methods, which has for long time been exclusively based on the identification of eggs in feces [1].

Abbreviata caucasica appears to be capable of living attached to the wall of the digestive tract between the esophagus and the small intestine in human and non-human primates [1,33]. However, clinical features of *A. caucasica* infestation in chimpanzees remain unknown at this time and further studies are needed to identify such features [6].

We developed a specific 12S qPCR-based assay for the detection of *A. caucasica* from biological samples and potential intermediate hosts, though the unique *Abbreviata* species DNA and target sequence from *A. caucasica* used to confirm the assay specificity may represent a limitation of the assay. In contrast, the newly *cox*1 *A. caucasica* specific PCR could be used to assess the identification of *A. caucasica* from hosts exposed to a wide range of nematode infestations. Since the PCR replicated the same result as the qPCR ($p = 0.25$), both tools were highly sensitive and specific in detecting *A. caucasica*,

even the presence of coinfestations, avoiding the hard diagnosis based on egg identification. These tools can resolve problems related to the detection of larval stages from the intermediate and paratenic hosts and therefore avoid the sequencing identification by nematode generic primers. A detection limit as low as 1.13×10^{-3} eggs per gram of positive feces, regardless of consistency, solves the problems associated with conventional protocols requiring fresh equipment [38]. Data generated by qPCR showed a rate ranging from 0.2 to 1.4 eggs/g according to the fecal consistency, the best record being 113 egg/g. Appleton and Henzi (1993), reported the same results from baboons in Natal, South Africa, where egg output of *A. caucasica* ranged from 0.32 to 1.48 eggs/g with 215 eggs/g as the best record [39]. These observations highlight the usefulness of the qPCR quantification protocol we developed to evaluate the load of *A. caucasica* eggs. We therefore developed a 5S pan-Nematoda qPCR for the global exploration of nematode infestations from different biological samples.

The absence of *A. caucasica* DNA from all environmental samples could be explained by the fact that they were not contaminated by the feces of infested hosts. However, despite the absence of *A. caucasica* DNA in the termite (*Isoptera* spp.) specimens that we tested, we cannot be sure if they are involved in the life cycle of *A. caucasica* or not. Termites (*Isoptera*) are the intermediate host of several nematodes such as *A. antarctica*, achanthocephalans (*Thorny-headed worms*), and *Heterakis gallinarum* [40–42].

Poinar and Quentin, (1972) experimentally demonstrated the ability of *Blatella germanica* and *Schistocerca gregaria* to develop the infective stage of *A. caucasica.* However, the life cycle of this nematode remains largely unknown. More than 28 paratenic and second intermediate hosts are also suspected [6]. However, we cannot be sure whether the environmental samples from species included in the diet of the chimpanzee population in our study, screened here, are not implemented in the life cycle of *A. caucasica* even in the absence of its DNA from all specimens. Termites are known to be the most prevalent arthropod in the chimpanzee diet [43].

4. Materials and Methods

4.1. Study Site and Study Subjects

Samples were collected at the Dindefelo Community Natural Reserve, located in the Kedougou region, southeastern Senegal, about 35 km from the town of Kedougou. The vegetation of the reserve is a sudano-guinean savanna woodland [44], one of the driest and more open habitats occupied by the species [45]. All chimpanzees live in multi-female/multi-male communities composed of flexible groups that fission and fuse [46]. At the time of data collection, some individuals were semi-habituated to observers, but the rest remained unhabituated and thus the exact community composition and size were unknown. Although the total home range of Dindefelo chimpanzees was not known, conspecifics living in savanna woodland habitats have extremely large home ranges (e.g., >85 km^2, [47]). Based on size, the fecal samples analyzed in this study were assumed to belong to adults.

4.2. Fecal, Worms, and Environmental Samples

Two expeditions to the Dindefelo Community Natural Reserve in Senegal were undertaken in order to collect the samples. During the first trip (August 2016), 49 fecal samples of the western chimpanzee (*Pan troglodytes verus*) (Figure 7A) were collected from three localities in the reserve: Locality 1, three decomposing "degraded" fecal samples (12.382539, −12.287977); Locality 2, six degraded fecal samples (12.381437, −12.290776); and Locality 3, thirty-eight fresh fecal samples (12.379919, −12.296830). The fecal samples were collected and stored at −80 °C. Two adult worms were recovered from two fresh feces in the field and stored in 70% ethanol. In shape and general appearance, these worms resembling *Ascaris* to the naked eye (Figure 7B). All samples were transported to our laboratory at the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection for further examination, and the adult worms were sent to the parasitology laboratory of the Department of Veterinary Medicine (University of Bari, Italy), where they were subjected to morphological identification. During the

second expedition (August 2019), we targeted the potential contamination of these parasitic nematodes for the chimpanzees (e.g., the possible intermediate hosts that chimpanzees could eat or their water sources). A total of 113 environmental samples including the main species from the chimpanzee's diet were collected in the vicinity of chimpanzee sleeping sites and other areas frequented by the apes. These included 47 termites, 42 soil samples from termite mounds, 21 plant species, one sample from a water source used by the chimpanzees, a centipede, and one maggot. All samples were preserved in 70% ethanol and were transported to our laboratory for analysis.

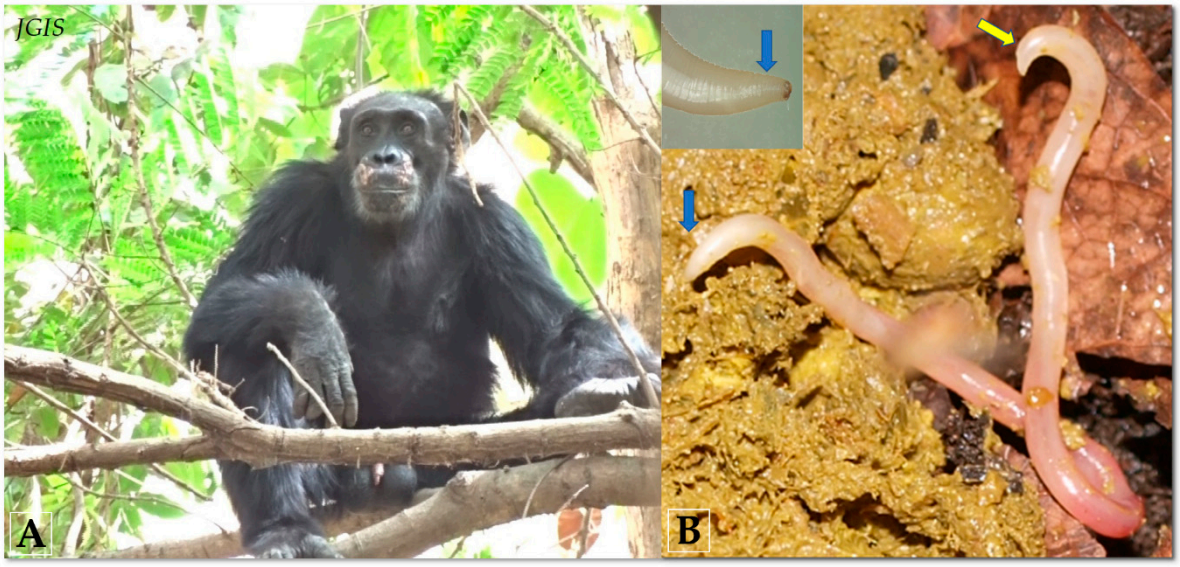


Figure 7. Adult of A. caucasica nematode found in the fecal matter of wild chimpanzee from the Dindefelo Community Natural Reserve, Senegal. (**A**) A chimpanzee (*Pan troglodytes verus*) in its natural habitat. (**B**) Adult female of *A. caucasica* looks like *Ascaris* with smooth and elastic body, strangled head, circular mouth (blue arrow), and incurved tail (yellow arrow).

4.3. Morphological Analysis of A. caucasica Adult Worms

The female worms were processed for morphometric analysis. The body of the nematodes were measured and then cut into three pieces. The central part was subjected to DNA extraction for molecular identification. The cephalic and caudal ends of the worms were fixed and stained in lactophenol solution to observe anatomical structures. Digital images and measurements were made with an optic microscope Leica® DM LB2 with differential interference contrast. The software Leica® LASAF 4.1 was used for the image analysis process including the measuring of nematodes, which are provided in micrometers. The identification was carried out following the description made by Schulz, (1926), Ortlepp, (1926) and Brede and Burger (1977) [8,9,21].

The observation of structures in the cephalic region, the stout size of the nematode, a thick cuticle finely striated, a large cephalic collarette, the total length, and the distance from the anterior end to the vulva all confirmed the identification of this helminth as *A. caucasica*.

4.4. Identification of A. caucasica Eggs from Positive Feces

The exploration of *A. caucasica* eggs was investigated from two fecal samples from which the adult worms were collected. A formol-ether sedimentation method of fecal concentration was used [48]. Egg identification was carried out according to the key of Fain and Vandepitte (1964) [7], while the differential diagnosis was performed as described elsewhere [49].

4.5. DNA Extraction

Genomic DNA was extracted from 200 mg of fecal samples, adult worms of *A. caucasica*, and environmental specimens using the QIAGEN DNA tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. The extraction was performed after two lysis steps: (i) mechanical lyses performed on a FastPrep-24™ 5G homogenizer using high speed stirring for 40 s in the presence of the powder glass, and (ii) enzymatic digestion using the proteinase K in the appropriate buffer (QIAGEN, Hilden, Germany) for 12 h at 56 °C. The extracted DNA was then eluted in a total volume of 100 µL and stored at −20 °C.

4.6. Molecular Characterization of Adult Worms

4.6.1. Development of PCR Primer Sets

The primer sets used in this study are listed in Table 4. First, sequences of the cytochrome *c* oxidase I (*cox*1), 16S rRNA, 12S rRNA, and the internal transcribed spacer 2 (ITS2) genes were used to design primer sets targeting nematodes. For each PCR system, a fasta file was constructed from nematode sequences retrieved from the GenBank database. Sequences were aligned using BioEdit v7.0.5.3 software [50]. The highly conserved areas were submitted in Primer3 software v. 0.4.0 [51]. PCRs standardization was performed as described elsewhere [52]. Primers designed are reported in Table 4.

Table 4. Primer sets used for the molecular characterization of *A. caucasica.*

Primer Name	Sequences 5′-3′	Target Gene	Size (bp)	Melting Tm	Elongation Time	Specificity	Ref.
Fwd-ITS-793	TCGATGAAGAACGCAGCTA	ITS2	420–750	57 °C	1′	Pan-Nematoda	This study
Rwd-ITS-1495	AGTTTCTTTTCCTCCGCTTAGTT						
Fwd-12S-Nem-1	AAGTTTGATTTTGGTTTTGGTTG	12S	680	58 °C	1′		
Rwd-12S-Nem-681	CCATTGACGGATGGTTTGTA						
Fwd-16S-Nem-488	GCAGCCTTAGCGTGATGG	16S	430	58 °C	1′		
Rwd-16S-Nem-918	TAAACCGCTCTGTCTCACGA						
dg.Fwd.COI.Nem.257	TTGGKGGTTTTGGWAATTGG	*Cox* 1	1069	52 °C	1′30″		
dg.Rwd.COI.Nem.1325	CCAGCAAAATGCAWAGGAAAA						
Fwd.18S.631	TCGTCATTGCTGCGGTTAAA	18S	1127–1155	54 °C	1′30″	Pan-Nematoda	[53]
Rwd.18S.1825	GGTTCAAGCCACTGCGATTAA						
Fwd.Abbrev.COI.51f	TGATCAGGGTTGGGAGCTT	*Cox* 1	550	53 °C	1′	*A. caucasica*	This study
Rwd.Abbrev.COI.601r	AAAAAGAACAATTAAAATTACGATCC						

In addition, primers Fwd.18S.631 and Rwd.18S.1825r, recently designed to amplify a partial fragment of the 18S rRNA gene of nematodes, were also used in this study (Table 4) [53]. These genes were chosen in order to compare the relatedness with *Physaloptera* and parasitic nematodes available in the GenBank database.

4.6.2. Polymerase Chain Reaction, Sequencing and Phylogenetic Analysis

All PCR reactions were carried out in a total volume of 50 µL, consisting of 25 µL of AmpliTaq Gold master mix (Thermo Fisher Scientific), 18 µL of ultra-purified water DNAse-RNAse free, 1 µL of each primer, and 5 µL of genomic DNA. PCR reactions with all systems were run using the following protocol: incubation step at 95 °C for 15 min, 40 cycles of 1 min at 95 °C, 30 s for the annealing at a different melting temperature for each PCR assay, and elongation for 45 s to 1 min and 30 s (Table 4) at 72 °C with a final extension for 5 min at 72 °C. PCR reactions were performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA).

The amplicons obtained from each gene examined were purified using the filter plate Millipore NucleoFast 96 PCR kit following the manufacturer's recommendations (Macherey Nagel, Düren, Germany). Purified DNAs were subjected to the second amplification using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Then, BigDye PCR products

were purified on the Sephadex G-50 Superfine gel filtration resin prior to sequencing on the ABI Prism 3130XL.

First, all nucleotide sequences were assembled and edited by ChromasPro 2.0.0. The absence of co-amplification of nuclear mitochondrial genes (numts) was verified for the *cox*1 DNA sequences, wherein the alignment was performed with the complete sequence of *cox*1 DNA from the *P. rara* mitochondrion sequence (MH931178) using the ClustalW application within Bioedit v.7.2.5. [50]. In addition, the visual verification of sequence chromatograms ambiguities, indels and stop codons of the translated sequences was performed using Chromas Pro 2.0.0 software as recommended [54]. Sequences amplified from the *cox*1, 16S, 12S, 18S rRNA, and ITS2 genes were subjected separately to a preliminary analysis using the Basic Local Alignment Search Tool (BLAST) [55].

The closely related sequences of *Physaloptera* and nematode species retrieved from the GenBank database were included in the study and a fasta file was constructed for each gene and then subjected to the alignment. In addition, alignment of nematode COI protein sequences was also performed. All alignments were conducted using the ClustalW application within Bioedit v.7.2.5. [50]. The conservation of amino acids between the COI sequences of *A. caucasica* relative to the sequences of *Physaloptera* was visualized on CLC Sequence Viewer 7 (CLC Bio Qiagen, Aarhus, Denmark).

From the alignment of each gene examined, the interspecific nucleotide pairwise distance (INPD) was evaluated to estimate the genetic divergence between all species included. Furthermore, the interspecific amino acid pairwise distance (IaaPD) was reproduced for COI-protein sequence alignment. standard errors were obtained by a bootstrap procedure with 1000 replicates using the maximum composite likelihood model [56] and Poisson correction model [57] for nucleotide and protein sequence alignments, respectively. Evolutionary analyses were inferred on MEGA6 software [58].

DNA sequences of *Necator americanus* (AJ920348) and *Ascaris* sp. (KC839986) were chosen as out-groups for18S rRNA and *cox*1, respectively, according to the fast-minimum evolution tree on BLAST [55]. The corresponding COI protein sequence of *Ascaris* sp. (AGN72537) was maintained as an out-group for the COI-protein alignment. The best model parameters with the lowest score were selected to generate phylogenetic trees of aligned 18S and *cox*1 sequences as well as COI protein sequences by running the MrBayes algorithm on each model using Topaliv2.5 software [59]. The Bayesian phylogenetic tree [60] was inferred for nucleotide sequence alignments using the K80 (+G) [61] and GTR (+G, +I) [62] models, respectively, while the Bayesian phylogenetic tree was inferred on the COI protein sequence alignment using Mtmam (+G) [63]. All phylograms were generated with five runs for 1,100,000 generations, 25% of burn-in length, and 1000 for sample frequencies.

In order to resolve the haplotype variations of *Physaloptera* species and *A. caucasica*, the Fasta file of the *cox*1 sequences was converted to the Nexus format using an online converter [64]. During the second time, a Templeton–Crandall–Sing (TCS) network phylogram [65] was inferred with a 95% connection limit and drawn with 1000 iterations using the PopArt software [66].

4.7. TaqMan qPCR for Nematoda Parasites Detection

The 5S rRNA gene was selected for the development of a TaqMan qPCR as an exploratory tool targeting nematode parasites. This choice was based on its conservation among nematodes species [67] and its tandem repetition of 110 times, which improves the assay sensitivity [68]. The partial sequences (XR002251414, JX489168, HM641830, M27961, JX117890, LS997562, U32120, AP018154, LK928622, X16226) representing nematode members from *Spirurina* and *Rhabditida* clades were aligned against the 5S gene of some plathelminth worms (*Schistosoma mansoni*: XR001974633, *Spirometra erinaceieuropaei*: LN313518, *Hymenolepis microstoma*: LR215994) and vertebrate hosts (human: AC275639, dog: XR003137316, chimpanzee: XR002941379, horse: XR002802613). Primers: qNem.5S.1f 5′-ACCACGTTGAAAGCACGMC-3′; qNem.5S.110r 5′-TGTCTACAACACCTSGRATTCC-3′; Eurogentec (Liège, Belgium), and a TaqMan probe qNem.5S.38p 6-FAM-5′-AGTTAAGCAACGTTGGGCC-3′-TAMRA; Applied Biosystems™, were chosen from the highly conserved region specific for nematodes.

4.8. Quantitative TaqMan Real-Time PCR (qPCR) for A. caucasica Detection

Sequences of the 12S rRNA gene amplified from the adult worms of *A. caucasica* were aligned with the closely related sequences of nematodes available in the GenBank using Bioedit v.7.2.5. [50]. The specific regions for 12S rRNA of *A. caucasica* were submitted in Primer3 software v. 0.4.0 [51] in order to design the following primers Phy.12S.f.204:5′-GAATTGGATTAGTACCCAAGTAAGTG-3′/Phy.12S.r.305: 5′-TGTTCCAAAAATCTTTCTAAGATCAG-3′ (Eurogentec, Liège, Belgium) and TaqMan probe: Phy.12S.242p. 6VIC-GCGGGAGTAAAGTTAAGTTTAAACC-TAMRA) (Applied Biosystems^{TM}), allowing the amplification of a fragment of 101 bp.

Both qPCRs were tested in silico within the DNA databases of metazoans (taxid: 33208), vertebrates (taxid: 7742), bacteria (taxid: 2), *Canidae* (taxid: 9608), Felidae (taxid: 9682), and humans (taxid: 9605). This experiment was performed for both combinations of forward-reward and probe-reverse of each qPCR using Primer-BLAST [69]. Subsequently, the specificity was also validated in vitro against the genomic DNA extracted from adult worms of *A. caucasica* and DNA database including several nematodes, arthropods, laboratory-maintained colonies, hemopathogens as well as human, monkey, donkey, horse, cattle, mouse, and dog as described elsewhere [52].

All qPCR reactions included 5 µL of DNA template, 10 µL of Master Mix Roche (Eurogentec), and 3 µL of ultra-purified water DNAse-RNAse free. Concentration of each primer, UDG, and each probe was 0.5 µL. The TaqMan reaction of both systems was run using the same cycling conditions. This included two hold steps at 50 °C and 95 °C for 2 and 15 min, respectively, followed by 40 cycles of two steps each (95 °C for 30 s and 60 °C for 30 s). The qPCR reaction was performed in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, CA, USA) after activating the appropriate dye readers for each qPCR system.

A protocol for the quantification of eggs has been established to assess the analytical sensitivity of qPCR in the detection of fecal infestation. A 10-fold serial dilution of DNA extracted from 200 mg of fecal matter containing 113 eggs (Figure 2) per gram (i.e., 22.6 eggs/100 µL of eluted DNA and 1.13 eggs/5 µL of qPCR reaction). Standard curves and derived parameters (PCR efficiency, Slope, Y-intercept, and correlation coefficient) were generated using CFX Manager Software Version 3 [70].

The molecular approaches described above were used to screen the presence of *A. caucasica* and other nematodes in chimpanzee fecal and environmental samples collected in a chimpanzee dormitory.

4.9. Conventional PCR Specific for A. caucasica

The use of universal pan-Nematoda primers does not allow for the identification of species-specific DNA sequences due to a non-specific amplification in co-infestations. A specific *cox*1-based PCR was developed in order to complete the identification of *A. caucasica* from fecal samples. The specific region for *A. caucasica* was analyzed for the design of the primers COI.51f and COI.601r, targeting 550 bp of the *cox*1 gene (Table 1). *A. caucasica cox*1 partial sequences herein amplified by the pan-Nematoda primers from the adult worms were aligned with *Heliconema longissimum* (AN: GQ332423) and *Gongylonema pulchrum* (AN: AP017685), representative members of *Physalopteroidea* and *Gongylonematidae*, respectively.

4.10. Molecular Survey of A. caucasica and Nematode Infestations in a Chimpanzee Population and the Environmental Samples

DNA from fecal samples of chimpanzee (n = 48) and environmental samples (n = 113) were screened for the DNA of *A. caucasica* and nematode using the 12S rRNA *A. caucasica* and the 5S rRNA pan-Nematoda qPCR assays, respectively. Positive samples for *A. caucasica* were also subjected to amplification and sequencing using the *cox*1 *A. caucasica*-specific primers.

4.11. Statistical Analysis

XLSTAT Addinsoft version 4.1 (XLSTAT 2019: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France) was used for the statistical analysis. Results of qPCRs analysis were used to set a database using the Microsoft Excel® program (Microsoft Corp., Redmond, Washington, USA). The effect of localities and fecal consistency on the infestation rates were tested using the Khi2 test or exact Fisher test. One-way analysis of variance (ANOVA) was performed to compare the predicted eggs from fresh and degraded feces. Negative samples and those with a studentized residual higher than 2.9 were removed before discarding the ANOVA test. McNemar's test was used to compare the detection accuracy of the qPCR and conventional PCR of *A. caucasica* from the chimpanzee samples. Significance level was considered at alpha ≤ 0.05 for all analyses.

5. Conclusions

A. caucasica measurements indicated the inconsistencies of certain indexes such as index a, b, and c (Table 1) within this nematode, while it remains distinguishable from other *Physaloptera* species by the morphological features of the anterior and posterior ends as well as the presence of four uteri with a common trunk. However, the phylogenetic analyses showed that *A. caucasica* are clustered together with other monophyletic species of the *Physaloptera* genus. In the absence of strong morphological and epidemiological data, the species of *Abbreviata* may be re-classified as *Physaloptera* and a revision of the genus is needed. We developed specific and reliable molecular tools for the detection and egg quantification of *A. caucasica* from fecal samples. The tests can ultimately help to identify possible intermediates as well as paratenic hosts involved in the life cycle of *A. caucasica*. We therefore investigated its prevalence in a chimpanzee population from Senegal. Further studies are needed to clarify the epidemiology, circulation, life cycle, and possible pathological effects of *A. caucasica*, and the role of paratenic hosts or arthropods as intermediate hosts.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/7/517/s1. Figure S1. *Abbreviata caucasica* partial COI-protein sequences alignment against *Physaloptera* species. The sequences of *A. caucasica* (selected box) obtained from adult worms were aligned against Physaloptera sequences available in the GenBank database. Residues were matched as dots. Conserved areas are indicated in blue, while the intensity of mutations is indicated by a foreground color (red to black). Figure S2. Quantification protocol of the 12S *A. caucasica*-specific qPCR. (**A**) Determination of detection limits and efficiency (eggs/g of fecal matter). (**B**) Standard curves generated from a serial 10-fold dilution of DNA.

Author Contributions: Conceptualization, O.M. and D.R.; Field study, O.M., D.R., B.D., G.D., and C.S.; Methodology, O.M. and Y.L.; Software, Y.L.; Validation, O.M., D.O., and D.R.; Molecular analysis, Y.L. and H.M.; Parasitological analysis, Y.L., M.S.L., and D.O.; Data curation, O.M. and Y.L.; Writing—original draft preparation, Y.L.; Writing—review and editing, B.D., D.R., D.O., O.M., R.A.H.-A., and A.B. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: We thank the Direction des Parcs Nationaux and the Direction des Eaux et Forests Chasses et de la Conservation des Sols for permission to work in Senegal. We thank Paula Alvarez Varona, Daouda Diallo, Mamadou F. Diallo, and Mamadou Samba Silla for help in the field in the Dindefelo Community Natural Reserve, Senegal.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Strait, K.; Else, J.G.; Eberhard, M.L. *Parasitic Diseases of Nonhuman Primates*, 2nd ed.; Elsevier Inc: Amsterdam, The Netherlands, 2012; ISBN 9780123813664.
2. Cleeland, L.M.; Reichard, M.V.; Tito, R.Y.; Reinhard, K.J.; Lewis, C.M. Clarifying prehistoric parasitism from a complementary morphological and molecular approach. *J. Archaeol. Sci.* **2013**, *40*, 3060–3066. [CrossRef] [PubMed]

3. Makki, M.; Dupouy-Camet, J.; Seyed Sajjadi, S.M.; Moravec, F.; Naddaf, S.R.; Mobedi, I.; Malekafzali, H.; Rezaeian, M.; Mohebali, M.; Kargar, F.; et al. Human spiruridiasis due to *Physaloptera* spp. (Nematoda: Physalopteridae) in a grave of the Shahr-e Sukhteh archeological site of the Bronze Age (2800–2500 BC) in Iran. *Parasite* **2017**, *24*, 18. [CrossRef] [PubMed]
4. Morgan, B.B. The Nematode genus *Abbreviata* (Travassos, 1920) Schulz, 1927. *Am. Midl. Nat.* **1945**, *34*, 485–490. [CrossRef]
5. Poinar, G.O.; Quentin, J.-C. The development of *Abbreviata caucasica* (Von Linstow) (Spirurida: *Physalopteridae*) in an intermediate host. *J. Parasitol.* **1972**, *58*, 23–28. [CrossRef] [PubMed]
6. Metzger, S. Gastrointestinal helminthic parasites of habituated wild chimpanzees (*Pan troglodytes verus*) in the Tai NP, Cote d'Ivoire-including characterization of cultured helminth developmental stages using genetic markers. Ph.D. Thesis, Freie University, Berlin, Germany, 2014.
7. Fain, A.; Vandepitte, J. Description des physaloptères (*Abbreviata caucasica* Linstow, 1902) récoltés chez l'homme au Congo. *Bull. Acad. R. Med. Belg.* **1964**, *4*, 663–682. [PubMed]
8. Schulz, R.-E. Sur la morphologie du *Physaloptera caucasica* von Linstow, 1902, de l'homme. *Ann. Parasitol. Hum. Comparée* **1926**, *4*, 74–84. [CrossRef]
9. Ortlepp, R.J. On the identity of *Physaloptera caucasica* v. Linstow, 1902, and *Physaloptera mordens* Leiper, 1908. *J. Helminthol.* **1926**, *4*, 199. [CrossRef]
10. Calle, P.P.; Ott Joslin, J. New world and old world monkeys. In *Fowler's Zoo and Wild Animal Medicine*; Miller, E., Fowler, M., Eds.; Elsevier Inc.: Amsterdam, The Netherlands, 2015; Volume 8, pp. 301–335.
11. Mul, I.F.; Paembonan, W.; Singleton, I.; Wich, S.A.; Van Bolhuis, H.G. Intestinal parasites of free-ranging, semicaptive, and captive *Pongo abelii* in Sumatra, Indonesia. *Int. J. Primatol.* **2007**, *28*, 407–420. [CrossRef]
12. McGrew, W.C.; Tutin, C.E.G.; Collins, D.A.; File, S.K. Intestinal parasites of sympatric *Pan troglodytes* and *Papio* spp. at two sites: Gombe (Tanzania) and Mt. Assirik (Senegal). *Am. J. Primatol.* **1989**, *17*, 147–155. [CrossRef]
13. Gillespie, T.R.; Lonsdorf, E.V.; Canfield, E.P.; Meyer, D.J.; Nadler, Y.; Raphael, J.; Pusey, A.E.; Pond, J.; Pauley, J.; Mlengeya, T.; et al. Demographic and ecological effects on patterns of parasitism in eastern chimpanzees (*Pan troglodytes schweinfurthii*) in Gombe National Park, Tanzania. *Am. J. Phys. Anthropol.* **2010**, *143*, 534–544. [CrossRef]
14. Howells, M.E.; Pruetz, J.; Gillespie, T.R. Patterns of gastro-intestinal parasites and commensals as an index of population and ecosystem health: The case of sympatric western chimpanzees (*Pan troglodytes verus*) and guinea baboons (*Papio hamadryas papio*) at Fongoli, Senegal. *Am. J. Primatol.* **2011**, *73*, 173–179. [CrossRef] [PubMed]
15. Kerr, K. Zoonoses: Infectious diseases transmissible from animals to humans. *J. Clin. Pathol.* **2004**, *57*, 1120. [CrossRef]
16. Petri, L.H. Life cycle of *Physaloptera rara* Hall and Wigdor, 1918 (Nematoda: Spiruroidea) with the Cockroach, *Blatella germanica*, serving as the intermediate host. *Trans. Kansas Acad. Sci.* **1950**, *63*, 331–337. [CrossRef]
17. Petri, L.H.; Ameel, D.J. Studies on the life cycle of *Physaloptera rara* Hall and Wigdor, 1918 and *Physaloptera praeputialis* Linstow, 1889. *J. Parasitol.* **1950**, *36*, 6.
18. Olsen, J.L. Life cycle of *Physaloptera rara* Hall and Wigdor, 1918 (Nematoda: Physalopteroidea) of canids and felids in definitive, intermediate and paratenic hosts. *Rev. Iber. Parasitol.* **1980**, *40*, 489–525.
19. Flynn, R.J. *Parasites of Laboratory Animals*; Iowa State University Press: Ames, IA, USA, 1973; p. 884.
20. Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [CrossRef]
21. Brede, H.D.; Burger, P.J. Physaloptera caucasica (=Abbreviata caucasica) in the South African baboon (Papio ursinus). *Arb Paul Ehrlich Inst Georg Speyer Haus Ferdinand Blum Inst Frankf A M.* **1977**, *71*, 119–122.
22. Harras, S.F.; Elmahy, R.A. New record of *Abbreviata leptosoma* Gervais, 1848 (Spirurida: *Physalopteridae*) infection in two species of lizards in north and south Sinai, Egypt. *Egypt. J. Zool.* **2019**, *72*, 1–10.
23. Chabaud, A.G. Essai de révision des Physaloptères parasites de reptiles. *Ann. Parasitol. Hum. Comparée* **1956**. [CrossRef]
24. Fortuner, R. Ratios and indexes in nematode taxonomy. *Nematologica* **1990**, *36*, 205–216. [CrossRef]
25. Vovlas, N.; Subbotin, S.A.; Troccoli, A.; Liébanas, G.; Castillo, P. Molecular phylogeny of the genus *Rotylenchus* (Nematoda, Tylenchida) and description of a new species. *Zool. Scr.* **2008**, *37*, 521–537. [CrossRef]

26. Avó, A.P.; Daniell, T.J.; Neilson, R.; Oliveira, S.; Branco, J.; Adão, H. DNA barcoding and morphological identification of benthic nematodes assemblages of estuarine intertidal sediments: Advances in molecular tools for biodiversity assessment. *Front. Mar. Sci.* **2017**, *4*, 66. [CrossRef]
27. Blaxter, M.; Mann, J.; Chapman, T.; Thomas, F.; Whitton, C.; Floyd, R.; Abebe, E. Defining operational taxonomic units using DNA barcode data. *Philos. Trans. R. Soc. B Biol. Sci.* **2005**, *360*, 1935–1943. [CrossRef] [PubMed]
28. Meldal, B.H.M.; Debenham, N.J.; De Ley, P.; De Ley, I.T.; Vanfleteren, J.R.; Vierstraete, A.R.; Bert, W.; Borgonie, G.; Moens, T.; Tyler, P.A.; et al. An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. *Mol. Phylogenet. Evol.* **2007**, *42*, 622–636. [CrossRef] [PubMed]
29. Ferri, E.; Barbuto, M.; Bain, O.; Galimberti, A.; Uni, S.; Guerrero, R.; Ferté, H.; Bandi, C.; Martin, C.; Casiraghi, M. Integrated taxonomy: Traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Front. Zool.* **2009**, *6*, 1. [CrossRef] [PubMed]
30. Pereira, F.B.; Alves, P.V.; Rocha, B.M.; de Souza Lima, S.; Luque, J.L. A new *Physaloptera* (Nematoda: *Physalopteridae*) Parasite of *Tupinambis merianae* (Squamata: *Teiidae*) from Southeastern Brazil. *J. Parasitol.* **2012**, *98*, 1227–1235. [CrossRef]
31. Kalyanasundaram, A.; Henry, C.; Brym, M.Z.; Kendall, R.J. Molecular identification of *Physaloptera* sp. from wild northern bobwhite (*Colinus virginianus*) in the Rolling Plains ecoregion of Texas. *Parasitol. Res.* **2018**, *117*, 2963–2969. [CrossRef]
32. Sao Luiz, J.; Simoes, R.; Torres, E.; Barbosa, H.; Santos, J.; Giese, E.; Rocha, F.; Maldonado-Junior, A. A new species of *Physaloptera* (Nematoda: *Physalopteridae*) from *Cerradomys subflavus* (Rodentia: *Sigmodontidae*) in the Cerrado Biome, Brazil. *Neotrop. Helminthol.* **2015**, *9*, 301–312.
33. Maldonado, A.; SimAes, R.O.; Luiz, J.S.; Costa-Neto, S.F.; Vilela, R.V. A new species of *Physaloptera* (Nematoda: *Spirurida*) from *Proechimys gardneri* (Rodentia: *Echimyidae*) from the Amazon rainforest and molecular phylogenetic analyses of the genus. *J. Helminthol.* **2019**, *94*, e68. [CrossRef]
34. De Oliveira, M.C.; Lima, V.F.; Pinto, C.L.D.M.; da Silva, É.G.; Teles, D.A.; Ferreira-Silva, C.; Almeida, W.D.O. New record of *Physaloptera* sp. (Nematoda: *Physalopteridae*) parasitizing *Philodryas nattereri* (Ophidia: *Dipsadidae*) in Brazil. *Herpetol. Notes* **2019**, *12*, 1031–1034.
35. Velarde-Aguilar, M.G.; Romero-Mayén, Á.R.; León-Règagnon, V. First report of the genus *Physaloptera* (Nematoda: *Physalopteridae*) in *Lithobates montezumae* (Anura: *Ranidae*) from Mexico. *Rev. Mex. Biodivers.* **2014**, *85*, 304–307. [CrossRef]
36. Ederli, N.B.; Gallo, S.S.M.; Oliveira, L.C.; de Oliveira, F.C.R. Correction to: Description of a new species *Physaloptera goytaca* n. sp. (Nematoda, *Physalopteridae*) from *Cerradomys goytaca* Tavares, Pessôa & Gonçalves, 2011 (Rodentia, *Cricetidae*) from Brazil. *Parasitol. Res.* **2018**, *117*, 2757–2766. [PubMed]
37. Leiper, B.Y.R.T. Observation on certain helminths of men. *Trans. R. Soc. Trop. Med. Hyg.* **1915**, *6*, 265–297. [CrossRef]
38. Pouillevet, H.; Dibakou, S.-E.; Ngoubangoye, B.; Poirotte, C.; Charpentier, M.J.E. A comparative study of four methods for the detection of nematode eggs and large protozoan cysts in mandrill faecal material. *Folia Primatol.* **2017**, *88*, 344–357. [CrossRef] [PubMed]
39. Appleton, C.C.; Henzi, S.P. Environmental correlates of gastrointestinal parasitism in montane and lowland baboons in Natal, South Africa. *Int. J. Primatol.* **1993**, *14*, 623–635. [CrossRef]
40. Anderson, R.C. Nematode parasites of vertebrates. In *Their Development and Transmission*, 2nd ed.; CABI Publishing: Wallingford, Oxon, UK, 2000; p. 650.
41. Martin, J. Australian mammals: Biology and captive management. *Austral. Ecol.* **2005**, *30*, 126–129. [CrossRef]
42. King, C.; Jones, H.I.; Tay, C.Y. Arthropod intermediate hosts of *Abbreviata antarctica* (Nematoda: *Physalopteridae*) in Australia. *J. Parasitol.* **2013**, *99*, 708–711. [CrossRef]
43. McGrew, W.C. *Chimpanzee Material Culture: Implications for Human Evolution*; Cambridge University Press: Cambridge, UK, 1993; p. 277.
44. Pacheco, L.; Fraixedas, S.; Fernández-Llamazares, Á.; Estela, N.; Mominee, R.; Guallar, F. Perspectives on sustainable resource conservation in community nature reserves: A case study from senegal. *Sustainability* **2012**, *4*, 3158–3179. [CrossRef]
45. McGrew, W.C.; Baldwin, P.J.; Tutin, C.E.G. Chimpanzees in a hot, dry and open habitat: Mt. Assirik, Senegal, West Africa. *J. Hum. Evol.* **1981**. [CrossRef]

46. Nishida, T. The social group of wild chimpanzees in the Mahali Mountains. *Primates* **1968**, *9*, 167–224. [CrossRef]
47. Rudicell, R.S.; Piel, A.K.; Stewart, F.; Moore, D.L.; Learn, G.H.; Li, Y.; Takehisa, J.; Pintea, L.; Shaw, G.M.; Moore, J.; et al. High Prevalence of Simian Immunodeficiency Virus Infection in a Community of Savanna Chimpanzees. *J. Virol.* **2011**. [CrossRef] [PubMed]
48. Gillespie, T.R. Noninvasive assessment of gastrointestinal parasite infections in free-ranging primates. *Int. J. Primatol.* **2006**, *27*, 1129–1143. [CrossRef]
49. Jessee, M.T.; Schilling, P.W.; Stunkard, J.A. Identification of intestinal helminth eggs in old world primates. *Lab. Anim. Care* **1970**, *20*, 83–87. [PubMed]
50. Hall, T.; Biosciences, I.; Carlsbad, C. BioEdit: An important software for molecular biology. *GERF Bull. Biosci.* **2011**, *2*, 60–61.
51. Koressaar, T.; Lepamets, M.; Kaplinski, L.; Raime, K.; Andreson, R.; Remm, M. Primer3_masker: integrating masking of template sequence with primer design software. *Bioinformatics* **2018**, *34*, 1937–1938. [CrossRef] [PubMed]
52. Laidoudi, Y.; Davoust, B.; Varloud, M.; Niang, E.H.A.; Fenollar, F.; Mediannikov, O. Development of a multiplex qPCR-based approach for the diagnosis of *Dirofilaria immitis*, *D. repens* and *Acanthocheilonema reconditum*. *Parasites Vectors* **2020**, *13*, 319. [CrossRef]
53. Laidoudi, Y.; Ringot, D.; Watier-grillot, S.; Davoust, B.; Mediannikov, O. A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France. *Parasite* **2019**, *26*, 72. [CrossRef]
54. Song, H.; Buhay, J.E.; Whiting, M.F.; Crandall, K.A. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13486–13491. [CrossRef]
55. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
56. Tamura, K.; Nei, M.; Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11030–11035. [CrossRef]
57. Zuckerkandl, E.; Pauling, L. Evolutionary divergence and convergence in proteins. *Evolv. Genes Proteins* **1965**. [CrossRef]
58. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef] [PubMed]
59. Milne, I.; Lindner, D.; Bayer, M.; Husmeier, D.; Mcguire, G.; Marshall, D.F.; Wright, F. TOPALi v2: A rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. *Bioinformatics* **2009**, *25*, 126–127. [CrossRef]
60. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **2001**, *17*, 754–755. [CrossRef] [PubMed]
61. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef]
62. Waddell, P.J.; Steel, M.A. General time-reversible distances with unequal rates across sites: Mixing Y and inverse Gaussian distributions with invariant sites. *Mol. Phylogenet. Evol.* **1997**, *8*, 398–414. [CrossRef]
63. Cao, Y.; Janke, A.; Waddell, P.J.; Westerman, M.; Takenaka, O.; Murata, S.; Okada, N.; Pääbo, S.; Hasegawa, M. Conflict among individual mitochondrial proteins in resolving the phylogeny of eutherian orders. *J. Mol. Evol.* **1998**, *47*, 307–322. [CrossRef]
64. Rice, P.; Longden, I.; Bleasby, A. EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet.* **2000**, *16*, 276–277. [CrossRef]
65. Clement, M.; Posada, D.; Crandall, K.A. TCS: A computer program to estimate gene genealogies. *Mol. Ecol.* **2000**, *9*, 1657–1659. [CrossRef]
66. Leigh, J.; Bryant, D.; Steel, M. PopART (Population Analysis with Reticulate Trees). 2015. Available online: http://popart.otago.ac.nz/index.shtml (accessed on 22 June 2020).
67. Bamuhiiga, J.; Williams, S.A.; Fischer, P.; Büttner, D.W. Detection of the filarial parasite *Mansonella streptocerca* in skin biopsies by a nested polymerase chain reaction-based assay. *Am. J. Trop. Med. Hyg.* **2017**, *58*, 816–820.
68. Huang, X.Y.; Hirsh, D. A second trans-spliced RNA leader sequence in the nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 8640–8644. [CrossRef] [PubMed]

69. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* **2012**, *13*, 134. [CrossRef] [PubMed]
70. Bio-Rad, L. *Real-Time PCR Applications Guide*; Bio-Rad Laboratories Inc.: Hercules, CA, USA, 2006; p. 41.

Publication N°5

Parasitic Infections in African Humans and Non-Human Primates

Hacène Medkour[1,2,3,†], Inestin Amona[2,4,5,†], **Younes Laidoudi**[1,2,3], Bernard Davoust[1,2], Idir Bitam[4,6], Anthony Levasseur[1,2], Jean Akiana[7], Georges Diatta[2,4,8], Liliana Pacheco[9], Slim Gorsane[10], Cheikh Sokhna[2,4,8], R. Adriana Hernandez-Aguilar[11,12], Amanda Barciela[11], Florence Fenollar[2,4], Didier Raoult[1,2] and Oleg Mediannikov[1,2,*]

[1]Aix Marseille Univ, IRD, AP-HM, MEPHI, Marseille 13385, France
[2]IHU Méditerranée Infection, Marseille 13385, France
[3]PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, El Khroub 25100, Algeria
[4]Aix-Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille 13385, France
[5]Faculté des Sciences et Techniques, Université Marien Ngouabi, Brazzaville, République du Congo
[6]Superior School of Food Sciences and Food Industries of Algiers 16004, Algeria
[7]Laboratoire National de Santé Publique, Brazzaville, République du Congo
[8]VITROME, IRD 257, Campus IRD/UCAD Hann, Dakar, Senegal
[9]Wara Conservation Project, Projet GALF-Guinée, Le Cannet, France
[10]Direction interarmées du Service de santé des armées des Forces françaises stationnées
[11]Jane Goodall Institute Spain and Senegal, Dindefelo Biological Station, Dindefelo, Kedougou, Senegal
[12]Department of Social Psychology and Quantitative Psychology, Faculty of Psychology, University of Barcelona, Passeig de la Vall d'Hebron 171, 08035 Barcelona, Spain
† These authors equally contributed to this work.

ARTICLE DE RECHERCHE

Publié dans : Pathogens, 2020, 9(7), 561

(doi.10.3390/pathogens9070561)

pathogens

MDPI

Article

Parasitic Infections in African Humans and Non-Human Primates

Hacène Medkour [1,2,3,†], Inestin Amona [2,4,5,†], Younes Laidoudi [1,2,3], Bernard Davoust [1,2], Idir Bitam [4,6], Anthony Levasseur [1,2], Jean Akiana [7], Georges Diatta [2,4,8], Liliana Pacheco [9], Slim Gorsane [10], Cheikh Sokhna [2,4,8], Raquel Adriana Hernandez-Aguilar [11,12], Amanda Barciela [11], Florence Fenollar [2,4], Didier Raoult [1,2] and Oleg Mediannikov [1,2,*]

1 Aix Marseille Univ, IRD, AP-HM, MEPHI, 13385 Marseille, France; hacenevet1990@yahoo.fr (H.M.); younes.laidoudi@yahoo.com (Y.L.); bernard.davoust@gmail.com (B.D.); anthony.levasseur@univ-amu.fr (A.L.); didier.raoult@gmail.com (D.R.)
2 IHU Méditerranée Infection, 13385 Marseille, France; amoninestin@gmail.com (I.A.); georges.diatta@ird.fr (G.D.); cheikh.sokhna@ird.fr (C.S.); florence.fenollar@univ-amu.fr (F.F.)
3 PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, El Khroub 25100, Algeria
4 Aix-Marseille Univ, IRD, AP-HM, SSA, VITROME, 13385 Marseille, France; idirbitam@gmail.com
5 Faculté des Sciences et Techniques, Université Marien Ngouabi, Brazzaville, Congo
6 Superior School of Food Sciences and Food Industries, Algiers 16004, Algeria
7 Laboratoire National de Santé Publique, Brazzaville, Congo; jakiana2000@yahoo.fr
8 VITROME, IRD 257, Campus IRD/UCAD Hann, Dakar, Senegal
9 Wara Conservation Project, Projet GALF-Guinée, 06110 Le Cannet, France; lilianaalbapacheco@gmail.com
10 Direction Interarmées du Service de Santé des Armées des Forces Françaises Stationnées, Djibouti, East Africa; slim.gorsane@intradef.gouv.fr
11 Jane Goodall Institute Spain and Senegal, Dindefelo Biological Station, Dindefelo, Kedougou, Senegal; r.a.hernandez-aguilar@ibv.uio.no (R.A.H.-A.); amanda.b@janegoodall.es (A.B.)
12 Department of Social Psychology and Quantitative Psychology, Faculty of Psychology, University of Barcelona, Passeig de la Vall d'Hebron 171, 08035 Barcelona, Spain
* Correspondence: olegusss1@gmail.com
† These authors equally contributed to this work.

Received: 8 June 2020; Accepted: 8 July 2020; Published: 11 July 2020

Abstract: Different protozoa and metazoa have been detected in great apes, monkeys and humans with possible interspecies exchanges. Some are either nonpathogenic or their detrimental effects on the host are not yet known. Others lead to serious diseases that can even be fatal. Their survey remains of great importance for public health and animal conservation. Fecal samples from gorillas (*Gorilla gorilla*) and humans living in same area in the Republic of Congo, chimpanzees (*Pan troglodytes*) from Senegal and one other from the Republic of Congo, Guinea baboons (*Papio papio)* from Senegal, hamadryas baboons (*Papio hamadryas*) from Djibouti and Barbary macaques (*Macaca sylvanus*) from Algeria, were collected. DNA was extracted and screened using specific qPCR assays for the presence of a large number of helminths and protozoa. Positive samples were then amplified in standard PCRs and sequenced when possible. Overall, infection rate was 36.5% in all non-human primates (NHPs) and 31.6% in humans. Great apes were more often infected (63.6%) than monkeys (7.3%). At least twelve parasite species, including ten nematodes and two protozoa were discovered in NHPs and five species, including four nematodes and a protozoan in humans. The prevalences of *Giarida lamblia*, *Necator americanus*, *Enterobius vermicularis*, *Strongyloides stercoralis* were similar between gorillas and human community co-habiting the same forest ecosystem in the Republic of Congo. In addition, human specific *Mansonella perstans* (5.1%) and other *Mansonella* spp. (5.1%) detected in these gorillas suggest a possible cross-species exchange. Low prevalence (2%) of *Ascaris lumbricoides*, *Enterobius vermicularis*, *Strongyloides stercoralis* were observed in chimpanzees, as well as a high prevalence of *Abbreviata caucasica* (57.1%), which should be considered carefully as this parasite can affect other NHPs, animals and humans. The Barbary macaques were less infected (7.2%) and *Oesophagostomum*

muntiacum was the main parasite detected (5.8%). Finally, we report the presence of *Pelodera* sp. and an environmental Nematoda DNAs in chimpanzee feces, *Nematoda* sp. and *Bodo* sp. in gorillas, as well as DNA of uncharacterized Nematoda in apes and humans, but with a relatively lower prevalence in humans. Prevalence of extraintestinal parasites remains underestimated since feces are not the suitable sampling methods. Using non-invasive sampling (feces) we provide important information on helminths and protozoa that can infect African NHPs and human communities living around them. Public health and animal conservation authorities need to be aware of these infections, as parasites detected in African NHPs could affect both human and other animals' health.

Keywords: nonhuman primates; humans; Nematoda; Mansonella; cross-species transmission; PCR

1. Introduction

Parasitic infections cause a tremendous burden of disease in both the tropics and subtropics as well as in more temperate climates. There are three main groups of parasites that can cause disease in humans: protozoa, helminths and ectoparasites [1,2]. Parasitism is one of the most common disease entities threatening non-human primates (NHPs). Numerous protozoan and metazoan genera have been described, can infect different great apes and monkeys [3]. Some are nonpathogenic—or at least their detrimental effects on the host are not yet elucidated. However, a large number can lead to physiologic disorders, nutritional loss or produce lesions that result in severe damages, sometimes allowing sondary infections that may be fatal. Infection can be promoted by immunosuppression and various stressors [4].

Using the primate–parasite network, the role of different NHPs was evaluated for the probability of sharing infectious diseases with humans. Apes, as well as monkeys, such as baboons and macaques were shown to be infected with many parasites identified as emerging infectious diseases in humans [5]. Other studies have shown that parasites are frequently transmitted from wild or captive NHPs to humans in a shared habitat [6–9]. The protozoan *Giardia lamblia*, an enteric flagellate, induces diarrhea in monkeys and children [10]. *Entamoeba histolytica* has been described mainly in Old World NHPs including some apes (gibbons, orangutans and chimpanzees) as a cause of severe enteric disease [11]. It can infect humans as well, leading to dysentery. Cutaneous leishmaniasis agents were identified in NHPs, *Leishmania major* in wild gorillas [12] and *L. infantum* (visceral leishmaniasis) in New World NHPs [13]. Old World NHPs, such as chimpanzees and mangabeys can carry either *Schistosoma mansoni* or *S. hematobium* [14]. Parasites from the genus *Plasmodium* are among the best-studied parasites in African humans and NHPs, as they are responsible for malaria, the deadliest vector-borne disease [15]. The origin of the human malaria parasite *Plasmodium* was attributed to African apes. *Laverania* spp., found in various apes, belong to lineages in eastern chimpanzees as well as western lowland gorillas. They are nearly identical to *P. falciparum* and *P. vivax* [16,17]. Of note, *P. cynomolgi, P. siminovale* and *P. inui* are related to *P. vivax, P. ovale* and *P. malariae* in humans, respectively. Cross infection of *P. knowlesi* has also been documented in humans and NHPs [18–21]. Several other parasites that can be found in NHPs, such as *Babesia, Cryptosporidium*, *Amoeba, Toxoplasma, Trypanosoma*, Coccidia, nematodes and cestodes, possibly constitute a risk for humans [4,22,23].

For millennia, indigenous groups that depended on wildlife for their survival were exposed to the risk of NHP pathogens' transmission. Inter species transmission of pathogens can occur through direct and indirect mechanisms. Direct mechanisms include hunting, fomites and wild meat consumption, keeping infected NHPs as pets or by eco-guardians, visitors or personal of primate center laboratories entering in direct contact with infected NHPs. Indirect mechanisms transmission of infectious pathogen stages through vectors such as blood sucking ticks, flies, fleas, sandflies, lice and tsetse flies transmitted pathogens such as *Trypanosoma* spp., *Bertiella* sp. tapeworm [3,24]. We still do not understand the dynamics of parasite interchange in detail but the increasing contact among species, may result in new

ways of parasite interchange. By the way, in Uganda a study on how human impact the use of the ground and patterns of parasitism of *Pan troglodytes* suggest that the creation of trails and the increase of presence of humans in the forest results in increase in the frequency of use of the ground and higher parasite richness and intensity of infections [25]. The same results were found comparing groups of long-tailed macaques (*Macaca fascicularis*) living close to human modified environments and within the forests in Thailand [26]. No parasites present in humans were found in the macaques, but the macaques living in habitats modified by humans presented parasites (*Strongyloides fuelleborni* and probably *Haplorchis* sp.) that were not present in more isolated macaque groups. These last results are of importance, as the presence of humans and human related activities may modify the parasite community of non-human primates resulting in still unknown effects over their survival [27]. In this regard, Conly and Johnston (2008) suggest that this uncontrolled exposure of macaques to humans may have the potential for novel cross-species transmission of different parasites [28].

Finally, the high burden of zoonotic diseases continues to undermine the efforts and investments made for social and economic development. Therefore, developing strategies to ameliorate human and animal health through capacity building is pivotal for socioeconomic transformation and ranks top in developing country's development agenda. Most zoonotic disease remains largely neglected in Africa and specially in sub Saharan Africa, probably because of distances existing between veterinary and medical professions. This lack of cooperation has in most cases left the burden of surveillance and control almost solely in the hands of veterinarian. Therefore, there is an urgent need for change of the status quo by adopting a holistic approach in controlling infectious diseases shared between humans and animals. As response to this need, in this study, we performed a survey of parasites (protozoa and helminths) using a fast typing technique by PCR in feces (noninvasive sampling method) of African NHPs. In an one health context and using the same approach, we examined a human population from the Republic of Congo living in the vicinity to gorillas in order to assess potential zoonotic transmission.

2. Results

In this study, the parasitic infection rate (i.e., presence of at least one parasitic infection) was 36.5% (62/170) in all NHPs. At least one infection was detected in 79.6% (39/49) of chimpanzees, 43.6% (17/39) of gorillas, 7.7% (1/13) of baboons and 7.2% (5/69) of macaques. Great apes were found to be more infested (63.6%) than monkeys (7.3%) (Z test, p-value < 0.0001) (Figure 1). In the Republic of Congo, infection rate was 43.6% (17/39) in gorillas and 31.6% (12/38) in the human community living close to them (Z test, p-value = 0.390), respectively.

At least one Nematoda infection was reported in 31.2% and 21.1% of NHPs and humans, respectively. Infection rates were (in NHPs and humans, respectively) 4.7% and 2.6% for *Filarioidea*, 2.9% and 13.2% for *Necator americanus*, 0.6% and 5.3% for *Ascaris lumbricoides*, 2.4% and 2.6% for *Strongyloides stercoralis*. *Abbreviata caucasica* von Linstow (Physalopteridae: Spirurida) (Syn *Physaloptera caucasica*) was detected only in Senegalese wild chimpanzees (57.1%), as reported in our previous study [29]. *Enterobius vermicularis* nematode was identified only in one gorilla and one chimpanzee, thus in 1.2% of NHPs.

DNA of *Mansonella* spp. has been detected in four gorilla samples from the Republic of Congo. Among them, two were identified as *M. perstans*. In this study, DNA of uncharacterized nematodes was detected in 12 (7.1%) NHPs, including 10 chimpanzees (20.4%), one gorilla (2.6%), one Guinea baboon (7.7%) and in one human (Table 1). Except for filarioid infections (Z test, p-value = 0.028), no significant difference was observed for the presence of Nematoda in gorillas and humans in the Republic of Congo.

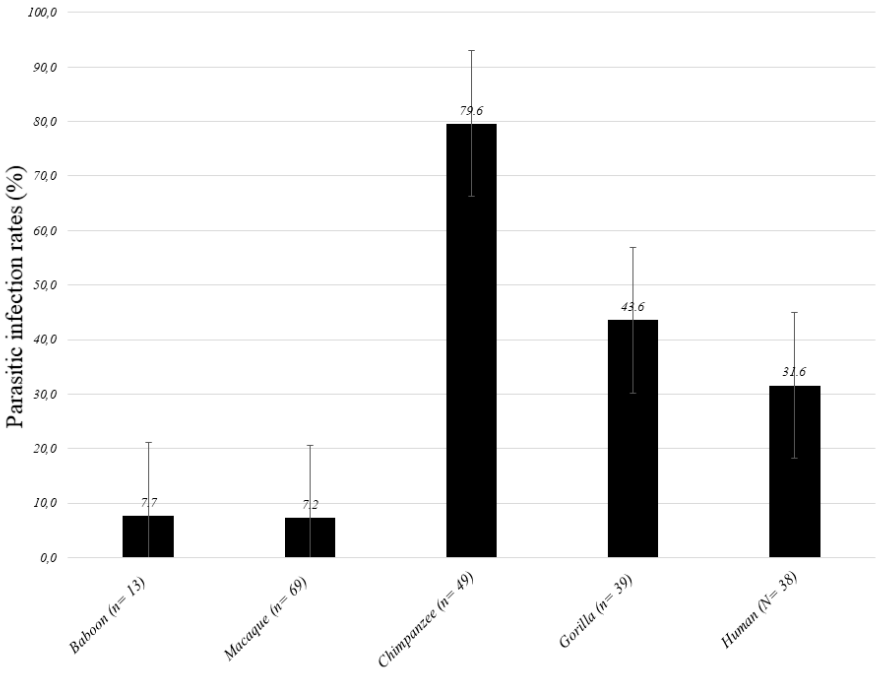


Figure 1. Results for parasite screening in feces from African humans and non-human primates (NHPs). Error bars represent the *SD*. Parasitic infection rates (%) mean the percentage of the presence of at least one infection.

Table 1. Parasites detected in the present study and their prevalence, *n* (%).

***Species* (*n*)**	**Gorilla (*n* = 39)**	**Chimpanzee (*n* = 49)**	**Baboon (*n* = 13)**	**Macaque (*n* = 69)**	**NHPs (N = 170)**	**Human (N = 38)**	***Difference:* *p*-Value**
Parasitic infections	17(43.6)	39 (79.6)	1 (7.7)	5 (7.2)	62 (36.5)	12 (31.6)	*0.390*
Nematoda	10 (25.6)	38 (77.6)	1 (7.7)	4 (5.8)	53 (31.2)	8 (21.1)	*0.836*
Filarioidea	8 (20.5)	0	0	0	8 (4.7)	1 (2.6)	*0.028*
Mansonella spp.	4 (10.3)	0	0	0	4 (2.4)	0	*0.115*
M. perstans	2 (5.1)	0	0	0	2 (1.2)	0	*0.474*
Abbreviata caucasica	0	28 (57.1)	0	0	28 (16.5)	0	*<0.0001*
Necator americanus	5 (12,8)	0	0	0	5 (2.9)	5 (13.2)	*1.000*
Ascaris lumbricoides	0	1 (2)	0	0	1 (0.6)	2 (5.3)	*0.462*
Enterobius vermicularis	1 (2.6)	1 (2)	0	0	2 (1.2)	0	*1.000*
Strongyloides stercoralis	3 (7.7)	1 (2)	0	0	4 (2.4)	1 (2.6)	*0.622*
Oesophagostomum muntiacum	0	0	0	4 (5.8)	4 (2.4)	0	*1.000*
Unknown Nematoda	1 (2.6)	10 (20.4)	1 (7.7)	0	12 (7.1)	1 (2.6)	*1.000*
Protozoa	9 (23.1)	0	0	1 (1.4)	10 (5.9)	4 (10.5)	*0.014*
Giardia lamblia	5 (12.8)	0	0	0	5 (2.9)	4 (10.5)	*1000*
Kinetoplastida *(Bodo sp.)*	4 (10.3)	0	0	1 (1.4)	5 (2.9)	0	*0.115*

p-value: between humans and NHPs from the Republic of Congo living in the same area.

Protozoan infections were also present in 5.9% of NHPs, including almost all in gorillas (23%) and in 10.5% of humans (Z test, *p*-value: 0.014). *Giardia lamblia* was detected in 12.5% of gorillas and in 10.5% of humans, while Kinetoplastida were detected in 10.3% of gorillas and in one macaque 1.4% (Figure 2, Table 1).

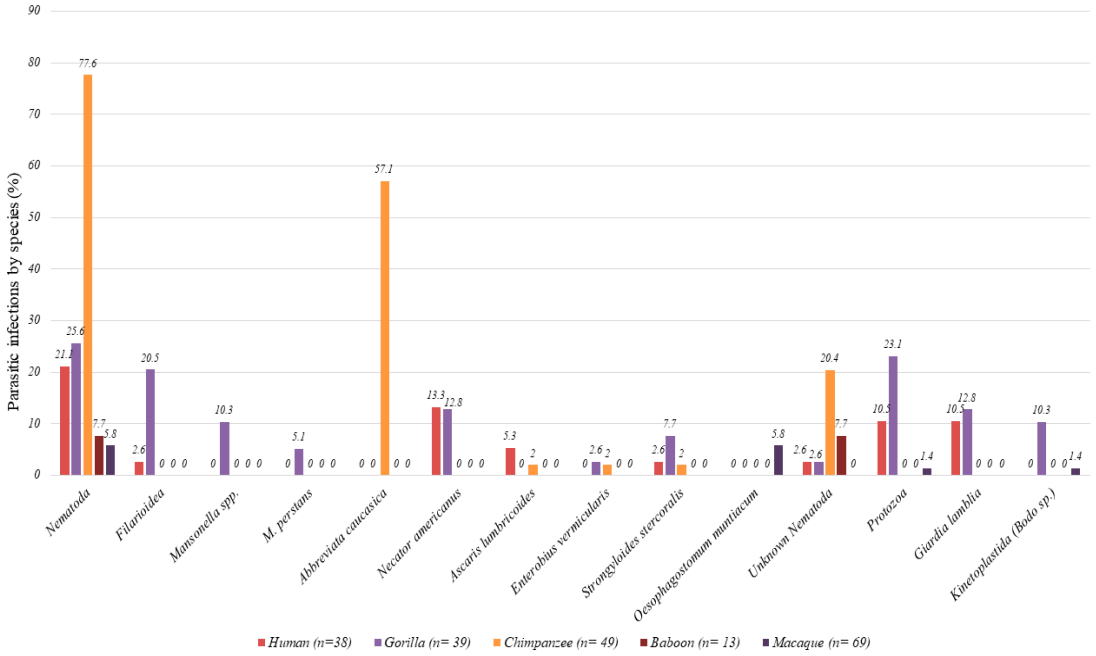


Figure 2. Parasitic infection by species of NHPs and humans.

Except for *A. caucasica* (Z test, *p*-value < 0.0001*), no significant differences in infection rates between humans and NHPs were observed. The other pathogens searched in this study were not detected.

Furthermore, after PCR/sequencing of qPCR positive samples for the partial Nematoda 18S gene (Figure 3), in addition to confirming the qPCR results, DNA from *Oesophagostomum* sp. was identified in four (4.2%) Barbary macaques. These sequences of ~1100 bps from 18S rRNA, were almost identical to each other and exhibited > 99.3 identity with *O. muntiacum* NSMT (LC415112) detected in large intestine of Reeves's muntjac (*Muntiacus reevesi*) from Izu-Oshima Island, Tokyo, Japan. Using PCR primers for 28S pan-helminths, *O. muntiacum* was confirmed by amplifying ~500 bps of the 28S gene from all positive samples by pan-Nematoda 18S PCR. These sequences were almost similar and allowed 99.7% similarity with *O. muntiacum* NSMT (LC415112). Almost all of the chimpanzee positive samples by qPCR for *A. caucasica* were amplified and ~1200 bps fragments were obtained. They were almost identical and were identified as *A. caucasica*. Simultaneously, the COI genes were amplified, they were almost identical to each other and were identified as *A. caucasica* as described previously [29].

The evolutionary history based on 18S rRNA partial gene was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 0.71539928 is shown. The confidence probability (multiplied by 100) that the inside length of the branches is greater than 0, as estimated by the bootstrap test (1000 replicates), is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Sequences obtained in this study are highlighted by red points in the beginning, Nematoda species, NHP species sample ID and country. They are compared to the related Nematoda sequences from GenBank database. The evolutionary distances were computed using the Tamura–Nei method and are in the units of the number of base substitutions per site. The analysis involved 38 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7.

Three other sequences of ~400 bps of 18S gene from gorillas were 100% identical to *N. americanus* (AJ920348), all of these samples were also positive by specific qPCR for *N. americanus*; (E) *vermicularis* qPCR-positive samples were confirmed by amplifying a 400-bps sequence showing 99.2% homology with *E. vermicularis* (JF934731). In addition, small good quality sequences of approximately 200 bps were obtained from positive gorilla *M. perstans* qPCR samples. They were similar and exhibited 98.5%

similarity with *M. perstans* isolate PlasNew649 (MN821068) found in the blood of a Gabonese man. Finally, a DNA sequence was obtained from one of the two positive human *A. lumbricoides* qPCR samples, it displayed 99.8% similarity to *A. lumbricoides* (LN600406).

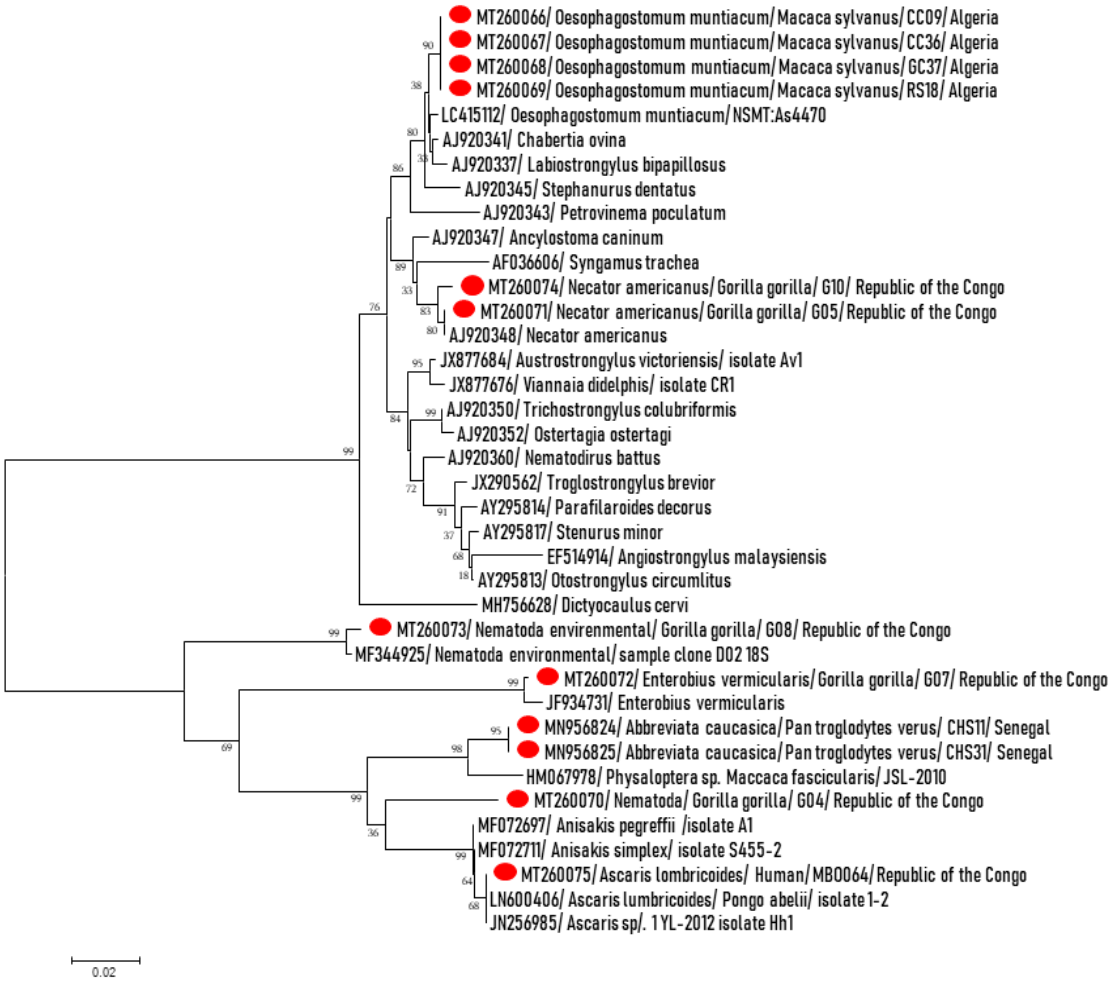


Figure 3. Phylogenetic tree representing *Nematoda* spp. detected in this study from African primates and humans.

We detected a *Rhabditidae* nematode on a chimpanzee. The 320-bps fragment of 28S partial gene amplified was 82% similar to saprophytic nematode *Pelodera cylindrica* (EU195994). A fragment of the 18S gene, approximately 400 bp in length, was obtained from a gorilla that did not show any nematode-like identity. The closest was only 94% of similarity with *Anisakis simplex* (MF072711) and *A. pegreffii* (MF072697). An environmental nematode showing 99.5% identity with Plectidae sp. MK-2017 (LC275886) was detected in a gorilla, we do not exclude environmental contamination since the fecal samples were taken from the soil.

All positive samples in qPCR pan-Kinetoplastida were identified by sequencing of 18S and 28S partial genes as *Bodo* spp., a free living nonpathogenic Kinetoplastida.

3. Discussion

Overall, when parasites (helminths and protozoa) were searched in the feces of NHPs and a nearby indigenous human population using molecular tools, a wide range of parasites belonging to the Nematoda and Kinetoplastida classes were detected. Parasitism rate was almost similar in both humans and gorillas co-habiting the same ecosystem forest in the Republic of Congo and nematode infections were the most predominant. The prevalence was 8.7-fold higher in great apes than monkeys.

In addition, sub-Saharan African primates were 7.3-fold more infected than macaques from North Africa. The raison of this difference is not clear, and since infectious diseases are modulated by ecosystems, we think that primates in sub-Saharan Africa are more exposed to these diseases because this region is characterized by the greatest infectious disease burden as well as by the weakest public health infrastructure in the world [30]. The method used here (PCR) was more sensitive than microscopy for parasite detection in patients without gastrointestinal symptoms [31]. By contrast, we are aware of the low sensitivity of specific PCR on feces for the detection of extraintestinal parasites, such *Plasmodium*, Filaria and Kinetoplastida etc., particularly parasites of blood. Consequently, prevalence of extraintestinal parasites is underestimated in this study.

Our results are complementary to a study conducted in Gabon in the Lopé Reserve, which revealed the presence of protozoa, trematodes and nematodes in 84% of gorilla samples and 56% of chimpanzee samples [32]. Another study, conducted in the Dzanga-Ndoki National Park in the Central African Republic, described both helminths and protozoa in fecal samples from great apes (gorillas, chimpanzees) and human populations (Ba'Aka, indigenous Bantu and western researchers) [33].

Giardia lamblia (syn *G. intestinalis, G. duodenalis*), a parasite of the protozoan group, has been found in feces of great apes (gorillas), as well as in humans living around them in this study, suggesting possible interactions. NHPs can be asymptomatic carriers and hence be a source to human infections, with infection via direct contact [34]. Evidence of possible fecal–oral transmission of *Giardia* between apes and their attendants was reported from the Kansas City Zoo. Clinical signs in Kansas City outbreak consisted of diarrhea and vomiting in both non-human and human patients [4]. Although work continues on speciation and host-specificity in *Giardia*, studies have shown that at least some species of *Giardia* are transmissible from animals to humans and vice versa, thus making it potentially both a zoonotic and an anthropozoonotic infection [35,36]. In the studies conducted by Nolan et al. in Uganda and Drakulovski et al. in Cameroon, *G. lamblia* was absent in mountain gorilla and chimpanzee samples, while 9.1% of humans were infected with this protozoan [37,38]. *Giardia*, once considered a harmless parasite for humans and animals, is now recognized as a pathogenic agent [4]. *G. lamblia* occurs worldwide and is a common inhabitant of the small intestine of humans, rhesus monkeys, cynomolgus monkeys, chimpanzees, gorillas and other NHPs [39–41]. We also detected in feces of NHPs *Bodo* spp., a free-living nonpathogenic flagellate. Other pathogenic protozoa reported in feces of non-human and human primates, such as *Plasmodium*, *Leishmania, Trypanosoma, Cryptosporidium, Toxoplasma* and *Babesia* [4,22] have not been detected in the present study. Except for *Cryptosporidium*, the other parasites have an extraintestinal localization, so do not found them in stools is not surprising and samples as blood will be more suitable for their surveillance.

As for helminths, at least ten *Nematoda* species were detected in NHPs versus four species in humans in this study. In gorillas, filariid infections (including *Mansonella* spp.) were the most prevalent, followed by *N. americanus* and *S. stercoralis* which were detected in human co-habited gorillas.

A wide range of filariid nematodes has been reported in great apes and monkeys [4]. Interestingly, we detected DNA of *M. perstans* in two gorilla stools and *Mansonella* spp. in two others. Several non-perstans *Mansonella* were reported in apes from Congo and neighboring countries. This includes: *Mansonella* (*Esslingeria*) (Chabaud & Bain, 1976), *M. (E.) leopoldi* from *Gorilla gorilla* in the Republic of the Congo [42] and Gabon [43], *M. (E.) lopeensis* from *Gorilla gorilla* in Gabon [43], *M. (E.) vanhoofi* from *Pan paniscus* in DR Congo [44,45] and from *Gorilla gorilla* in the Republic of Congo [42]; (M) *(E.) streptocerca* and *M. (E.) rodhaini* are two filariid parasites reported from chimpanzees and gorillas [46–48]. Chimpanzees in Central Africa are the reservoir of *M. rodhaini*, which was identified in skin biopsy samples from several villagers in Gabon [49]. *M. (E.) vanhoofi*, a filariid parasite of the chimpanzee, inhabits the mesenteries and the connective tissue adjacent to the gallbladder, bile duct, liver, pancreas and kidney and the loose connective tissues and lymphatics surrounding the hepatic blood vessels [4,46]. Three *Mansonella* species often infect humans: *M. perstans, M. ozzardi* and *M. streptocerca*. Whereas *M. perstans* has never been reported to infect NHPs, except a report in *Gorilla gorilla* and *Pan troglodytes* in Cameroon (Reichenow 1917) [50]. *M. streptocerca* has been found in primates [48]. Based on our

findings, it will be very important to study *Mansonella* infections in great apes to achieve a better understanding of this genus.

Several other filariids have been reported from the great apes, including *Dirofilaria immitis* from the heart of an orangutan [51] and in the abdominal cavity of another orangutan [52] and *Loa loa* and *Onchocerca volvulus* from chimpanzees and gorillas [4,42,53], but none of them were detected in this study.

In our study, the identified infection rate for *S. stercoralis* was lower compared with that reported by Lilly et al. (2002) (82–94% in NHPs and 30–93% in humans) [33]. It was also lower than the prevalence 74.3% and 100% observed in two chimpanzee communities in Gombe National Park, Tanzania [53] and one in Kibale National Park, Uganda [54], respectively. In contrast, the prevalence of *Strongyloides* in gorillas in our study was higher than the prevalence (1.4%) in gorilla populations from Rwanda [22] and almost similar to the prevalence (10.9%) in chimpanzees from Rubondo National Park, Tanzania [55]. These results contrast with those of Hasegawa et al. who reported the presence of *S. stercoralis* in fecal samples from local human populations and the absence of this parasite in wild western lowland gorillas (*Gorilla gorilla*) and a central chimpanzee (*Pan troglodytes troglodytes*) living in the Dzanga-Sangha Protected Areas (DSPA), Central African Republic and in eastern chimpanzees (*Pan troglodytes schweinfurthii*) living in degraded forest fragments on agricultural land in Bulindi, Uganda [37,56]. Conversely, a study carried out in 2017 in Thailand revealed the presence of *S. stercoralis* in human communities in contact with long-tailed macaques with a prevalence of 8.92% [57].

In the case of *N. americanus*, the prevalence observed in this study in great apes and humans is lower than the rates of 86% in wild lowland gorillas in Cameroon [58] and 44% in chimpanzees from Uganda [37]. Our study concords with the study of Hasegawa et al. conducted in Central African Republic. It has been shown that *Necator* hookworms are shared by humans and great apes co-habiting the same tropical forest ecosystems [59]. Another nematode detected in both apes (Chimpanzee from Rep. of Congo) and humans in our study was *A. lumbricoides* with low infection rates. These rates are lower than the rates of *Ascaroides* observed by Lilly et al. (2002) in apes (14–88%) and humans (0–15%). *E. vermicularis* was detected with a low prevalence only in apes (2.3%). *E. vermicularis* and other *Enterobius* species found in Old World monkeys and great apes as well as humans, *E. anthropopitheci* in the chimpanzees and in several species of prosimian primates. These parasites are considered cosmopolitan in their geographical distribution [4]. Naturally infected NHPs could be sources of infection for humans. In addition, captive primates can acquire *E. vermicularis* infection from humans and then can act as reservoirs to reinfect humans [39,60].

A. caucasica has been detected with high prevalence (57%) only in wild Senegalese wild chimpanzees as reported in Laidoudi et al. (submitted). Physalopteriasis, is a disease caused by members of the genus *Physaloptera*. Nine species of physalopterids have been reported to occur in the upper gastrointestinal tract of NHPs [51]. *A. caucasica* has been found in the esophagus, stomach and small intestine of the rhesus macaques, baboons and orangutans [39,42,61–63]. This parasite can be transmitted to humans, but complete life-cycle is not identified until now. It has been reported from humans in Brazil, Colombia, Congo Republic (Zaire), India, Indonesia, Israel, Namibia, Panama, Zambia and Zimbabwe [4]. In one case, in an Indonesian woman, adult worms were recovered from the bile duct, where they had caused biliary pain, jaundice and fever [4].

In addition, *O. muntiacum* was detected in 4.3% of North African macaques in the present study. Oesophagostomiasis is caused by infection of nematodes from the genus *Oesophagostomum*, the nodular worm. Eggs are shed in the feces of the definitive host and may be indistinguishable from the eggs of *Necator* and *Ancylostoma*. Eggs hatch into rhabditiform (L1) larvae in the environment, given appropriate temperature and level of humidity. In the environment, the larvae will undergo two molts and become infective filariform (L3) larvae. Worms can go from eggs to L3 larvae in a matter of a few days, given appropriate environmental conditions. Definitive hosts become infected after ingesting infective L3 larvae. After ingestion, L3 larvae burrow into the submucosa of the large or small intestine and induce cysts. Within these cysts, the larvae molt and become L4 larvae.

These L4 larvae migrate back to the lumen of the large intestine, where they molt into adults. Eggs appear in the feces of the final host approximately one month after ingestion of infectious L3 larvae (https://www.cdc.gov/dpdx/oesophagostomiasis/index.html). These parasites are considered the most common nematodes found in Old World monkeys and great apes, which constitute the definitive hosts. They have been described in baboons, mangabeys, guenons, macaques, chimpanzees and gorillas [4,64,65]. They are rare in New World monkeys [4,66,67]. *Oesophagostomum* spp. parasite normally ruminants, pigs and monkeys and occasionally humans [68]. It has been shown that multiple cryptic forms of *Oesophagostomum* circulate in populations of primates in western Uganda, and that parasitic clades differ in terms of host range and potential transmission between species [69].

Finally, using the high-sensitive PCR systems, we detected—among other things—three unidentifiable genotypes. Of them, two may belong to the environmental nematodes such *Pelodera* sp. and an environmental *Nematoda* sp. in chimpanzee feces. This may be an environmental contamination since samples were collected on the ground. One *Nematoda* sp. we detected in gorillas remains difficult to interpret. On one hand, the gene does not correspond to any known species deposited in the GenBank, on the other hand, it is grouped among the other pathogens of the phylogenetic tree (Figure 2), it can thus belong to a pathogenic species but not described or not characterized. Further, we detected DNA of uncharacterized Nematoda in apes and humans, but with a relatively lower prevalence in humans. Characterization of these parasites by standard PCR-sequencing was not possible due to co-infections with more nematodes in the same sample and/or small amounts of DNA since most of the collected NHPs samples are degraded. In addition, DNA sequences of wild parasite range are not available, which makes the molecular characterization difficult. This requires further molecular studies, parasite characterization and database enrichments.

The present study remains interesting on the knowledge of parasites of NHPs and humans in Africa. One of its strengths that we performed a large PCR screening on samples from different countries using a non-invasive sampling method. In addition, different great ape and monkey species were involved, as well as humans in contact with great apes to look at the zoonotic risk. Nevertheless, it has some limitations such as: the low number of samples for some species (baboons and green monkeys), underestimated prevalence of extraintestinal parasites (*Plasmodium*, Filaria, Kinetoplastida etc.), since feces are not the preferred sampling method. These limits require further investigation in future studies using other samples such blood.

4. Material and Methods

4.1. Animals and Study Area

Feces from humans and NHPs were collected (Table 2). The sampling was non-invasive and did not harm the wild fauna. For NHPs, fecal samples were collected at sleeping sites, feeding sites and places where the primates had been observed. Gorilla and macaque samples, ten from chimpanzees and samples from hamadryas baboons were degraded stored in absolute alcohol. Thirty-eight chimpanzee and all human stool samples were collected in the fresh state. All degraded samples were stored in absolute alcohol, and fresh samples and human samples were first stored at −20 °C before being sent from the Republic of Congo to France for analysis.

All humans that participated in the study were apparently healthy, the health status of animals is unknown. All samples collected were brought to the IHU Méditerranée Infection laboratory, Marseille, France, where they were stored at −20 °C or −80 °C until further analysis.

Table 2. Study subjects and study sites.

Species	Country	Region	Coordinates	Number (date)
Non-human primates				160 (2015–2018)
Chimpanzee (*Pan troglodytes*)	Senegal	Kédougou	12°22′57.1404″N 12°17′16.7172″W	3 (2016)
			12°22′53.1732″N 12°17′26.7936″W	7 (2016)
			12°22′47.7084″N 12°17′48.588″W	38 (2016)
	Rep. Congo	Odzala-Kokoua NP	1.3206°″N 14.8455°″E	1 (2017)
Gorillas (*Gorilla gorilla*)	Rep. Congo	Lésio-Louna NP	2°58′33.1″S 15°28′33.4″E	16 (2015), 12 (2017)
		Odzala-Kokoua NP	1.3206°″N, 14.8455°″E	10 (2017)
		Nouabale-Ndoki NP	2.5857°″N, 16.6291°″E	1 (2017)
Baboons (*Papio papio*)	Senegal	Niokolo-Koba NP	13°04′28.6″N 12°43′18.2″W	7 (2015)
Baboons (*Papio hamadryas*)	Djibouti	Oueah	11°29′56.1″N 42°51′14.8″E	6 (2017)
Barbary macaques (*Macaca sylvanus*)	Algeria	Chréa NP	36°23′42.9″N 2°45′53.6″E	30 (2018)
		Cap Carbon	36°46′31.6″N 5°06′11.2″E	39 (2018)
Humans	Rep. Congo	Mbomo village	1.3206°″N, 14.8455°″E	35 (2017)
		Lésio-Louna (Eco-guards)	2°58′33.1″S 15°28′33.4″E	3 (2017)

NP: national park

4.2. Ethic Statement

Study authorizations were obtained from the Direction National of Parks (DNP) in the Senegalese Ministry of the Environment (DNP, No. 1302, Oct 16, 2015) for Senegalese primates, the Ministry of Health (No 208/MSP/CAB.15 of 20 August 2015) and the Forest Economy and Sustainable Development (No 94/MEFDD/CAB/DGACFAP-DTS of 24 August 2015) of the Rep. of Congo for humans and gorillas respectively, the Center for Studies and Research of Djibouti for baboons (*Papio hamadryas*) and the management of the Chréa National Park (CNP) in Blida Province, Algeria, for Barbary macaques. No experimentation was conducted on NHPs, as fecal samples were collected from the soil. Human samples were taken after obtaining verbal consents of participants due to their low literacy.

4.3. DNA Extraction

DNA extraction was performed using the EZ1®DNA tissue kit (Qiagen, Hiden, Germany) on BIOROBOT EZ1 (Qiagen, Hiden, Germany), according to the manufacturer's instructions. Initially, we mixed in tubes about 200 mg of stool with 360 µL of G2 lysis buffer (Qiagen, Hiden, Germany). This was mechanically lysed with tungsten beads (Qiagen, Hiden, Germany) using FastPrep-24TM 5G Grinder for 40 s. After 10 min of incubation at 100 °C to allow for complete lysis, tubes were centrifuged at 10,000× *g* for 1 min. Subsequently, 200 µL of supernatant was enzymatically digested using 20 µL of proteinase K (20 mg/mL, Qiagen) and incubated overnight at 56 °C. DNA was extracted from 200 µL of sample, eluted in 200 µL volume, then aliquoted in individual tubes of: pure extracted DNA, diluted to 1:10 and finally DNA diluted to 1:100.

To control the extraction quality and the absence of PCR inhibitors, universal eubacterial qPCR targeting the 16S rRNA bacterial genes, named "qPCR all bacteria" [70], was performed on pure DNA, dilutions to1:10 and to 1:100. By comparison of Ct values obtained for pure and diluted DNAs, the dilution to1:10 were chosen for parasite screening. DNA tubes were stored at −20 °C until use.

4.4. Molecular Screening for Parasites by Real-Time PCR Assays (qPCR)

DNA dilutions to 1:10 were screened for parasites in order to evade the nonspecific PCR inhibition. We have used single target real-time PCR assays specific for each pathogen. Eight qPCR assays targeting a large number of parasite members (class, order or genus) and seventeen different species—specific primers and Taqman probes (hydrolysis probes), all known for their specificity and sensitivity, were used in multiparallel assays, as shown in Table 3.

The qPCR amplifications were performed in a CFX96 Real-time system (BioRad Laboratories, Foster City, CA, USA). Reactions were performed in a volume of 20 µL, containing 5 µL of DNA template, 10 µL of Master Mix Roche (Eurogentec, Seraing, Belgium), 0.5 µL each primer per reaction at the concentration of 20 µM, 0.5 µL UDG and 0.5 µL of each probe at the concentration of 5 µM. The TaqMan cycling conditions included two hold steps at 50 °C for 2 min, followed by 95 °C for 15 min, and 40 cycles of two steps each (95 °C for 30 s and 60 °C for 30 s). Each PCR plate contains 96-wells. Known DNAs or plasmids were used as positive controls and master mixtures as a negative control in each reaction.

4.5. Genetic Amplification by Standard PCR, Sequencing and Phylogeny

In addition to species-specific qPCR screening, we performed screening by broad-range qPCRs for pan-Nematoda and for pan-Kinetoplastida parasites, followed by PCR/ sequencing. Positive samples in pan-Nematoda qPCR were subjected to standard PCRs targeting 18S rRNA gene of Nematoda and the 28S rRNA gene of helminths. Positive ones for Kinetoplastida qPCR were amplified using primer pairs for 18S and 28S genes of Kinetoplastida (Table 3). Amplifications were carried out in a total volume of 50 µL, consisting of 25 µL of AmpliTaq Gold master mix, 18 µL of ultra-purified water DNAse-RNAse free, 1 µL of primers (20 µM of concentration) and 5 µL of DNA template. The thermal cycling conditions amplifications were as follows: incubation step for 15 min at 95 °C, 40 cycles of: one minute at 95 °C, 30 s for at the annealing temperature (Table 3), an elongation step at 72 °C. Finally, an extension step for five minutes at 72 °C. This was performed in a Peltier PTC-200 model thermal cycler (MJ Research, Inc., Watertown, MA, USA) and visualized on 2% agarose gel. In a second time, amplicons were purified using NucleoFast 96 PCR plates (Macherey–Nagel EURL, Hoerdt, France) as per the manufacturer's instructions and sequenced using the Big Dye Terminator Cycle Sequencing Kit (PerkinElmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). Generated electropherograms were assembled and edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) and compared with those available in the GenBank database by NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The fragments obtained were compared with each other and with related fragments available in the GenBank database. The phylogenetic analyses were inferred using neighbor joining methods and tree reconstructions were performed using MEGA software version 7 (https://www.megasoftware.net/). Bootstrap analyses were conducted using 1000 replicates.

Table 3. Primer and probe sequences used in this study.

Parasite	Target Gene	Primer Name	Sequence (5′-3′)	Source
Kinetoplastida	28s	F. 24a.5198	AGTATTGAGCCAAAGAAGG	[71]
		R. 24a.5412	TTGTCACGACTTCAGGTTCTAT	
		P. 24a.5345	FAM-TAGGAAGACCGATAGCGAACAAGTAG-TAMRA	
Leishmania spp.	18s	F	GGTTTAGTGCGTCCGGTG	[72]
		R	ACGCCCCAGTACGTTCTCC	
		P	FAM-CGGCCGTAACGCCTTTTCAACTCA-TAMRA	
Trypanosoma spp.	5.8s	F. 5.8 S Tryp 3874	CAACGTGTCGCGATGGATGA	
		R. 5.8 S Tryp 3935	ATTCTGCAATTGATACCACTTATC	
		S. 5.8 S Tryp 3911	FAM-GTTGAAGAACGCAGCAAAGGCGAT-TAMRA	
Piroplasmida	5.8s	5.8S-F5	TCGCAGRAGTCTKCAAGTC	[73]
		5.8S-R	AYYKTYAGCGRTGGATGTC	
		5.8S-S	FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB	
Cyclospora cayetanensis	18s	Cyclo250F	TAGTAACCGAACGGATCGCATT	[74]
		Cyclo350R	AATGCCACGTAGGCCAATA	
		Cyclo281T	FAM-CCGGCGATAGATCATTCAAGTTTCTGACC-TAMRA	
Plasmodium spp.	Cox	Plasmo_cox_15_F	AGGAACTCGACTGGCCTACA	[75]
		Plasmo_cox_16_R	CCAGCGACAGCGGTTATACT	
		Plasmo-cox_P	FAM-CGAACGCTTTTAACGCCTGACATGG-TAMRA	
Toxoplasma gondii	ITS1	Tgon_ITS1_F	GATTTGCATTCAAGAAGCGTGATAGTA	[76]
		Tgon_ITS1_R	AGTTTAGGAAGCAATCTGAAAGCACATC	
		Tgon_ITS1_P	FAM-CTGCGCTGCTTCCAATATTGG-TAMRA	

Table 3. *Cont.*

Parasite	Target Gene	Primer Name	Sequence (5′-3′)	Source
Cryptosporidium parvum; C. hominis	hsp70 gene	1PSF	AACTTTAGCTCCAGTTGAGAAAGTACTC	[77]
		1PSR	AACTTTAGCTCCAGTTGAGAAAGTACTC	
		Crypt P	FAM-AATACGTGTAGAACCACCAACCAATACAACATC-TAMRA	
Giardia lamblia (intestinalis or duodenalis)	18s	Giardia-80F	GACGGCTCAGGACAACGGTT	[78]
		Giardia-127R	TTGCCAGCGGTGTCCG	
		Giardia-105T	FAM-CCCGCGGCGGTCCCTGCTAG-TAMRA	
Entamoeba histolytica	18s	Ehf	AACAGTAATAGTTTCTTTGGTTAGTAAAA	[79]
		Ehr	CTTAGAATGTCATTTCTCAATTCAT	
		Ehp	FAM-ATTAGTACAAAATGGCCAATTCATTCA-TAMRA	
Nematoda	5s	qNem.5S.1f	ACCACGTTGAAAGCACGMC	[29]
		qNem.5S.110r	TGTCTACAACACCTSGRATTCC	
		qNem.5S.38p	FAM-AGTTAAGCAACGTTGGGCC-TAMRA	
Filariae	28S	qFil-28S-F	TTGTTTGAGATTGCAGCCCA	[80]
		qFil-28S-R	GTTTCCATCTCAGCGGTTTC	
		qFil-28S-S	FAM-CAAGTACCGTGAGGGAAAGT-TAMRA	
Mansonella spp.	ITS1	Forward	CCTGCGGAAGGATCATTAAC	[81]
		Reverse	ATCGACGGTTTAGGCGATAA	
		Probe	FAM-CGGTGATATTCGTTGGTGTCT-TAMRA	
Mansonella perstans	ITS1	Forward	AGGATCATTAACGAGCTTCC	
		Reverse	CGAATATCACCGTTAATTCAGT	
		Probe	FAM-TTCACTTTTATTTAGCAACATGCA-TAMRA	

Table 3. *Cont.*

Parasite	Target Gene	Primer Name	Sequence (5′-3′)	Source
Loa loa	LL20 15-kDa ladder antigen	15r3-5	CGAAAAATTATAGGGGGAAAC	[82]
		15r3-6	TCGTAGACCAAACTGCGAAC	
		15r3-P	FAM-TCAAGAGCCGATATACTGAAAGCTATC-TAMRA	
Abbreviata caucasica	12s	Phy.12S.f.204	GAATTGGATTAGTACCCAAGTAAGTG	[29]
		Phy.12S.r.305	TGTTCCAAAAATCTTTCTAAGATCAG	
		Phy.12S.242p	VIC-GCGGGAGTAAAGTTAAGTTTAAACC-TAMRA	
		Cyclo350R	AATGCCACGTAGGCCAATA	
		Cyclo281T	FAM-CCGGCGATAGATCATTCAAGTTTCTGACC-TAMRA	
Necator americanus	ITS2	Na58F	CTGTTTGTCGAACGGTACTTGC	[83]
		Na158R	ATAACAGCGTGCACATGTTGC	
		Na81T	FAM-CTGTACTACGCATTGTATAC-MGB	
Ancylostoma duodenale	ITS2	Ad125F	GAATGACAGCAAACTCGTTGTTG	
		Ad195R	ATACTAGCCACTGCCGAAACGT	
		Ad155-XS	FAM-ATCGTTTACCGACTTTAG-MGB	
Schistosoma mansoni	Tandem repeat units M61098	SRA1	CCACGCTCTCGCAAATAATCT	[84]
		SRS2	CAACCGTTCTATGAAAATCGTTGT	
		SRP	FAM-TCCGAAACCACTGGACGGATTTTTATGAT-TAMRA	
Taenia solium	ITS	Tsol_145F	ATGGATCAATCTGGGTGGAGTT	[85]
		Tsol_230R	ATCGCAGGGTAAGAAAAGAAGGT	
		Tsol_169Tq	FAM-TGGTACTGCTGTGGCGGCGG-TAMRA	
Taenia saginata	ITS	Tsag_F529	GCGTCGTCTTTGCGTTACAC	
		Tsag_R607	TGACACAACCGCGCTCTG	
		Tsag_581Tq	FAM-CCACAGCACCAGCGACAGCAGCAA-TAMRA	

Table 3. *Cont.*

Parasite	Target Gene	Primer Name	Sequence (5′-3′)	Source
Ascaris lumbricoides	ITS1	Alum96F	GTAATAGCAGTCGGCGGTTTCTT	[86]
		Alum183R	GCCCAACATGCCACCTATTC	
		Alum124T	FAM-TTGGCGGACAATTGCATGCGAT-TAMRA	
Trichuris trichiura	18s	TrichF	TTGAAACGACTTGCTCATCAACTT	[87]
		TrichR	CTGATTCTCCGTTAACCGTTGTC	
		TrichP	FAM-CGATGGTACGCTACGTGCTTACCATGG-TAMRA	
Strongyloides stercoralis	18s	Stro-1530F	GAATTCCAAGTAAACGTAAGTCATTAGC	[88]
		Stro-1630R	TGCCTCTGGATATTGCTCAGTTC	
		Stro-1586T	FAM-ACACACCGGCCGTCGCTGC-TAMRA	
Enterobius vermicularis	5s	EnterF	TTTCCAAGCCACAGACTCAC	[31]
		EnterR	ATTGCTCGTTTGCCGATTAT	
		EnterP	TCATGTCTGAGCCGGAACGAGA	
Nematoda	18s	Fwd.18S.631	TCGTCATTGCTGCGGTTAAA	[89]
		Rwd.18S.1825r	GGTTCAAGCCACTGCGATTAA	
Helminths	28s	Hspec.28S. 5748f	GGTAAGGGAAGTCGGCAAAT	This study
		Hspec.28S.6394r	TAGGGACAGTGGGAATCTCG	
Abbreviata caucasica	COI	F.Abbrev.COI.51f	TGATCAGGGTTGGGAGCTT	[29]
		R.Abbrev.COI.601r	AAAAAGAACAATTAAAATTACGATCC	

5. Conclusions

This study provides data on different helminths and protozoa that infect NHPs in Africa and human communities living around them. Parasites known to infect both humans and NHPs have been detected in humans and gorillas living in the same tropical forest ecosystem, suggesting possible interactions. In addition, human specific parasites, such as *Mansonella perstans*, causative agent of one of the major human neglected tropical diseases, was detected in gorillas, suggesting an exchange between humans and NHPs and other investigations are required at this stage for better understanding these findings. However, prevalence of extraintestinal parasites remain underestimated since feces are not the preferable sampling method and samples such as blood can give more information. In addition, public health and animal conservation authorities need to be aware of these infections, as the parasites observed in African NHPs could affect both human and animal health.

Author Contributions: H.M., I.A., B.D., D.R. and O.M. designed the study. H.M., I.A., Y.L., B.D., J.A., I.B., A.L., G.D., C.S., S.G.; D.R. and O.M. collected the samples. H.M., I.A. performed the lab work. H.M., I.A., Y.L., F.F. and O.M. carried out the data analysis. H.M. and I.A. drafted the manuscript. J.A., B.D., I.B., G.D., L.P., R.A.H.-A., A.B., F.F., D.R. and O.M. reviewed and edited the manuscript. All authors read and approved the final manuscript.

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI, Service de Coopération et d'Action Culturelle (SCAC) de l'Ambassade de la France en République du Congo, Laboratoire National de Santé Publique de Brazzaville.

Acknowledgments: We are grateful to Ismail Lafri, Rahal Mohamed and Fayçal Zeroual, Direction National of Parks (DNP), in Senegalese Ministry of the Environment, Agence Congolaise de la Faune et des Aires Protégées (Republic of Congo), Ministry of Health and Population (the Republic of Congo) for their valuable assistance in this work. We thank the Direction des Parcs Nationaux and the Direction des Eaux et Forests Chasses et de la Conservation des Sols for permission to work in Senegal. We thank Paula Alvarez Varona, Daouda Diallo, Mamadou F. Diallo y Mamadou Samba Silla for help in the field in the Dindefelo Community Natural Reserve, Senegal.

Conflicts of Interest: The authors declare no conflicts of interest. The funding sponsors had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript and in the decision to publish the results.

Consent for Publication: Not applicable.

Availability of Data and Materials: The data supporting the conclusions of this article are included within the article.

References

1. Cox, F.E.G. History of human parasitic diseases. *Infect. Dis. Clin. N. Am.* **2004**, *18*, 171–188. [CrossRef] [PubMed]
2. Cao, B.; Guiton, P.S. Important Human Parasites of the Tropics. *Front. Young Minds* **2018**, *6*, 1–10. [CrossRef]
3. Devaux, C.A.; Mediannikov, O.; Medkour, H.; Raoult, D. Infectious Disease Risk Across the Growing Human-Non Human Primate Interface: A Review of the Evidence. *Front. Public Health* **2019**, *7*, 1–22. [CrossRef] [PubMed]
4. Strait, K.; Else, J.G.; Eberhard, M.L. Parasitic diseases of non human primates. In *Nonhuman Primates in Biomedical Research*, 2nd ed.; Elsevier Inc.: Cambridge, MA, USA, 2012. [CrossRef]
5. Gómez, J.M.; Nunn, C.L.; Verdú, M. Centrality in primate-parasite networks reveals the potential for the transmission of emerging infectious diseases to humans. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 7738–7741. [CrossRef] [PubMed]
6. Ashford, R.W.; Reid, G.D.F.; Butynski, T.M. The intestinal faunas of man and mountain gorillas in a shared habitat. *Ann. Trop. Med. Parasitol.* **1990**, *84*, 337–340. [CrossRef] [PubMed]
7. Muriuki, S.M.K.; Murugu, R.K.; Munene, E.; Karere, G.M.; Chai, D.C. Some gastro-intestinal parasites of zoonotic (public health) importance commonly observed in old world non-human primates in Kenya. *Acta Trop.* **1998**, *71*, 73–82. [CrossRef]

8. Nizeyi, J.; Cranfield, M.; Graczyk, T. Cattle near the Bwindi Impenetrable National Park, Uganda, as a reservoir of *Cryptosporidium parvum* and *Giardia duodenalis* for local community and free-ranging gorillas. *Parasitol. Res.* **2002**, *88*, 380–385. [CrossRef]
9. Leendertz, F.H.; Pauli, G.; Maetz-Rensing, K.; Boardman, W.; Nunn, C.; Ellerbrok, H.; Jensen, S.A.; Junglen, S.; Christophe, B. Pathogens as drivers of population declines: The importance of systematic monitoring in great apes and other threatened mammals. *Biol. Conserv.* **2006**, *131*, 325–337. [CrossRef]
10. Mohammed Mahdy, A.K.; Lim, Y.A.L.; Surin, J.; Wan, K.L.; Al-Mekhlafi, M.S.H. Risk factors for endemic giardiasis: Highlighting the possible association of contaminated water and food. *Trans. R. Soc. Trop. Med. Hyg.* **2008**, *102*, 465–470. [CrossRef]
11. Vlčková, K.; Kreisinger, J.; Pafčo, B.; Čížková, D.; Tagg, N.; Hehl, A.B.; Modrý, D. Diversity of *Entamoeba* spp. in African great apes and humans: An insight from Illumina MiSeq high-throughput sequencing. *Int. J. Parasitol.* **2018**, *48*, 519–530. [CrossRef]
12. Hamad, I.; Forestier, C.L.; Peeters, M.; Delaporte, E.; Raoult, D.; Bittar, F. Wild gorillas as a potential reservoir of *Leishmania major*. *J. Infect. Dis.* **2015**, *211*, 267–273. [CrossRef]
13. Medkour, H.; Davoust, B.; Levasseur, A.; Mediannikov, O. Molecular Evidence of *Leishmania infantum* and *Leishmania guyanensis* in Red Howler Monkey (*Alouatta seniculus*) From French Guiana. *Vector Borne Zoonotic Dis.* **2019**, *19*, 1–5. [CrossRef] [PubMed]
14. Else, J.G.; Satzger, M.; Sturrock, R.F. Natural infections of schistosoma mansoni and S. Haematobium in cercopithecus monkeys in kenya. *Ann. Trop. Med. Parasitol.* **1982**, *76*, 111–112. [CrossRef] [PubMed]
15. Faust, C.; Dobson, A.P. Primate malarias: Diversity, distribution and insights for zoonotic *Plasmodium*. *One Health* **2015**, *1*, 66–75. [CrossRef]
16. Liu, W.; Li, Y.; Learn, G.H.; Rudicell, R.S.; Robertson, J.D.; Keele, B.F.; Ndjango, J.-B.N.; Sanz, C.M.; Morgan, D.B.; Locatelli, S.; et al. Origin of the human parasite *Plasmodium falciparum* in gorillas. *Nature* **2010**, *467*, 420–425. [CrossRef] [PubMed]
17. Liu, W.; Li, Y.; Shaw, K.S.; Learn, G.H.; Plenderleith, L.J.; Malenke, J.A.; Sundararaman, S.A.; Ramirez, M.A.; Crystal, P.A.; Smith, A.G.; et al. African origin of the malaria parasite *Plasmodium vivax*. *Nat. Commun.* **2014**, *5*, 3346. [CrossRef] [PubMed]
18. Lambrecht, F.; Dunn, F.; Eyles, D. Isolation of *Plasmodium knowlesi* from Philippine Macaques. *Nature* **1961**, *191*, 1117–1118. [CrossRef] [PubMed]
19. Singh, J.; Ray, A.; Nair, C. Isolation of a new strain of *Plasmodium knowlesi*. *Nature* **1953**, *172*, 122. [CrossRef]
20. Chin, W.; Contacos, P.G.; Coatney, G.R.; Kimball, H.R. A naturally acquired quotidian-type malaria in man transferable to monkeys. *Science* **1965**, *149*, 865. [CrossRef]
21. Müller, M.; Schlagenhauf, P. *Plasmodium knowlesi* in travellers, update 2014. *Int. J. Infect. Dis.* **2014**, *22*, 55–64. [CrossRef]
22. Sleeman, J.M.; Meader, L.L.; Mudakikwa, A.B.; Foster, J.W.; Patton, S. Gastrointestinal Parasites of Mountain Gorillas (*Gorilla Gorilla Beringei*) in the Parc National Des Volcans, Rwanda. *J. Zoo Wildl. Med.* **2000**, *31*, 322–328. [CrossRef] [PubMed]
23. Bhagwant, S. Human *Bertiella studeri* (family *Anoplocephalidae*) infection of probable Southeast Asian origin in Mauritian children and an adult. *Am. J. Trop. Med. Hyg.* **2004**, *70*, 225–228. [CrossRef] [PubMed]
24. Estrada, A.; Garber, P.A.; Rylands, A.B.; Roos, C.; Fernandez-Duque, E.; Di Fiore, A.; Anne-Isola Nekaris, K.; Nijman, V.; Heymann, E.W.; Lambert, J.E.; et al. Impending extinction crisis of the world's primates: Why primates matter. *Sci. Adv.* **2017**, *3*. [CrossRef] [PubMed]
25. Zommers, Z.; Macdonald, D.W.; Johnson, P.J.; Gillespie, T.R. Impact of human activities on chimpanzee ground use and parasitism (*Pan troglodytes*). *Conserv. Lett.* **2013**, *6*, 264–273. [CrossRef]
26. Wenz-Mücke, A.; Sithithaworn, P.; Petney, T.N.; Taraschewski, H. Human contact in fl uences the foraging behaviour and parasite community in long-tailed macaques. *Parasitology* **2013**, *140*, 709. [CrossRef] [PubMed]
27. Kowalewski, M.M.; Gillespie, T.R. *Primatology, Biocultural Diversity and Sustainable Development in Tropical Forests*; UNESCO Office: Mexico City, Mexico, December 2018.
28. Conly, J.M.; Johnston, B.L. The infectious diseases consequences of monkey business. *Can. J. Infect. Dis. Med. Microbiol.* **2008**, *19*, 12–14. [CrossRef]
29. Laidoudi, Y.; Medkour, H.; Latrofa, M.S.; Davoust, B.; Sokhna, C.; Barciela, A.; Hernandez-Aguilar, R.A.; Raoult, D.; Otranto, D.; Mediannikov, O. Zoonotic *Abbreviata caucasica* in Wild Chimpanzees (*Pan troglodytes verus*) from Senegal. *Pathogens* **2020**, *9*, 517. [CrossRef]

30. Fenollar, F.; Mediannikov, O. Emerging infectious diseases in Africa in the 21st century. *New Microbes New Infect.* **2018**, *26*, S10–S18. [CrossRef]
31. Sow, D.; Parola, P.; Sylla, K.; Ndiaye, M.; Delaunay, P.; Halfon, P.; Camiade, S.; Dieng, T.; Tine, R.C.K.; Faye, B.; et al. Performance of real-time polymerase chain reaction assays for the detection of 20 gastrointestinal parasites in clinical samples from Senegal. *Am. J. Trop. Med. Hyg.* **2017**, *97*, 173–182. [CrossRef]
32. Landsoud-Soukate, J.; Tutin, C.E.G.; Fernandez, M. Intestinal parasites of sympatric gorillas and chimpanzees in the Lope Reserve, Gabon. *Ann. Trop. Med. Parasitol.* **1995**, *89*, 73–79. [CrossRef]
33. Lilly, A.A.; Mehlman, P.T.; Doran, D. Intestinal parasites in gorillas, chimpanzees, and humans at Mondika research site, dzanga-ndoki national park, Central African Republic. *Int. J. Primatol.* **2002**, *23*, 555–573. [CrossRef]
34. Renquist, D.M.; Whitney, R.A. Zoonoses acquired from pet primates. *Vet. Clin. N. Am. Small Anim. Pract.* **1987**, *17*, 219–240. [CrossRef]
35. Nygård, K.; Schimmer, B.; Søbstad, Ø.; Walde, A.; Tveit, I.; Langeland, N.; Hausken, T.; Aavitsland, P. A large community outbreak of waterborne giardiasis—delayed detection in a non-endemic urban area. *BMC Public Health* **2006**, *6*, 1–10. [CrossRef] [PubMed]
36. Gillespie, T.R.; Greiner, E.C.; Chapman, C.A. Gastrointestinal Parasites of the Guenons of Western Uganda. *J. Parasitol.* **2004**, *90*, 1356–1360. [CrossRef] [PubMed]
37. Nolan, M.J.; Unger, M.; Yeap, Y.T.; Rogers, E.; Millet, I.; Harman, K.; Fox, M.; Kalema-Zikusoka, G.; Blake, D.P. Molecular characterisation of protist parasites in human-habituated mountain gorillas (*Gorilla beringei beringei*), humans and livestock, from Bwindi impenetrable National Park, Uganda. *Parasites Vectors* **2017**, *10*, 1–12. [CrossRef]
38. Drakulovski, P.; Bertout, S.; Locatelli, S.; Butel, C.; Pion, S.; Mpoudi-Ngole, E.; Delaporte, E.; Peeters, M.; Mallié, M. Assessment of gastrointestinal parasites in wild chimpanzees (*Pan troglodytes troglodytes*) in southeast Cameroon. *Parasitol. Res.* **2014**, *113*, 2541–2550. [CrossRef]
39. Baker, D.G. Parasites of non-human primates. In *Flynn's Parasites of Laboratory Animals*, 2nd ed.; Blackwell Publishing: Ames, IA, USA, 2008; pp. 693–743. [CrossRef]
40. Ruch, T.C. Diseases of Laboratory Primates. *Ann. Surg.* **1961**, *153*, 137.
41. Karim, M.R.; Wang, R.; Yu, F.; Li, T.; Dong, H.; Li, D.; Zhang, L.; Li, J.; Jian, F.; Zhang, S.; et al. Multi-locus analysis of *Giardia duodenalis* from nonhuman primates kept in zoos in China: Geographical segregation and host-adaptation of assemblage B isolates. *Infect. Genet. Evol.* **2015**, *30*, 82–88. [CrossRef] [PubMed]
42. Van Den Berghe, L.; Chardome, M.; Peel, E. The Filarial Parasites of the Eastern Gorilla in the Congo. *J. Helminthol.* **1964**, *38*, 349–368. [CrossRef] [PubMed]
43. Fernando Caserta Tencatt, L.; Ribeiro de Britto, M.; Simone Pavanelli, C. Revisionary study of the armored catfish *Corydoras paleatus* (Jenyns, 1842) (Siluriformes: Callichthyidae) over 180 years after its discovery by Darwin, with description of a new species. *Neotrop. Ichthyol. J.* **2016**, *14*, 315–322. [CrossRef]
44. Pommier de Santi, V.; Briolant, S.; Mahamat, A.; Ilcinkas, C.; Blanchet, D.; de Thoisy, B.; Reynaud, Y.; Hyvert, G.; Lou Marié, J.; Edouard, S.; et al. Q fever epidemic in Cayenne, French Guiana, epidemiologically linked to three-toed sloth. *Comp. Immunol. Microbiol. Infect. Dis.* **2018**, *56*, 34–38. [CrossRef] [PubMed]
45. Thompson, P. Overview of Rift Valley Fever. Available online: https://www.merckvetmanual.com/generalized-conditions/rift-valley-fever/overview-of-rift-valley-fever (accessed on 11 July 2020).
46. Mohan, S.; Mohan, H.; Mohan, S. Infectious and Parasitic Diseases. *Essent. Pathol. Dent. Stud.* **2017**, *166*, 29. [CrossRef]
47. Chalifoux, L.V. *Filariasis, New World Primates*; Springer: Berlin/Heidelberg, Germany, 1993; pp. 206–214. [CrossRef]
48. Bain, O.; Mutafchiev, Y.; Junker, K.; Guerrero, R.; Martin, C.; Lefoulon, E.; Uni, S. Review of the genus *Mansonella* Faust, 1929 sensu lato *(Nematoda: Onchocercidae*), with descriptions of a new subgenus and a new subspecies. *Zootaxa* **2015**, *3918*, 151–193. [CrossRef] [PubMed]
49. Mediannikov, O.; Ranque, S. Mansonellosis, the most neglected human filariasis. *New Microbes New Infect.* **2018**, *26*, S19–S22. [CrossRef]
50. Agerholm, J.S. *Coxiella burnetii* associated reproductive disorders in domestic animals-a critical review. *Acta Vet. Scand.* **2013**, *55*, 13. [CrossRef]
51. Sandosham, A.A. On Two Helminths from the Orang Utan, *Leipertrema rewelli* n.g., n.sp. and *Dirofilaria immitis* (Leidy, 1856). *J. Helminthol.* **1951**, *25*, 19–26. [CrossRef]

52. Rodhain, J. Corollaire à l'étude de E. Peel et M. Chardome sur les filaridés des chimpanzés au Congo belge. *Ann. Soc. Belge Med. Trop.* **1947**, *26*, 157–160.
53. Park, N.; Gillespie, T.R.; Lonsdorf, E.V.; Canfield, E.P.; Meyer, D.J.; Nadler, Y.; Raphael, J.; Pusey, A.E.; Pond, J.; Pauley, J.; et al. Demographic and ecological effects on patterns of parasitism in eastern chimpanzees (*Pan troglodytes schweinfurthii*) in Gombe National Park, Tanzania. *Am. J. Phys. Anthropol.* **2014**, *143*, 534–544. [CrossRef]
54. Muehlenbein, M.P. Parasitological analyses of the male chimpanzees (*Pan troglodytes schweinfurthii*) at Ngogo, Kibale National Park, Uganda. *Am. J. Primatol.* **2005**, *65*, 167–179. [CrossRef]
55. Petrželkov, K.J.; Hasegawa, H.; Appleton, C.C.; Huffman, M.A.; Archer, C.E.; Moscovice, L.R.; Mapua, M.I.; Singh, J.; Kaur, T. Gastrointestinal parasites of the chimpanzee population introduced onto Rubondo Island National Park, Tanzania. *Am. J. Primatol.* **2010**, *72*, 307–316. [CrossRef]
56. Hasegawa, H.; Sato, H.; Fujita, S.; Nguema, P.P.M.; Nobusue, K.; Miyagi, K.; Kooriyama, T.; Takenoshita, Y.; Noda, S.; Sato, A.; et al. Molecular identification of the causative agent of human strongyloidiasis acquired in Tanzania: Dispersal and diversity of *Strongyloides* spp. and their hosts. *Parasitol. Int.* **2010**, *59*, 407–413. [CrossRef] [PubMed]
57. Thanchomnang, T.; Intapan, P.M.; Sanpool, O.; Rodpai, R.; Tourtip, S.; Yahom, S.; Kullawat, J.; Radomyos, P.; Thammasiri, C.; Maleewong, W. First molecular identification and genetic diversity of *Strongyloides stercoralis* and *Strongyloides fuelleborni* in human communities having contact with long-tailed macaques in Thailand. *Parasitol. Res.* **2017**, *116*, 1917–1923. [CrossRef] [PubMed]
58. Hamad, I.; Keita, M.B.; Peeters, M.; Delaporte, E.; Raoult, D.; Bittar, F. Pathogenic eukaryotes in gut microbiota of western lowland gorillas as revealed by molecular survey. *Sci. Rep.* **2014**, *4*, 1–9. [CrossRef] [PubMed]
59. Hasegawa, H.; Modrý, D.; Kitagawa, M.; Shutt, K.A.; Todd, A.; Kalousová, B.; Profousová, I.; Petrželková, K.J. Humans and Great Apes Cohabiting the Forest Ecosystem in Central African Republic Harbour the Same Hookworms. *PLoS Negl. Trop. Dis.* **2014**, *8*, 1–10. [CrossRef] [PubMed]
60. Levine, N.D. Diseases of Man Acquired from His Pets. *Am. J. Trop. Med. Hyg.* **1968**, *17*, 906–907. [CrossRef]
61. Yamaguti, S. *Systema Helminthum. The Nematodes of Vertebrates*; Interscience (Wiley): New York, NY, USA, 1962; Volume 3, p. 1261.
62. Rodrigo, R.K.; Perera, M.S.J.; Perera, N.; Peries, T.N.; de Silva, S.; Bambaradeniya, C.N.B. A preliminary survey on the herpetofauna in the Anawilundawa wetland sanctuary: The sond Ramsar site of Sri Lanka. *Tigerpaper* **2008**, *35*, 27–32.
63. Essa, A. Worms and human disease. *J. Clin. Pathol.* **2004**, *57*, 110–111. [CrossRef]
64. Vyas, R. Preliminary survey of herpetofauna of Narayan sarovar Sanctuary, Gujarat. *Zoos' Print J.* **2002**, *17*, 812–814. [CrossRef]
65. Remfry, J. The incidence, pathogenesis and treatment of helminth infections in rhesus monkeys (*Macaca mulatta*). *Lab. Anim.* **1978**, *12*, 213–218. [CrossRef]
66. Toft, J.D. The pathoparasitology of nonhuman primates: A review. In *Primates*; Benirschke, K., Ed.; Proceedings in Life Sciences; Springer: New York, NY, USA, 1986.
67. Yamashita, J. Ecological relationships between parasites and primates—I. Helminth Parasites and Primates. *Primates* **1963**, *4*, 1–96. [CrossRef]
68. Polderman, A.M.; Blotkamp, J. *Oesophagostomum* infections in humans. *Parasitol. Today* **1995**, *11*, 451–456. [CrossRef]
69. Ghai, R.R.; Chapman, C.A.; Omeja, P.A.; Davies, T.J.; Goldberg, T.L. Nodule Worm Infection in Humans and Wild Primates in Uganda: Cryptic Species in a Newly Identified Region of Human Transmission. *PLoS Negl. Trop. Dis.* **2014**, *8*, 39. [CrossRef] [PubMed]
70. Dridi, B.; Henry, M.; El Khéchine, A.; Raoult, D.; Drancourt, M. High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *PLoS ONE* **2009**, *4*. [CrossRef] [PubMed]
71. Medkour, H.; Varloud, M.; Davoust, B.; Mediannikov, O. New Molecular Approach for the Detection of Kinetoplastida Parasites of Medical and Veterinary Interest. *Microorganisms* **2020**, *8*, 356. [CrossRef]
72. Medkour, H.; Laidoudi, Y.; Athias, E.; Bouam, A.; Dizoé, S.; Davoust, B.; Mediannikov, O. Molecular and serological detection of animal and human vector-borne pathogens in the blood of dogs from Côte d'Ivoire. *Comp. Immunol. Microbiol. Infect. Dis.* **2020**, *69*, 101412. [CrossRef]

73. Dahmana, H.; Amanzougaghene, N.; Davoust, B.; Normand, T.; Carette, O.; Demoncheaux, J.P.; Mulot, B.; Fabrizy, B.; Scandola, P.; Chik, M.; et al. Great diversity of Piroplasmida in Equidae in Africa and Europe, including potential new species. *Vet. Parasitol. Reg. Stud. Rep.* **2019**, *18*, 100332. [CrossRef]
74. Verweij, J.J.; Laeijendecker, D.; Brienen, E.A.T.; Van Lieshout, L.; Polderman, A.M. Detection of *Cyclospora cayetanensis* in travellers returning from the tropics and subtropics using microscopy and real-time PCR. *Int. J. Med. Microbiol.* **2003**, *293*, 199–202. [CrossRef]
75. Mourembou, G.; Fenollar, F.; Socolovschi, C.; Lemamy, G.J.; Nzoughe, H.; Kouna, L.C.; Toure-Ndouo, F.; Million, M.; Mbiguino, A.N.; Lekana-Douki, J.B.; et al. Molecular detection of fastidious and common Bacteria as well as *Plasmodium* spp. in Febrile and Afebrile children in Franceville, Gabon. *Am. J. Trop. Med. Hyg.* **2015**, *92*, 926–932. [CrossRef]
76. Jauregui, L.H.; Higgins, J.; Zarlenga, D.; Dubey, J.P.; Lunney, J.K. Development of a real-time PCR assay for detection of *Toxoplasma gondii* in pig and mouse tissues. *J. Clin. Microbiol.* **2001**, *39*, 2065–2071. [CrossRef]
77. Garcés-Sanchez, G.; Wilderer, P.A.; Munch, J.C.; Horn, H.; Lebuhn, M. Evaluation of two methods for quantification of hsp70 mRNA from the waterborne pathogen *Cryptosporidium parvum* by reverse transcription real-time PCR in environmental samples. *Water Res.* **2009**, *43*, 2669–2678. [CrossRef]
78. Verweij, J.J.; Blange, R.A.; Templeton, K.; Schinkel, J.; Brienen, E.A.T.; Van Rooyen, M.A.A.; Van Lieshout, L.; Polderman, A.M. *Cryptosporidium parvum* in fecal samples by using Multiplex real-time PCR. *J. Clin. Microbiol.* **2004**, *42*, 1220–1223. [CrossRef] [PubMed]
79. Roy, S.; Kabir, M.; Mondal, D.; Ali, I.K.M.; Petri, W.A.; Haque, R. Real-time-PCR assay for diagnosis of *Entamoeba histolytica* infection. *J. Clin. Microbiol.* **2005**, *43*, 2168–2172. [CrossRef] [PubMed]
80. Laidoudi, Y.; Davoust, B.; Varloud, M.; Niang, E.H.A.; Fenollar, F.; Mediannikov, O. Development of a multiplexed qPCRs-based approach for the diagnosis of *Dirofilaria immitis, D. repens, Acanthocheilonema reconditum* and the others filariosis. *bioRxiv* **2020**, 842575. [CrossRef]
81. Bassene, H.; Sambou, M.; Fenollar, F.; Clarke, S.; Djiba, S.; Mourembou, G.; Alioune Badara, L.Y.; Raoult, D.; Mediannikov, O. High prevalence of *Mansonella perstans* filariasis in rural Senegal. *Am. J. Trop. Med. Hyg.* **2015**, *93*, 601–606. [CrossRef] [PubMed]
82. Touré, F.S.; Bain, O.; Nerrienet, E.; Millet, P.; Wahl, G.; Toure, Y.; Doumbo, O.; Nicolas, L.; Georges, A.J.; McReynolds, L.A.; et al. Detection of *Loa loa*-specific DNA in blood from occult-infected individuals. *Exp. Parasitol.* **1997**, *86*, 163–170. [CrossRef] [PubMed]
83. Verweij, J.J.; Brienen, E.A.T.; Ziem, J.; Yelifari, L.; Polderman, A.M.; Van Lieshout, L. Simultaneous detection and quantification of *Ancylostoma duodenale, Necator americanus*, and *Oesophagostomum bifurcum* in fecal samples using multiplex real-time PCR. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 685–690. [CrossRef]
84. Wichmann, D.; Panning, M.; Quack, T.; Kramme, S.; Burchard, G.D.; Grevelding, C.; Drosten, C. Diagnosing schistosomiasis by detection of cell-free parasite DNA in human plasma. *PLoS Negl. Trop. Dis.* **2009**, *3*. [CrossRef]
85. Praet, N.; Verweij, J.J.; Mwape, K.E.; Phiri, I.K.; Muma, J.B.; Zulu, G.; van Lieshout, L.; Rodriguez-Hidalgo, R.; Benitez-Ortiz, W.; Dorny, P.; et al. Bayesian modelling to estimate the test characteristics of coprology, coproantigen ELISA and a novel real-time PCR for the diagnosis of taeniasis. *Trop. Med. Int. Health* **2013**, *18*, 608–614. [CrossRef]
86. Wiria, A.; Indonesia, J.; Leiden, T.; Prasetyani, M.; Hamid, F.; University, M.; Wammes, L.; Lell, B.; Hospital, A.; Ariawan, I.; et al. Does treatment of intestinal helminth infections influence malaria? Background and methodology of a longitudinal study of clinical, parasitological and immunological parameters in Nangapanda, Flores, Indonesia (ImmunoSPIN Study). *BMC Infect. Dis.* **2010**, *10*, 77. [CrossRef]
87. Liu, J.; Gratz, J.; Amour, C.; Kibiki, G.; Becker, S.; Janaki, L.; Verweij, J.J.; Taniuchi, M.; Sobuz, S.U.; Haque, R.; et al. A laboratory-developed taqman array card for simultaneous detection of 19 enteropathogens. *J. Clin. Microbiol.* **2013**, *51*, 472–480. [CrossRef]
88. Verweij, J.J.; Canales, M.; Polman, K.; Ziem, J.; Brienen, E.A.T.; Polderman, A.M.; van Lieshout, L. Molecular diagnosis of *Strongyloides stercoralis* in faecal samples using real-time PCR. *Trans. R. Soc. Trop. Med. Hyg.* **2009**, *103*, 342–346. [CrossRef] [PubMed]
89. Laidoudi, Y.; Ringot, D.; Watier-Grillot, S.; Davoust, B.; Mediannikov, O. A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France. *Parasite* **2019**, *26*, 72. [CrossRef] [PubMed]

Publication N°6

New Molecular Data on Filaria and its *Wolbachia* from Red Howler Monkeys (*Alouatta macconnelli*) in French Guiana — A Preliminary Study

Younes Laidoudi[1,2], Hacène Medkour[1,2], Anthony Levasseur[1,2], Bernard Davoust[1,2] and Oleg Mediannikov[1,2,*]

[1] IRD, AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France
[2] Aix Marseille Univ, 19–21, Bd Jean Moulin, 13005 Marseille, France

ARTICLE DE RECHERCHE

Publié dans : Pathogens, 2020, 9(8), 626

(doi.10.3390/pathogens9080626)

pathogens

MDPI

Article

New Molecular Data on Filaria and its *Wolbachia* from Red Howler Monkeys (*Alouatta macconnelli*) in French Guiana—A Preliminary Study

Younes Laidoudi [1,2], Hacène Medkour [1,2], Anthony Levasseur [1,2], Bernard Davoust [1,2] and Oleg Mediannikov [1,2,*]

1 IRD, AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, 19–21, Bd Jean Moulin, 13005 Marseille, France; younes.laidoudi@yahoo.com (Y.L.); hacenevet1990@yahoo.fr (H.M.); anthony.levasseur@univ-amu.fr (A.L.); bernard.davoust@gmail.com (B.D.)

2 Aix Marseille Univ, 19–21, Bd Jean Moulin, 13005 Marseille, France

* Correspondence: olegusss1@gmail.com; Tel.: +3304-1373-24-01

Received: 10 June 2020; Accepted: 29 July 2020; Published: 31 July 2020

Abstract: Previous studies have reported filarial parasites of the genus *Dipetalonema* and *Mansonella* from French Guiana monkeys, based on morphological taxonomy. In this study, we screened blood samples from nine howler monkeys (*Alouatta macconnelli*) for the presence of filaria and *Wolbachia* DNA. The infection rates were 88.9% for filaria and 55.6% for wolbachiae. The molecular characterization, based on the *18S* gene of filariids, revealed that *A. macconnelli* are infected with at least three species (*Mansonella* sp., *Brugia* sp. and an unidentified Onchocercidae species.). Since the *18S* and *cox1* generic primers are not very effective at resolving co-infections, we developed ITS genus-specific PCRs for *Mansonella* and *Brugia* genus. The results revealed coinfections in 75% of positives. The presence of *Mansonella* sp. and *Brugia* sp. was also confirmed by the *16S* phylogenetic analysis of their associated *Wolbachia*. *Mansonella* sp., which close to the species from the subgenus *Tetrapetalonema* encountered in New World Monkeys, while *Brugia* sp. was identical to the strain circulating in French Guiana dogs. We propose a novel *ITS1 Brugia* genus-specific qPCR. We applied it to screen for *Brugia* infection in howler monkeys and 66.7% were found to be positive. Our finding highlights the need for further studies to clarify the species diversity of neotropics monkeys by combining molecular and morphological features. The novel *Brugia* genus-specific qPCR assays could be an effective tool for the surveillance and characterization of this potential zoonosis.

Keywords: *Mansonella* sp.; *Brugia* sp.; Onchocercidae sp.; *Wolbachia*; neotropic monkeys; reservoir; zoonosis

1. Introduction

Filariasis unites diseases are caused by arthropod-borne filariids and nematodes belonging to the Onchocercidae family. Several species can be encountered in human and animals with some zoonotic aspects. Morphologically, the adult filariids are long, string-like, white-to-cream-colored worms [1]. They appear to be capable of living inside various tissues and cavities outside the gastrointestinal tract. Once mature, the adult females produce blood or cutaneous microfilariae, where they are available to arthropod vectors [2]. Species having a predilection for subcutaneous tissues are less or completely avirulent in comparison to those found in cavities, such as *Dipetalonema* species (*D. gracile, D. graciliformis, D. caudispina, D. robini* and *D. freitasi, D. vanhoofi*), *Macacanema formosana* where they induce serious disease manifestations such as pleuritis, fibrinopurulent peritonitis and fibrinous adhesion, resulting in the entrapment of worms [3,4]. Furthermore, species found in the circulatory system (e.g., *Dirofilaria immitis* and *D. pongoi, Edesonfilaria malayensis*), as well as those present in

the lymphatic system, such as Brugian filariids (*B. malayi, B. pahangi, B. timori* and *B. tupaiae*) and *Wuchereria bancrofti*, disrupt blood and lymphatic drainage, leading to serious and often irreversible vascular damage [4–9]. These filariids, along with *Onchocerca volvulus*, the agent of river blindness, constitute the most thread-like filarial worms and have affected up to 893 million people in 49 countries worldwide [10].

Several filariids of the subfamilies Onchocercinae and Dirofilariinae are associated with an endosymbiotic intracellular bacterium of the genus *Wolbachia* [11], which is present in all developmental stages of filariids that harbor *Wolbachia*, leading to their long-term survival [12]. The parasites' endosymbiotic *Wolbachia* are implicated in severe inflammatory-mediated filarial diseases [13–16]. Anti-wolbachial therapies, based on the administration of antibiotics, are known to be effective against the most common filariasis caused by *Brugia* spp., i.e., *W. bancrofti, Mansonella perstans* and *D. immitis* [17–19]. The *Wolbachia*-filaria relationship is species-specific, wherein each filariid has a specific genotype of *Wolbachia* [11], thus providing an additional target suitable for the diagnosis of filarial infections [20], especially when occurring in dead-end hosts, as is the case in *D. immitis* in human and cats [21,22]. Recently, the simultaneous detection of both filarial and wolbachial DNAs from infected hosts is used as an improvement tool for the diagnosis of filarial infections [23–25].

Filariasis is one of the most neglected tropical diseases selected, but it is included in the Mass Drug Administration (MDA) program to achieve its elimination by 2020 [26–28]. Human filariasis was almost eliminated from Latin America [29,30]. Thanks to the MDA program, river blindness (onchocerciasis caused by *O. volvulus*) transmission is currently limited to the Amazon rainforest on the Venezuelan–Brazilian border, while the lymphatic filariasis caused by *W. bancrofti* only occurs in four countries: Brazil, the Dominican Republic, Guyana, and Haiti [31]. Another human sympatric filariasis caused by *M. ozzardi* and *M. perstans* occurs today in a small foci in South America (Amazon Basin, Yucatan, Panama and Haiti) [32–34]. In Latin America, domestic and wild animals seem to be the foci of some neglected filariasis potentially zoonotic such as *Brugia guyanensis* (Orihel 1964) from the lymphatic system of the coatimundi (*Nasua nasua vittata*) in French Guiana [35] and some unidentified Brugian filariids in dogs and ring-tailed coatis (*Nasua nasua*) [25,36], and the zoonotic canine filariasis (e.g., *D. immitis* and *Acanthocheilonema reconditum*) from Brazil and French Guiana [25,37].

New world monkeys are a diverse group of arboreal primates inhabiting the tropical forest environments of southern Mexico, Central and South America [38]. These primates are the natural hosts for several filariids belonging to the genus *Dipetalonema* and *Mansonella*, where they are often present as co-infected [3,39]. Howler, monkeys (*Alouatta* spp., Atelidae, Primata) have a wide distribution, from Mexico to northern Argentina. Only a few species of this group have been genetically characterized [40]. The red howler monkey (*Alouatta macconnelli*, Linnaeus 1766—Elliot 1910) is one of eight species of primates found in the French Guiana forest [41]. They are medium sized (10 kg) and about 84 cm (head and body) with a prehensile tail [38]. They live in small groups of four to eight individuals. The primary forest in the canopy high strata is often frequented by these primates who are mainly found in the north of South America and the Amazonia (Suriname, Guyana, Trinidad, French Guiana, Venezuela and Brazil). Their diet is low in energy (leaves and sometimes fruits and seeds) [40]. Population density is estimated to be 13 individuals/km^2 along the Approuague River, which is the location in which we conducted our investigation [42]. Nowadays, little molecular data are available on filarial parasites in howler monkeys from French Guiana. The aims of the present study are mainly to determine, at the molecular level, the presence of filarial parasites and the status of their endosymbiotic *Wolbachia* in red howler monkeys. To this end, we examined blood samples obtained from a game that was hunted by the natives of French Guiana [43].

2. Results

2.1. Host Identification

Folmer's primers allowed for the amplification of DNA sequences from all blood samples, but despite several attempts, a high-quality DNA sequence of the vertebrate *cox1* gene was only obtained in one from among the nine samples tested, suggesting the presence of a non-specific amplification from the latter. The partial nucleotide sequence (558 bp) of the *cox1* gene obtained in this study was deposited in the GenBank under accession number MT193011. Blast analysis showed that the *cox1* sequence of howler monkeys in our study had an identity of 96.06% with *Alouatta seniculus* (HQ644333), 95.88% with *Alouatta caraya* (KC757384) and 95.34% with *Alouatta guariba* (KY202428) and a query cover of 100%. Accordingly, the phylogenetic analysis using the Maximum Likelihood (ML) method showed that the specimen of howler monkeys (*Alouatta macconnelli*) is monophyletic with other *Alouatta* species (Figure 1).

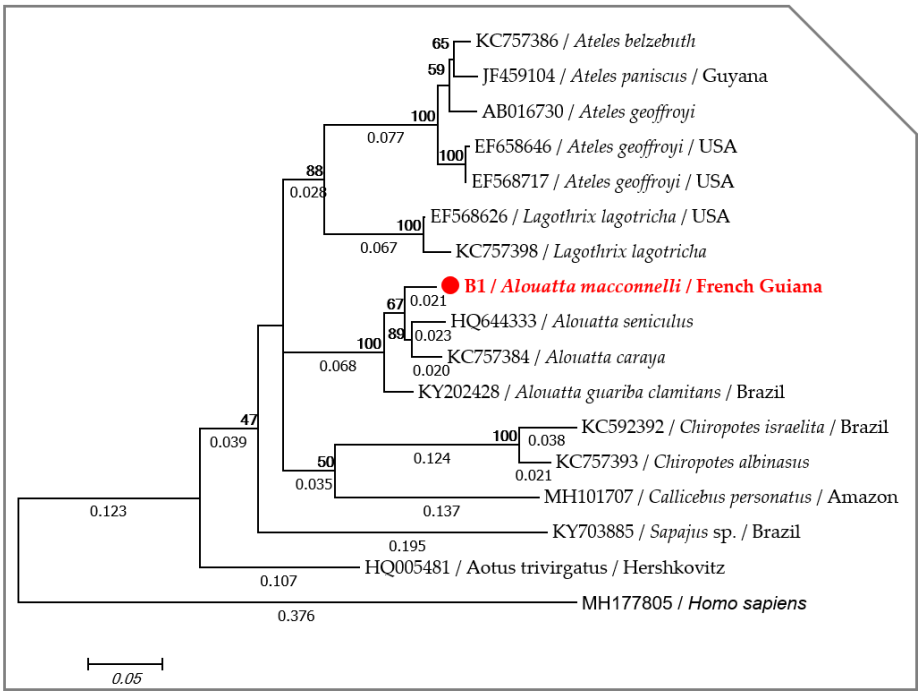


Figure 1. Phylogram generated by maximum likelihood method from 17 partial (521 bp) *cox1* sequences showing the position of *Alouatta macconnelli* through the neotropics monkeys. A discrete Gamma distribution was used to model evolutionary rate differences among the sites (5 categories (+*G*, parameter = 0.4575)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 57.2649% sites). Likelihood was −2676.5239. Numbers above and below the branches display the nod statistics and branch length, respectively. Geographical location (when available) and GenBank accession numbers are indicated in each node.

2.2. Molecular Screening for Filarial and Wolbachia DNAs in Howler Monkeys

Filarial and *Wolbachia* DNAs were detected by qPCR assays in eight out of nine samples tested and six out of nine samples tested, which correspond to a frequency of infection of 88.9% and 66.7% for filaria and *Wolbachia*, respectively. This is the first molecular report of filaria and its *Wolbachia* from the howler monkeys of French Guiana.

2.3. Molecular Characterization of Filarial Species

To identify filaria detected by qPCR. we performed standard polymerase chain reaction (PCR) screening with primers targeting the small subunit rRNA (*18S*), the internal transcribed spacer 1 (*ITS1*)

and the cytochrome c oxidase subunit I (*cox1*) genes. A nearly full-length DNA sequence of the *18S* rRNA gene was obtained from all eight samples, was positive in qPCR and was split into three isolates according to the blast results. (i) Six sequences were obtained from the monkeys B2, B3, B4, B6, B7 and B8. These amplicon sequences were identical to each other, showing an identity and query cover of 100% with *Dipetalonema* sp. (DQ531723) isolated from an owl monkey (*Aotus nancymaae*) captured in Peru and 99.6% of identification with the *Mansonella* species (MN432520, MN432519). (ii) One *18S* sequence obtained from sample B5 was very close to the Onchocercidae members (*Onchocerca cervicalis:* DQ094174, and *Loa loa*: DQ094173), where the identification was 99.9% and 100% of the query cover. Further sequence comparisons showed that the Adenine and Thymine mutated into Cytosine at the position 304 and 879 with *O. cervicalis* (DQ094174) and *L. loa* (DQ094173), respectively (Figure S1). (iii) One sequence from sample B9 showed an identification of 100% with *B. malayi* (AF036588) and 99.9% with *Brugia* sp. (MN795087), isolated from dogs in French Guiana.

Mansonella genus-specific PCR, based on the amplification of the *ITS1*, allowed us to obtain ITS sequences of *Mansonella* sp. from seven monkeys (B2, B3, B4, B5, B6, B7 and B8). They were almost identical and displayed an identity ranging from 83.47% to 93.49% and a query cover ranging from 62% to 83% with *Mansonella* species (*M. ozzardi*: KR952332, *M. perstans*: MN432520, *M. mariae*: AB362562, *M. streptocerca*: KR868771, *M. dunni*: KY434312 and *Mansonella* sp.: MN821052). Furthermore, *Brugia* sp. was identified in five samples (B2, 3, 4, 7 and 9) using the *Brugia*-specific qPCR and ITS sequences were obtained for four of them. These sequences were similar and were close to the *Brugia* species, wherein the identity ranged from 88.81% to 91.98% with *B. malayi* (JQ327147, EU419333) and from 89.10% to 91.19% with *B. pahangi* (EU373633, EU419348).

Primers targeting the *cox1* gene amplified the expected DNA amplicon size from all the filaria-positive samples. However, only two sample (B8 and B9) sequences provided good quality electropherograms. Several overlapping peaks (double peaks) within samples B2, B3, B4, B5, B6 and B7 suggested co-infection with two or more filarial species. Blast analysis showed that the specimen amplified from monkey B8 had an identity of 88.2% with *Mansonella perstans* (MN890111). While the *cox1* sequence amplified from monkey B9 was very close to Brugian filariids, with an identity of 99.6% with *Brugia* sp. (MT193074), isolated from dogs in French Guiana, 95.4% with *Brugia timori* (AP017686) and 94.9% with *Brugia malayi* (MN564741).

Phylogenetic analysis using the maximum likelihood method of the *18S* rRNA gene showed that howler monkeys from French Guiana are infected with at least three filarial species belonging to the Onchocercidae clade, namely ONC 5. The *18S* sequences amplified from monkeys B2, 3, 4, 6, 7 and 8 clustered in a separate branch with *Mansonella* species, while the sequence obtained from monkey B5 appeared paraphyletic with respect to *L. loa* (ADBU02009332) and *O. volvulus* (ADBW01003330), suggesting an unknown onchocercid. Finally, the sequence from monkey B5 clustered with the *B. pahangi* strain (UZAD01013810 and JAAVKF010000006) (Figure 2).

The ML tree, based on the concatenated rRNA sequences (*18S* and *ITS1*), showed that the specimens amplified from monkeys B2, 3, 4, 6, 7 and 8 clustered with other monophyletic species of the genus *Mansonella*, while the specimen amplified from monkey B9 clustered with the *Brugia* species (Figure 3). Interestingly, the *cox1* phylogram replicated the same results, though with a greater degree of accuracy. The species amplified in this study belong to the clade 5 of the Onchocercidae family. More precisely, the species amplified from monkey B8 belong to the genus *Mansonella* and the subgenus *Tetrapetalonema* encountered in New World Primates [44], while the species from monkey B9 clustered with *Brugia* sp. (MT193074), isolated from dogs in French Guiana [45] and are monophyletic with other Brugian filariids (Figure 4). Interspecific nucleotide distances (IND) of the *cox1* sequences ranged between 0.08 and 0.13 between *Mansonella* sp. from the monkey B8 and most species from the genus *Mansonella* (MN890075, MN890115, MN890111 and KY434309), while the IND ranged from 0 to 0.03 between *Brugia* sp. amplified from monkey B9 and Brugian filariids (Figure 5, Table S1).

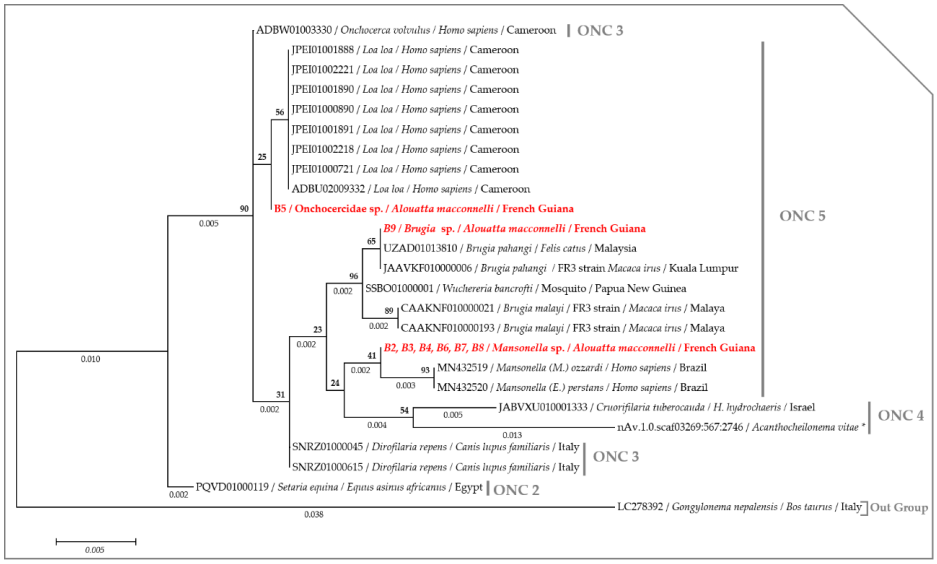


Figure 2. Phylogram generated by Maximum Likelihood (ML) method based on 24 partial (941 bps) rRNA sequences showing the position of filariids from howler monkeys Onchocercidae clades (ONC). A discrete Gamma distribution was used to model evolutionary rate differences among the sites (5 categories (+*G*, parameter = 0.1000)). The likelihood was −1770.1752. Numbers above and below the branches display nod statistics and branch lengths, respectively. Geographical location (when available) and GenBank accession numbers are indicated in each node. (*) indicates sequences retrieved from the Worm parasites database.

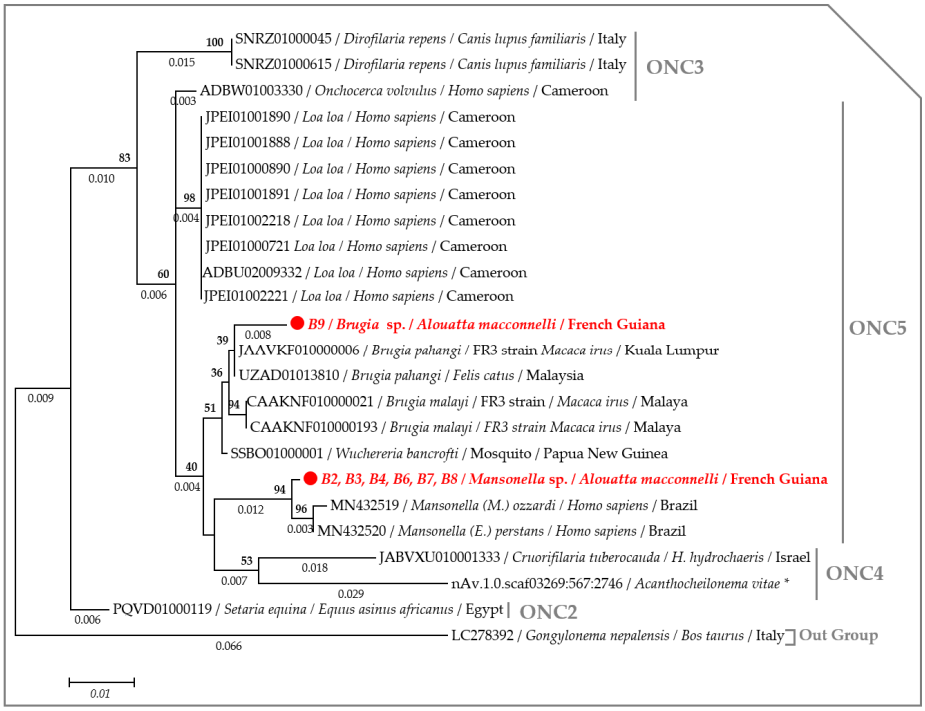


Figure 3. Phylogram generated by ML method based on 24 partitioned concatenated rRNA sequences (*18S* ad *ITS1*) showing the position of *Brugia* sp. and *Mansonella* sp. through Onchocercidae clades (ONC). The total length was 1221 bp, the rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 29.0648% sites). Likelihood was −3034.4989. Numbers above and below the branches display nod statistics and branch lengths, respectively. Geographical location (when available) and GenBank accession numbers are indicated in each node. (*) indicates sequences retrieved from Worm parasites database.

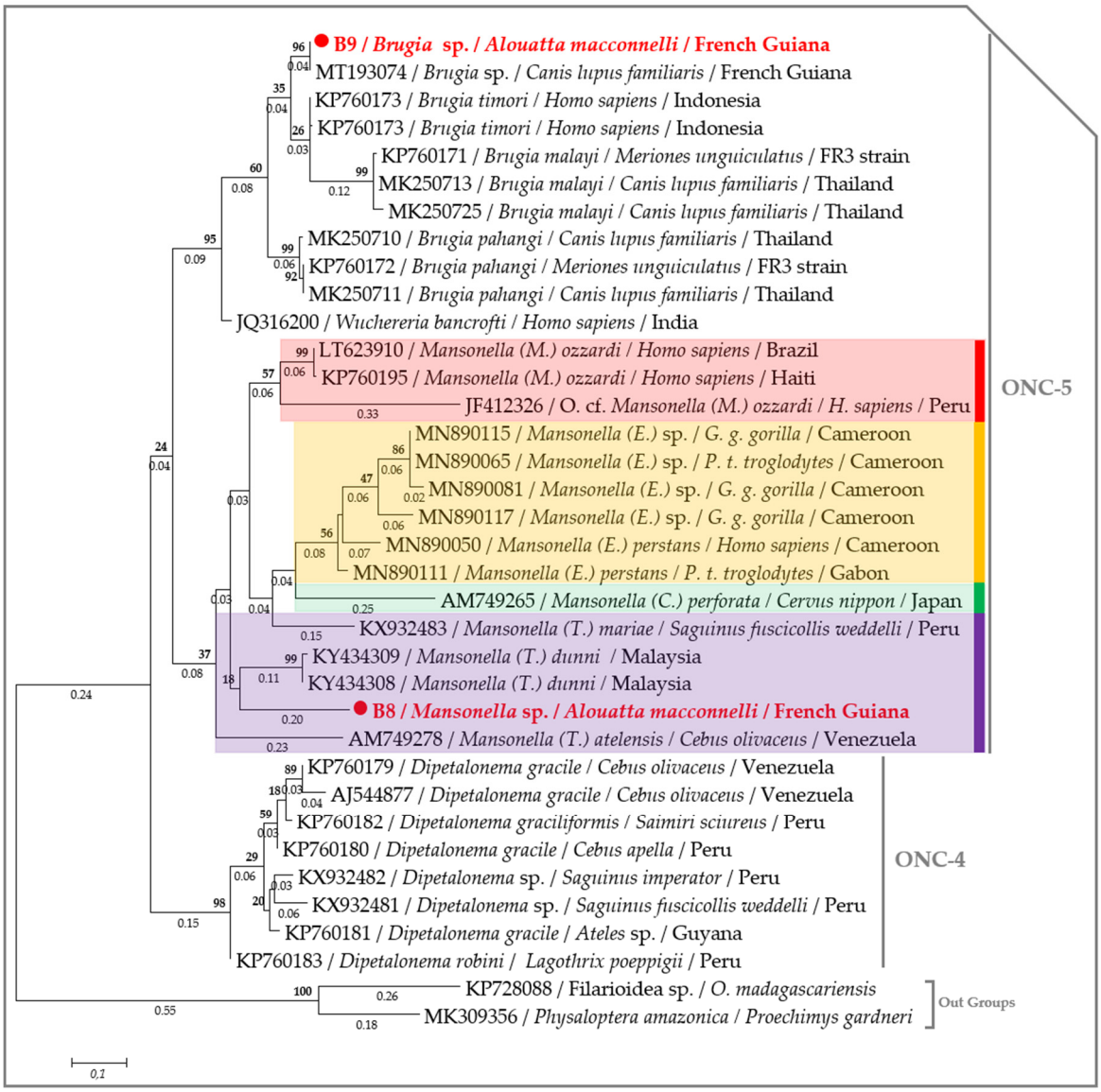


Figure 4. Phylogram generated by ML method based on 36 *cox1* partial sequences (266 bp) showing the position of *Brugia* sp. and *Mansonella* sp. through Onchocercidae clades (ONC). A discrete Gamma distribution was used to model evolutionary rate differences among the sites (five categories (+*G*, parameter = 0.4964)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 0.000% sites). The likelihood was −2194.0587. Numbers above and below the branches display nod statistics and branch lengths, respectively. Host, geographical location (when available) and GenBank accession numbers are indicated in each node. *Mansonella* species are color-coded according to their subgenus.

Importantly, the *cox1* DNA sequences were aligned correctly to the reference mitogenome of *M. ozzardi* (KX822021) [45], and when translated, there were no stop codons in the amino acid sequences, suggesting the absence of co-amplified numts. Finally, translated protein sequences of the cytochrome c oxidase subunit I (COI) showed three amino acid changes between *Mansonella* sp. from monkey B8 and the other *Mansonella* species from GenBank, namely, from threonine to alanine, threonine to isoleucine and aspartic acid to valine (Figure 6A). While *Brugia* sp. from monkey B9 showed a deletion of one amino acid instead of tryptophan, in comparison to Brugian filariids from GenBank (Figure 6B).

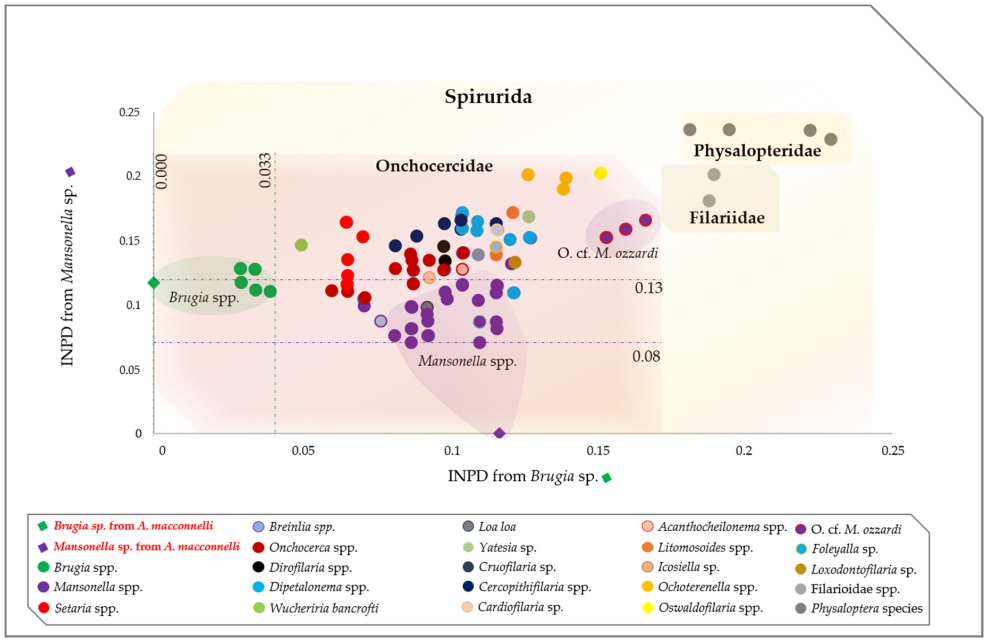


Figure 5. Scatter chart showing the interspecific pairwise distance between the cox1 sequences of *Brugia* sp. (abscissa) and *Mansonella* sp. (ordinate) from *A. macconnellii* and the representative members of Onchocercidae clades. The analyses involved 112 partial (266 bp) cox1 sequences with a total of 216 positions in the final dataset. All positions containing gaps and missing data were eliminated.

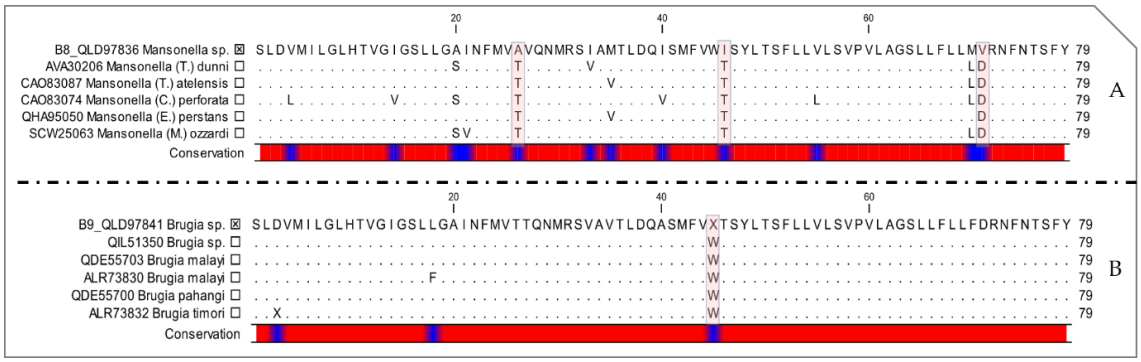


Figure 6. Cytochrome c oxidase subunit I protein sequences (COI) alignment showing the conservation of amino acid within (**A**) *Mansonella* spp., (**B**) *Brugia* spp. Protein Id and species name are indicated for each sequence. Selected boxes represent species obtained in this study.

A partial DNA sequence of the *Wolbachia 16S* gene (295 bps) was obtained from five out of six samples that tested positive for *Wolbachia* DNA through the qPCR. Three identical sequences revealed 99.32% identity with *Wolbachia* of *M. atelensis amazonae* (FR827940) and 98.64% with both *Wolbachia* of *M. perstans* (AY278355) and *M. ozzardi* (AJ279034). These sequences were obtained from filaria-positive monkeys, including monkey B4, which was co-infected with *Mansonella* sp. and *Brugia* sp., monkey B5 co-infected with an unidentified Onchocercidae species and *Mansonella* sp. and monkey B8, which was mono-infected with *Mansonella* sp. The two remaining sequences were amplified from two filaria-positive samples, one for *Mansonella* sp. (B6) and the other for *Brugia* sp. (B9). These sequences were identical with each other and were 100% identical with all *Wolbachia* genotypes associated to *Brugia* species (CP050521, CP034333, AJ012646 and MT231956). Accordingly, the ML inference indicates that the *Wolbachia* genotype from monkeys B4, 5 and 8 belong to the Clade F of *Wolbachia* lineage infecting *Mansonella* species, while the genotype obtained from monkeys B6 and B9 clustered together with *Wolbachia* endosymbiont of Brugian filariids within Clade D of the *Wolbachia* lineage (Figure 7).

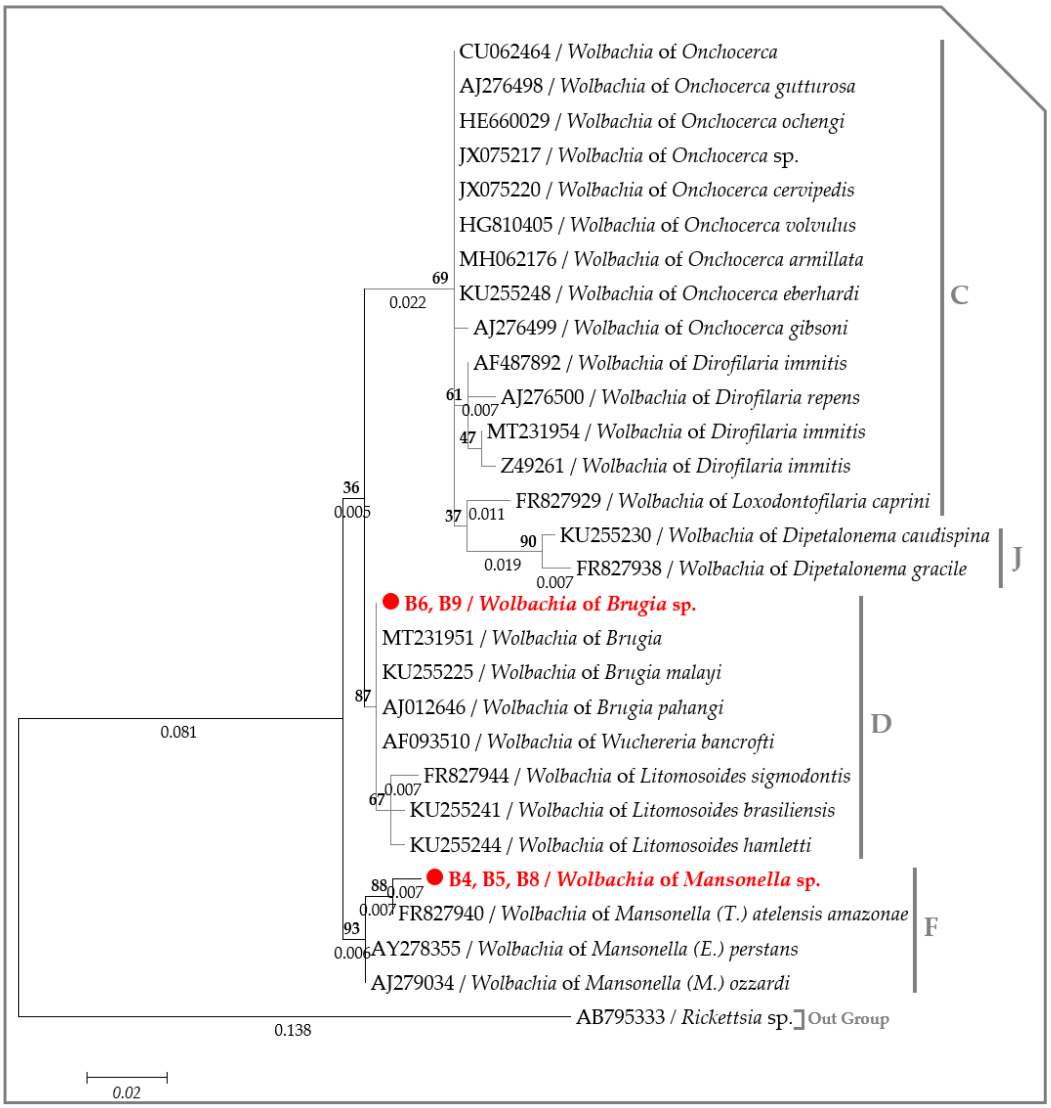


Figure 7. Phylogram generated by the maximum likelihood method based on 29 nucleotide sequences of the partial (295 bp) *16S* gene showing the position of *Wolbachia* of *Brugia* sp. and *Mansonella* sp. through *Wolbachia* of filarial nematodes. The likelihood was −777.8125. A discrete Gamma distribution was used to model evolutionary rate differences among the sites (5 categories (+*G*, parameter = 0.2802)). Numbers above and below the branches display nod statistics and branch lengths, respectively. Filarial host and GenBank accession numbers are indicated in each node.

Finally, by combining all the molecular results for filaria and *Wolbachia* detection, we concluded six cases (75%) of co-infections in monkeys, including *Mansonella* sp.—*Brugia* sp. co-infection in five and *Mansonella* sp.—unidentified Onchocercidae species in one. Two other monkeys (25%) presented mono-infections, one with *Mansonella* sp. and the other with *Brugia* sp. (Table 1).

Table 1. Results of molecular assays used for the identification of filariids and their associated *Wolbachia* in the blood of red howler monkeys from French Guiana.

Sample Code	Filarial DNA					*Wolbachia* DNA		Decision
	Filariids			ITS genus-specific PCRs		*Wolbachia* 16S-specific PCRs		
	28S qPCR	***18S* PCR**	***COI* PCR**	***Mansonella* spp. *PCR***	***Brugia* spp. PCR**	***Brugia* spp. qPCR**	***Wolbachia 16S* qPCR**	***Wolbachia 16S* PCR**	**Combined Assays**
B1	N/A	N/A	N/A	N/A	N/A	Neg.	Neg.	N/A	Negative.
B2	Pos.	*Mansonella* sp. [MT336169]	O/P	*Mansonella* sp. [MT341515]	N/A	Pos.	Neg.	N/A	*Mansonella* sp. + *Brugia* sp.
B3	Pos.	*Mansonella* sp. [MT336170]	O/P	*Mansonella* sp. [MT341516]	*Brugia* sp. [MT341511]	Pos.	Pos.	O/P	*Mansonella* sp. + *Brugia* sp.
B4	Pos.	*Mansonella* sp. [MT336171]	O/P	*Mansonella* sp. [MT341517]	*Brugia* sp. [MT341512]	Pos.	Pos.	*W-Mansonella sp.* [MT231961]	*Mansonella* sp. + *Brugia* sp.
B5	Pos.	unidentified Onchocercidae species [MT336175]	O/P	*Mansonella* sp. [MT341518]	N/A	Neg.	Pos.	*W-Mansonella sp.* [MT231962]	*Mansonella* sp. + unidentified Onchocercidae species
B6	Pos.	*Mansonella* sp. [MT336172]	O/P	*Mansonella* sp. [MT341519]	N/A	Neg.	Pos.	*W-Brugia* sp. [MT231964]	*Mansonella* sp. + *Brugia* sp.
B7	Pos.	*Mansonella* sp. [MT336173]	O/P	*Mansonella* sp. [MT341520]	*Brugia* sp. [MT341513]	Pos.	Neg.	N/A	*Mansonella* sp. + *Brugia* sp.
B8	Pos.	*Mansonella* sp. [MT336174]	*Mansonella* sp. [MT724663]	*Mansonella* sp. [MT341521]	N/A	Neg.	Pos.	*W-Mansonella sp.* [MT231963]	*Mansonella* sp.
B9	Pos.	*Brugia* sp. [MT336168]	*Brugia* sp. [MT724693]	N/A	*Brugia* sp. [MT341514]	Pos.	Pos.	*W-Brugia* sp. [MT231965]	*Brugia* sp.

N/A: no amplification, O/P: overlapping peaks on the electropherograms, Pos: positive reaction, Neg: negative reaction, *W-Mansonella* sp.: *Wolbachia* endosymbiont of *Mansonella* sp., *W-Brugia* sp.: *Wolbachia* endosymbiont of *Brugia* sp. GenBank accession numbers are given in square brackets.

3. Discussion

This is the first molecular report of filaria and *Wolbachia* infection from red howler monkeys (*Alouatta macconnelli*, Linnaeus 1766—Elliot 1910) in French Guiana. These monkeys were morphologically considered as a distinct species from *A. seniculus* and they are not a subspecies [46]. Our data confirmed that, molecularly, both species can be distinguished by their *cox1* sequences. The wide distribution of howler monkeys (from Mexico to northern Argentina) constitutes a non-negligible reservoir for zoonotic disease [43] and should be monitored. Our study is limited in the number of species and samples, due to the difficulties encountered in the field. The number of monkeys tested was much lower than those tested in Reference [47], where 1353 free-ranging mammals, including 114 howler monkeys (*A. seniculus*) and 84 red handed tamarins (*Saguinus midas*) from the neotropical primary rainforest in French Guiana were studied for haemoparasites and microfilariae. However, the prevalence of filarial infection we recorded using molecular assays is close to that reported in tamarins and howler monkeys using blood smear, where the infection rates were 80% and 92% of filaria infections (*Dipetalonema* and *Mansonella* (*Tetrapetalonema*) species), respectively [47]. Our data indicate that the prevalence of filarial infection was higher than that of sloths, anteaters and porcupines in French Guiana, where the infection rate of 40% was reported using blood smears test [47]. The higher prevalence observed in monkeys may be related to the lower host specificity of filariids [48] and/or similar biotope of potential vectors [49]. Another hypothesis is that the lifestyle of these animals increases the risk of vector-borne disease transmission between infected and non-infected individuals in the monkey colony. Therefore, the highest mixed-infection detected in our study corroborates previous reports [50], but it is still unknown whether it is geographical or host-specific. Several species of filariids are reported from a wide range of neo-tropical primates based on morphological taxonomy (Table 2). Most of them belong to the genus *Dipetalonema* and *Mansonella* (*Tetrapetalonema*). However, data in DNA barcoding of these species is lacking.

The use of two (or more) different molecular markers for species delimitation remained necessary for the accurate identification of nematode species [51]. In the present study, our molecular approach, based on generic and genus specific primers, permits the detection and characterization of filarial infections and resolved the co-infections. This is due to the ability of ITS genus-specific PCR assays to separately amplify DNA amplicons depending on their specificity. Filarial nematodes could be misclassified when the *18S* gene is used alone as a barcode. This gene is often limited to the genus level and has proven to be inconclusive for the molecular taxonomy of nematodes [52], while the ITS 1 gene appears to be a satisfactory barcode in resolving taxonomic relationships among species [53–55]. Furthermore, as suggested by previous authors [56], the use of partitioned concatenated DNA sequences enables the accurate identification of filarial nematodes. We used both the *18S* and the partitioned concatenated rRNA (*18S* and *ITS1*) gene, which confirmed the presence of at least three potential new species from clade 5 of the Onchocercidae family present in howler monkeys in French Guiana, including *Mansonella* sp., *Brugia* sp. and an unidentified Onchocercidae species.

The *cox1* gene enabled the accurate identification of the *Mansonella* species from wild non-human primates from Cameroon and Gabon [57], and has been proven to be a satisfactory discrimination between filarial species. This gene was described by its low nucleotide distances (from 0 to 0.02) within filarial species [58] and a larger variation between congeneric species (i.e., 0.098 to 0.2) [58,59]. In the present study, we used two different phylogenetic methods for the analysis of *cox1*, together with the alignment of COI protein sequences, which confirmed that species from monkeys B8 and B9 clustered, respectively, with *Mansonella Tetrapetalonema* subgenus and *Brugia* species, with the distance ranging between 0.02 and 0.2, suggesting unidentified or potential new species from these genera.

Wolbachia are host-specific, and each genotype is associated with a specific filarial species [11,60]. Bacterial genotype-specific identification was previously proposed for the speciation of *Brugia* parasites that infect humans [9]. Several studies showed the utility of the specific detection of *Wolbachia* in determining the subject as infected or not with filarial species (e.g., *D. immitis, D. repens, B. pahangi* and *B. malayi*) from domestic animals [14,21,23–25,61,62]. Accordingly, the phylogenetic analysis of the

Wolbachia 16S DNA sequences demonstrated the presence of two bacterial genotypes belonging to the supergroup F and D encountered in *Mansonella* and *Brugia* species, thus corroborating with filaria phylogenies. The inconsistency between the bacterial genotype and filaria species was observed in monkey B6. The presence of *Mansonella* sp. and *Wolbachia* of *Brugia* sp. DNAs highlights a co-infection with both filarial species. However, the absence of *Wallachia* of *Mansonella* sp. could be explained by a weaker infection density in this species, while the absence of *Brugia* sp. DNA, despite the presence of its *Wolbachia*, could be result to an amicrofilaremic infection due to single sex infection, an earlier infection stage or any other causes. Such inconsistencies were previously reported between *Brugia* and *Dirofilaria* species in dogs [63]. *Wolbachia*-filaria interactions within co-infected hosts are not well understood. Despite the presence of both parasites in co-infected dogs with *D. immitis* and *D. repens*, the single detection of *Wolbachia* of *D. immitis* is frequent [24] and may result in an unexplained suppression effect on the production of *D. immitis* microfilariae induced by the presence of *D. repens* [64,65].

Our findings extend the presence of *Brugia* sp. and an unidentified Onchocercidae species to the New World Monkeys (e.g., *Alouatta macconnelli*). Several species of filariae have been described from these primates and they all belong to the genus *Dipetalonema* or *Mansonella* subgenus *Tetrapetalonema* [4] (Table 2). The genus *Dipetalonema* (Diesing 1861) is restricted to non-human primates (NHPs) of the neotropics, according to the phylogenetic study conducted by Lefoulon et al. [56]. Adult worms are prevalent in the serous cavities of the hosts. A high species diversity of this genus was observed in a wide range of New World monkeys. *D. gracile* (Rudolphi 1819), *D. graciliformis* (Freitas 1964) and *D. caudispina* (Molin 1858) are the main species found in Guiana monkeys, using a morphological taxonomy (Table 2).

The subgenus *Mansonella* (*Tetrapetalonema*) is one of the five subgenera derived from the genus *Mansonella*. Adult filariids are small, slender and can be found in subcutaneous tissues. The *Tetrapetalonema* subgenus comprises 13 species (Table 2), which have been restricted to platyrrhine (neotropical) primates [66]. Human mansonellensiasis across South America regions are caused by *M. ozzardi* type species of *Mansonella* (*Mansonella*) subgen. n. [44,45] causing fever, pruritis, arthralgias, headache, rashes, lymphadenopathy, edema, and pulmonary symptoms and a common eosinophilia mainly associated with corneal lesions [67–70]. *M. perstans* type species of *Mansonella* (*Esslingeria*, Chabaud and Bain 1976) subgen. n. [44] is another agent of human mansonellensiasis in some neotropical regions of Central and South America that causes the bung-eye diseases [71]. These species have been found in both humans and non-human primates [4,44]. However, the possibility that the *Mansonella* sp. we have detected here is one of the 13 *Mansonella* (*Tetrapetalonema*) species or a new species from this subgenus cannot be ruled out in the absence of morphological identification.

Brugia spp. are incidental filariids that parasitize non-human vertebrates [72]. The classical brugian filariids involved in lymphatic filariasis are found in Asia, while species reported from North and South America constitute the most zoonotic species of this genus [73]. In Latin America, *Brugia* sp. infection was reported from the ring-tailed coatis (*Nasua nasua nasua*) in Brazil [36], *Brugia guyanensis* from the lymphatic system of the coatimundi (*Nasua nasua vittata*) in British Guiana [35] and *Brugia* sp. from domestic dogs in French Guiana [25]. Our findings indicate that *Brugia* sp. detected from howler monkeys is the same as that recently detected in domestic dogs [25]. Unlike Asian primates in which infection with *B. malayi* and *B. pahangi* has been reported [74], Brugian filariid has not been reported in neotropical primates [75]. Cases of human infection by *Brugia* sp. have been reported in several localities (Amazon, Peru, Colombia) in South America, but the reservoir of the parasites is unknown [72,73]. However, the possibility that the *Brugia* sp. we detected from howler monkeys and dogs in our previous study [25] is of the same species circulating in humans cannot be ruled out in the absence of molecular data.

Table 2. Filarial parasites and host diversity from neotropic monkeys.

Genera	Species	Host	References
Mansonella (Faust, 1929), *Mansonella (Tetrapetalonema)* comb. n. (Faust 1935)	*Mansonella (T.) marmosetae* (Faust 1935)	*Saguinus geoffroyi*, *Saimiri oerstedii oerstedii*, *Ateles paniscus*, *Saimiri boliviensis*, *Saimiri sciureus* and *Alouatta* spp.	[44,66,67,76]
	Mansonella (T.) zakii (Nagaty 1935)	*Leontopithecus (= Leontocebus) rosalia*	
	Mansonella (T.) panamensis (McCoy 1936)	*Cebus capucinus*, *Saimiri oerstedii oerstedii*, *Aotus lemurinus zonalis*, *C. apella* and *A. trivirgatus*	
	Mansonella (T.) atelensis atelensis (McCoy 1935)	*Ateles geoffroyi*, *A. fusciceps rufiventris*	
	Mansonella (T.) atelensis amazonae (Bain and Guerrero 2015)	*Cebus olivaceus*	
	Mansonella (T.) parvum (McCoy 1936)	*Cebus capucinus*, *Saimiri oerstedii oerstedii*	
	Mansonella (T.) obtusa (McCoy 1936)	*Cebus capucinus*, *C. capucinus*, *C. albifrons*, *Saimiri oerstedii oerstedii*	
	Mansonella (T.) tamarinae (Dunn and Lambrecht 1963)	*Saguinus (= Tamarinus) nigricollis*	
	Mansonella (T.) barbascalensis (Esslinger and Gardiner 1974)	*Aotus trivirgatus*	
	Mansonella (T.) mystaxi (Eberhard 1978)	*Saguinus mystax mystax*	
	Mansonella (T.) saimiri (Esslinger 1981)	*Saimiri sciureus*	
	Mansonella (T.) peruviana (Bain, Petit and Rosales-Loesener 1986)	*Saimiri sciureus*	
	Mansonella (T.) colombiensis (Esslinger 1982)	*Saimiri sciureus*, *Cebus apella*	
	Mansonella (T.) mariae (Petit, Bain and Roussilhon 1985)	*Saimiri sciureus*	
Dipetalonema (Diesing 1861)	***D. gracile* (Rudolphi 1819)**	*Saimiri sciureus*, *Cebus albifrons*, *A. geoffroyi*, *Aotus lemurinus*, *Ateles chamek*, *Ateles fusciceps*, *Ateles geoffroyi*, *Ateles paniscus*, *Cebus apella*, *Cebus capucinus*, *Cebus* spp., *Lagothrix lagothricha*, *Saguinus mystax*, *Saguinus nigricollis*, *Saimiri oerstedii*, *Saimiri sciureus*, *Saimiri sciureus*, *Sapajus macrocephalus*, *B. arachnoïdes*, *L. rosalia*, *Leontopithecus chrysopygus*, *Saguinus bicolor*, *Cebus albifrons*	[76–82]
	***D. graciliformis* (Freitas 1964)**	*Saguinus midas*	
	D. robini (Petit et al. 1985)	*Saimiri sciureus*, *Sapajus nigritus*, *Saimiri boliviensis*, *Cebus* spp.	
	D. freitasi (Bain, Diagne and Muller 1987)	*Cebus capucinus*	
	***D. caudispina* (Molin 1858)**	*Alouatta seniculus*, *Ateles paniscus*, *Brachyteles arachnoides*, *Cebus albifrons*, *Cebus apella*, *Lagothrix lagotricha*, *Leontopithecus rosalia*, *Saimiri sciureus*, *Saimiri sciureus*, *Sapajus macrocephalus*	
	D. obtusa (McCoy 1936)	*Cebus albifron*, *Cebus capucinus*	
	D. yatesi (Julians 2007)	*Ateles chamek*	

Species in bold are occurring in French Guiana monkeys.

4. Materials and Methods

4.1. Samples and Ethic Statement

In January 2016, we obtained samples from howler monkeys that were legally hunted by two Amerindian hunters for family consumption of meat. The International Union for Conservation of Nature conservation status for this species is a "least concern" [83,84]. The hunters applied the provisions of the prefectural decree regulating the quotas of species that can be taken by a person in the department of Guiana (No. 583/DEAL of 12 April 2011). The hunt took place in the deep forest (4°01'39.5" N 52°31'32.5" W), near the Approuague River, 50 km from the village of Regina. We were able to examine corpses of nine hunted howler monkeys (five females and four males). Blood was collected by a heart-puncture in sterile tubes containing Ethylene-Diamine-Tetra-Acetic acid (EDTA) and was kept in a cooler before being frozen at −20 °C until further analysis.

4.2. DNA Extraction

Genomic DNA was extracted from 200 µL of each blood samples. The extraction was performed using QIAGEN DNA tissues kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. Two lysis steps were applied before the extraction procedure: (i) mechanical lyses performed on FastPrep-24™ 5G homogenizer using high speed stirring for 40 s in the presence of glass powder, (ii) enzymatic digestion of proteins with buffer G2 and proteinase K for 12 h at 56 °C. The extracted DNA was eluted in a total volume of 100 µL and stored at −20 °C.

4.3. Host Identification

The universal *cox1* DNA barcoding region of metazoans [85] was targeted using the degenerated primers of Folmer, as described elsewhere [86]. The PCR products were purified, sequenced and edited, as described below, and were then aligned against *cox1* sequences of *Alouatta* spp. (HQ644333, KC757384, KY202428), *Ateles* spp. (AB016730, KC757386, JF459104, EF658646, EF568717), *Callicebus personatus* (MH101707), *Chiropotes israelita* (KC592392, KC757393), *Lagothrix lagotricha* (EF568626, KC757398), *Sapajus* spp. (KY703885) and *Aotus trivirgatus* (HQ005481) as representative New World monkeys [46]. The sequence (MH177805) of human *cox1* was used as an out-group. Finally, the Hasegawa-Kishino-Yano (*+G, +I*) [87] was selected as a best fit model according to the Akaike Information Criterion (AIC) option in MEGA6 [88]. The maximum likelihood (ML) phylogenetic inference was used with 1000 bootstrap replicates to generate the phylogenetic tree using the same software.

4.4. Molecular Screening for Filaria and Wolbachia

First, all blood samples were screened for the presence of filaria and *Wolbachia* DNAs using, respectively, the pan-filarial [Pan-fil 28S] and pan-*Wolbachia* [All-Wol 16S] qPCRs, as described elsewhere [24].

4.5. Molecular Characterization of Filariids and their Associated Wolbachia Using Generic Primers

Samples positive for filaria and *Wolbachia* by qPCR were subjected to amplification and sequencing analysis using the pan-Nematoda-*18S* primers [61] and pan-filarial *cox1* based PCR [Pan-fil *cox*1] [24] to generate 1127–1155 bp and 509 bp from the filarial *18S* and *cox1* genes, respectively. The third PCR system [W16S-Spec] PCR [89] was used to amplify 438 bp from the *16S* gene of *Wolbachia* spp. (Table 3).

Table 3. The primers and probes used in this study.

System Name	Target Gene	Primer and Probe Name	Sequence (5′–3′)	Amplicon Size (bp)	Tm/Elongation Time	Assay Specificity	Ref.
Pan-fil 28S qPCR-based system	*LSU* rRNA *(28S)*	qFil-28S-F qFil-28S-P qFil-28S-R	TTGTTTGAGATTGCAGCCCA 6FAM-CAAGTACCGTGAGGGAAAGT-TAMRA GTTTCCATCTCAGCGGTTTC	151	60 °C/30″	Filariids	[24]
All-Wol 16S qPCR-based system	*16S* rRNA gene	all.Wol.16S.301-F all.Wol.16S.347-P all.Wol.16S.478-R	TGGAACTGAGATACGGTCCAG 6FAM-AATATTGGACAATGGGCGAA-TAMRA GCACGGAGTTAGCCAGGACT	177	61 °C/30″	*Wolbachia*	
16S W-Spec		W-Specf W-Specr	CATACC TATTCGAAGGGATAG AGCTTCGAGTGAA ACCAATTC	438	60 °C/1′		[89]
Brug-gen-spec qPCR	Internal Transcribed Spacer 1 (*ITS1*)	Brug.ITS.f.260 Brug.ITS.p.307 Brug.ITS.r.421	AGCGATAGCTTAATTAATTTTACCATTT 6FAM- GCATTTATGCTAGATATGCTACCAA-TAMRA CCACCGCTAAGAGTTAAAAAAATT	161	61 °C/30″	*Brugia* spp.	This study
Brug-gen-spec PCR		Fil.ITS.f: Brug.ITS.r	GAACCTGCGGAAGGATCA CCACCGCTAAGAGTTAAAAAAATT	417–441	54 °C/30″		
Manso-gen-spec PCR		Fil.ITS.f: Manso.ITS.r	GAACCTGCGGAAGGATCA TGTGTATTTATTTGTTGGTAGCATATT	333–345	55 °C/30″	*Mansonella* spp.	
	SSU rRNA *(18S)*	Fwd.18S.631 Rwd.18S.1825r	TCGTCATTGCTGCGGTTAAA GGTTCAAGCCACTGCGATTAA	1127–1155	54 °C/1′30″	Nematoda	[61]
Pan-fil *cox1* PCR	Cytochrome c oxidase subunit 1 gene (*cox1*)	Fwd.957 Rwd.1465	ATRGTTTATCAGTCTTTTTTTATTGG GCAATYCAAATAGAAGCAAAAGT	509	52 °C/1′	Filariids	[24]
dg-Folmer′s primers		dgLCO-1490 dgHCO-2198	GGTCAACAAATCATAAAGAYATYGG TAAACTTCAGGGTGACCAAARAAYCA	708	44 °C/40″	Metazoans	[86]

4.6. Molecular Characterization of Filariids Using Genus Specific PCR Assays

4.6.1. Design of Oligonucleotides

In order to complete the molecular characterization of filariids detected by the *18S* and *cox1* genes, we targeted the Internal Transcribed Spacer 1 (*ITS1*) gene to design genus-specific PCR assays targeting *Brugia* and *Mansonella* species. The choice for this gene was based on the following criteria: a higher divergence between filarial species especially among *Brugia* species [90], its tandem repeat that increases PCR sensitivity [91] and its availability in the GenBank database for these species. Three PCR assays were designed by the alignment of *ITS1* sequences of *Brugia* sp. (HE856316), *B. malayi* (EU419346, JQ327149), *B. timori* (AF499132), *B. pahangi* (EU373628), *M. ozzardi* (MN432519, LT623912, AF228559), *M. perstans* (MN432520, KJ631373, EU272184) and *M. mariae* (KX932484) against 33 sequences (data not showed) from a representative member of Onchocercidae using the MUSCLE application within DNAstar software [92]. Three genus specific PCR systems were proposed (Table 3). This includes two PCRs: one specific for *Brugia* spp. [Brug-gen-spec] and the other specific for *Mansonella* spp. [Manso-gen-spec], and qPCR system [Brug-gen-spec qPCR] targeting *Brugia* spp.

Assay specificity was confirmed in silico and in vitro for each system, as described elsewhere [24]. Briefly, the in silico validation was conducted using Primer-BLAST [93]. Genomic DNA of *M. perstens* was used to validate the PCR for *Mansonella*, while the *B. malayi* DNA was used to validate both the qPCR and PCR for *Brugia* spp. Moreover, all PCR assays were challenged against the genomic DNA of filariids other than *Brugia* and *Mansonella,* as well as several nematodes, arthropods, vertebrate hosts (e.g., human, monkey, donkey, horse, cattle, mouse and dog) and laboratory-maintained colonies [24].

4.6.2. Amplification, Sequencing and Run Protocol

All blood samples from howler monkeys were screened for the presence of *Mansonella* and *Brugia* DNA using the genus specific PCR. The PCR reactions were carried out in a total volume of 50 µL, comprising 25 µL of AmpliTaq Gold master mix (Thermo Fisher Scientific, Saint Herblain, France), 18 µL of ultrapure water free of DNAse-RNAse, 1 µL of each primer and 5 µL of genomic DNA. PCR reactions were run under the following protocol: the incubation step at 95 °C for 15 min, 40 cycles of one minute at 95 °C, 30 s for the annealing at a different melting temperature for each PCR assays (Table 3), and 72 °C of elongation step (Table 3) with a final extension step of five minutes at 72 °C. PCR reactions were performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA).

DNA amplicons generated throughout each PCR reaction were purified using NucleoFast® 96 PCR DNA purification plate (Macherey Nagel EURL, Hoerdt, France). Purified DNAs were subjected to the second amplification using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA), then the BigDye PCR products were purified on the Sephadex G-50 Superfine gel filtration resin prior to sequencing on the ABI Prism 3130XL (Applied Biosystems, Courtaboeuf, France).

4.6.3. Molecular Screening for Brugia

In order to reveal the infection rate of *Brugia* spp., all the samples were subjected to the amplification using the genus-specific qPCR. The qPCR reaction was performed in a total volume of 20 µL including 5 µL of DNA template, 10 µL of Master Mix Roche (Eurogentec France, Angers, France), 3 µL of ultra-purified water DNAse-RNAse free and 0.5 µL of each primer, UDG and each probe. The TaqMan reaction of both systems was run using the same cycling conditions. This included two hold steps at 50 °C and 95 °C for 2 and 15 min, respectively, followed by 40 cycles of two steps each (f 95 °C for 30 s and 60 °C for 30 s). The qPCR reaction was performed in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, CA, USA).

4.7. Phylogenetic Analysis

First, nucleotide sequences of the filarial *cox1*, *18S* and *ITS1* genes, as well as the *16S* gene of *Wolbachia*, were assembled and edited by Chromas-Pro 2.0.0 (http://technelysium.com.au/wp/chromaspro/). The absence of co-amplification of nuclear mitochondrial genes (numts) was verified by aligning the obtained *cox1* sequences with the *Mansonella ozzardi* mitogenome (KX822021) [45]. Furthermore, ambiguities in the sequence chromatograms, stop codons and indels were visually verified, as recommended in Reference [94]. All the sequences were subjected separately to a preliminary analysis using Basic Local Alignment Search Tool (BLAST) [95].

Both the nuclear *18S* rRNA alone or concatenated with the *ITS1* (if amplified) gene from each filarial species generated through the present study were separately aligned against the previously published sequences from the complete rRNA sequences or draft/complete genomes from the Onchocercidae clade ONC2, ONC3, ONC4 and ONC5 [56]. While, the *cox1* sequences were aligned against the representative members of the clade ONC4 and ONC5 encountered in primates [56]. The *Wolbachia 16S* DNA sequences were aligned against the representative members of *Wolbachia* lineages (C, D, F and J) infecting filarial parasites [11,16]. MAFFT alignment was performed on the concatenated nuclear (*18S* rRNA and *ITS1*) sequences using DNAstar software [92], while the *18S*, the *cox1* and the *16S* DNA sequences were aligned using ClustalW application within Bioedit v.7.2.5. [96]. The Akaike Information Criterion (AIC) option in MEGA6 [88] was used to establish the best nucleotide substitution model adapted to each sequence alignment. The Kimura 2-parameter model (+*G*) [97] was used to generate the *18S* and the *16S* trees, while the Tamura 3-parameter model (+*I*) [98] and the General Time Reversible model (+*G*, +*I*) [98] were, respectively, used for the concatenated rRNA (*18S* and *ITS1*) and the *cox1* alignments. A maximum likelihood (ML) phylogenetic inference was used with 1000 bootstrap replicates to generate the phylogenetic tree in MEGA6 [88]. *Gongylonema nepalensis* (LC278392) rRNA sequence, both Filarioidea species (KP728088) and *Physaloptera amazonica* (MK309356) *cox1* sequences and the *16S* DNA sequence of *Rickettsia* sp. (AB795333) were used as out groups to root the trees.

In addition, we generated another *cox1* alignment, including the representative members of all the Onchocercidae clades (ONC1, ONC2, ONC3, ONC4 and ONC5) [56]. Two Filariidae and four Physalopteridae sequences were included as out-groups. The interspecific nucleotide pairwise distance (IND) was used to estimate the evolutionary divergence between *cox1* sequences among Onchocercidae. Standard error was obtained by a bootstrap procedure with 1000 replicates. Analyses were inferred on MEGA6 software [88], based on the Maximum Composite Likelihood model [99]. A scatter chart based on the IND between Onchocercidae members and the *cox1* sequences generated in the present study was drowned using XLSTAT Addinsoft version 4.1 (XLSTAT 2019: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France).

Finally, COI protein sequences of *Brugia* species (Protein Id: QIL51350, QDE55703, ALR73830, QDE55700 and ALR73832) and those of *Mansonella* species (Protein Id: CAO83087, QHA95050, AVA30206, CAO83074 and SCW25063) were retrieved from the GenBank database and aligned against the COI sequences obtained from monkeys B9 and B8, respectively. The alignment was performed using the ClustalW application within Bioedit v.7.2.5. [96]. Amino acids conservation between the COI sequences from this study comparatively to GenBank sequences was visualized on the CLC Sequence Viewer 7 (CLC Bio Qiagen, Aarhus, Denmark).

5. Conclusions

In this study, we phylogenetically describe filarial parasites belonging to three distinct genera: *Mansonella* sp. *Brugia* sp. and an unidentified Onchocercidae species. Funding extends the presence of *Brugia* sp. and the unidentified Onchocercidae species to Guiana monkeys. In addition, phylogenetic analyses highlight the necessity of completing the classification of filariasis of neo-tropical monkeys by combining morphological and molecular-based identification for an integrative taxonomical perspective. Filaria associated *Wolbachia* can be used as diagnostic markers since they are genus specific endosymbionts. Regarding the presence of *Brugia* sp. in Guiana monkeys, the same genotype

circulates in French Guiana dogs, suggesting host diversity of this filariids. We therefore developed a novel qPCR assay that could be useful for the surveillance of brugian filariasis in vectors, animals, and humans. Further studies will be needed to shed light on the life cycle, epidemiology and circulation of this potentially zoonotic parasite.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/8/626/s1, Figure S1: *18S* sequences alignment showing the nucleotide conservation of the unidentified Onchocercidae species obtained from howler monkey against the GenBank sequences of *O. volvulus* and *L. loa*, Table S1: Estimates of the evolutionary divergence between the cytochrome c oxidase subunit I (*cox1*) sequences of *Mansonella* sp. and *Brugia* sp. obtained in this study comparatively with Onchocercidae members from GenBank database.

Author Contributions: Conceptualization: B.D., Y.L., H.M., O.M.; Formal analysis and investigation: Y.L., H.M., B.D., A.L.; Writing—original draft preparation: B.D., Y.L.; Writing-review: O.M.; Supervision: O.M. and B.D. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: We especially thank Christophe B., Amélie V., and Coarasi S. for their significant help in providing samples.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Pritt, S.; Cohen, K.; Sedlacek, H. Parasitic Diseases. In *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents*, 2nd ed.; American College of Laboratory Animal Medicine; Academic Press: London, UK, 2012; pp. 415–446. ISBN 9780123809209.
2. Anderson, R.C. *Nematode parasites of vertebrates. Their development and transmission*; C.A.B. International: Wallingford, UK, 1992; p. 578.
3. Strait, K.; Else, J.G.; Eberhard, M.L. Parasitic Diseases of Nonhuman Primates. In *Nonhuman Primates in Biomedical Research*, 2nd ed.; Academic Press Elsevier Inc.: London, UK, 2012; Volume 2, pp. 197–297. ISBN 9780123813664.
4. Orihel, T.C.; Seibold, H.R. Nematodes of the bowel and tissues. In *Pathology of Simian Primates*; Fiennes, R.N., Ed.; Karger Publisher: Basel, Switzerland, 1972; pp. 1366–1368.
5. Gardiner, C.H.; Nold, J.B.; Sanders, J.E. Diagnostic exercise. *Edesonfilaria malayensis* infection in two cynomolgus monkeys. *Lab. Anim. Sci.* **1982**, *32*, 601–602.
6. Lowenstine, L.J.; Osborn, K.G. Respiratory system diseases of nonhuman primates. In *Nonhuman Primates in Biomedical Research*, 2nd ed.; Academic Press Elsevier Inc.: London, UK, 2012; Volume 2, pp. 413–481. ISBN 9780123813664.
7. Bennuru, S.; Nutman, T.B. Lymphangiogenesis and lymphatic remodeling induced by filarial parasites: Implications for pathogenesis. *PLoS Pathog.* **2009**, *5*, e1000688. [CrossRef] [PubMed]
8. Bowman, D.D.; Atkins, C.E. Heartworm biology, treatment, and control. *Vet. Clin. Small Anim. Pract.* **2009**, *39*, 1127–1158. [CrossRef]
9. Fischer, P.; Supali, T.; Maizels, R.M. Lymphatic filariasis and *Brugia timori*: Prospects for elimination. *Trends Parasitol.* **2004**, *20*, 351–355. [CrossRef] [PubMed]
10. World Health Organization. Lymphatic Filariasis. Available online: https://www.who.int/news-room/fact-sheets/detail/lymphatic-filariasis (accessed on 12 May 2020).
11. Bandi, C.; Anderson, T.J.C.; Genchi, C.; Blaxter, M.L. Phylogeny of *Wolbachia* in filarial nematodes. *Proc. R. Soc. B Biol. Sci.* **1998**, *265*, 2407–2413. [CrossRef]
12. Martin, C.; Gavotte, L. The bacteria *Wolbachia* in filariae, a biological Russian dolls' system: New trends in antifilarial treatments. *Parasite* **2010**, *17*, 79–89. [CrossRef]
13. Taylor, M.J. *Wolbachia* in the inflammatory pathogenesis of human filariasis. *Ann. N. Y. Acad. Sci.* **2003**, *990*, 444–449. [CrossRef]
14. Dingman, P.; Levy, J.K.; Kramer, L.H.; Johnson, C.M.; Lappin, M.R.; Greiner, E.C.; Courtney, C.H.; Tucker, S.J.; Morchon, R. Association of *Wolbachia* with heartworm disease in cats and dogs. *Vet. Parasitol.* **2010**, *170*, 50–60. [CrossRef]

15. Bouchery, T.; Lefoulon, E.; Karadjian, G.; Nieguitsila, A.; Martin, C. The symbiotic role of *Wolbachia* in Onchocercidae and its impact on filariasis. *Clin. Microbiol. Infect.* **2013**, *19*, 131–140. [CrossRef]
16. Hise, A.G.; Gillette-Ferguson, I.; Pearlman, E. The role of endosymbiotic *Wolbachia* bacteria in filarial disease. *Cell. Microbiol.* **2004**, *6*, 97–104. [CrossRef]
17. Debrah, L.B.; Phillips, R.O.; Pfarr, K.; Klarmann-Schulz, U.; Opoku, V.S.; Nausch, N.; Owusu, W.; Mubarik, Y.; Sander, A.L.; Lämmer, C.; et al. The efficacy of doxycycline treatment on *Mansonella perstans* infection: An open-label, randomized trial in Ghana. *Am. J. Trop. Med. Hyg.* **2019**, *101*, 84–92. [CrossRef] [PubMed]
18. Sulaiman, W.A.W.; Kamtchum-Tatuene, J.; Mohamed, M.H.; Ramachandran, V.; Ching, S.M.; Lim, S.M.S.; Hashim, H.Z.; Mat, L.N.I.; Hoo, F.K.; Basri, H. Anti-*Wolbachia* therapy for onchocerciasis & lymphatic filariasis: Current perspectives. *Indian J. Med. Res.* **2019**, *149*, 706–714.
19. McCall, J.W.; Genchi, C.; Kramer, L.; Guerrero, J.; Dzimianski, M.T. Heartworm and *Wolbachia:* Therapeutic implications. *Vet. Parasitol.* **2008**, *158*, 204–214. [CrossRef]
20. Punkosdy, G.A.; Dennis, V.A.; Lasater, B.L.; Tzertzinis, G.; Foster, J.M.; Lammie, P.J. Detection of serum IgG antibodies specific for *Wolbachia* surface protein in Rhesus monkeys infected with *Brugia malayi*. *J. Infect. Dis.* **2001**, *184*, 385–389. [CrossRef] [PubMed]
21. Turb, M.E.; Zambon, E.; Zannoni, A.; Russo, S.; Gentilini, F. Detection of *Wolbachia* DNA in blood for diagnosing filaria-associated syndromes in cats. *J. Clin. Microbiol.* **2012**, *50*, 2624–2630. [CrossRef]
22. Simón, F.; Siles-Lucas, M.; Morchón, R.; González-Miguel, J.; Mellado, I.; Carretón, E.; Montoya-Alonso, J.A. Human and animal dirofilariasis: The emergence of a zoonotic mosaic. *Clin. Microbiol. Rev.* **2012**, *25*, 507–544. [CrossRef]
23. Maia, C.; Altet, L.; Serrano, L.; Cristóvão, J.M.; Tabar, M.D.; Francino, O.; Cardoso, L.; Campino, L.; Roura, X. Molecular detection of *Leishmania infantum*, filariae and *Wolbachia* spp. in dogs from southern Portugal. *Parasit. Vectors* **2016**, *9*, 170. [CrossRef]
24. Laidoudi, Y.; Davoust, B.; Varloud, M.; Niang, E.H.A.; Fenollar, F.; Mediannikov, O. Development of a multiplexed qPCRs-based approach for the diagnosis of *Dirofilaria immitis, D. repens, Acanthocheilonema reconditum*. *Parasites Vectors Dev.* **2019**, *13*, 319. [CrossRef]
25. Laidoudi, Y.; Marié, J.L.; Tahir, D.; Watier-Grillot, S.; Mediannikov, O.; Davoust, B. Detection of canine vector-borne filariasis and their *Wolbachia* endosymbionts in French Guiana. *Microorganisms* **2020**, *8*, 770. [CrossRef]
26. Ichimori, K.; King, J.D.; Engels, D.; Yajima, A.; Mikhailov, A.; Lammie, P.; Ottesen, E.A. Global programme to eliminate lymphatic filariasis: The processes underlying programme success. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3328. [CrossRef]
27. Webster, J.P.; Molyneux, D.H.; Hotez, P.J.; Fenwick, A. The contribution of mass drug administration to global health: Past, present and future. *Philos. Trans. R. Soc. B Biol. Sci.* **2014**, *369*, 20130434. [CrossRef] [PubMed]
28. World Health Organization. *Lymphatic Filariasis: Practical Entomology—A Handbook for National Elimination Programmes*; No. WHO/HTM/NTD/PCT/2013.10.; World Health Organization: Geneva, Switzerland, 2013; p. 92.
29. Hotez, P.J.; Bottazzi, M.E.; Franco-Paredes, C.; Ault, S.K.; Periago, M.R. The neglected tropical diseases of Latin America and the Caribbean: A review of disease burden and distribution and a roadmap for control and elimination. *PLoS Negl. Trop. Dis.* **2008**, *2*, e300. [CrossRef] [PubMed]
30. Schneider, M.C.; Aguilera, X.P.; da Silva Junior, J.B.; Ault, S.K.; Najera, P.; Martinez, J.; Requejo, R.; Nicholls, R.S.; Yadon, Z.; Silva, J.C.; et al. Elimination of neglected diseases in Latin America and the Caribbean: A mapping of selected diseases. *PLoS Negl. Trop. Dis.* **2011**, *5*, e964. [CrossRef] [PubMed]
31. Pan American Health Organisation and World Health Organisation. Latin American countries gather to work towards the elimination of lymphatic filariasis (elephantiasis). Available online: https://www.paho.org/hq/index.php?option=com_content&view=article&id=8784:2013-paises-latinoamerica-reunen-avanzar-hacia-eliminacion-filariasis-linfatica-elefantiasis&Itemid=40282&lang=en (accessed on 22 June 2020).
32. Raccurt, C.P. *Mansonella ozzardi* and its vectors in the New World: An update with emphasis on the current situation in Haiti. *J. Helminthol.* **2018**, *92*, 655–661. [CrossRef] [PubMed]
33. Ta-Tang, T.H.; Luz, S.L.B.; Merino, F.J.; De Fuentes, I.; López-Vélez, R.; Almeida, T.A.P.; Lanza, M.; Abrahim, C.M.M.; Rubio, J.M. Atypical *Mansonella ozzardi microfilariae* from an endemic area of Brazilian amazonia. *Am. J. Trop. Med. Hyg.* **2016**, *95*, 629–632. [CrossRef]

34. Tavares da Silva, L.B.; Crainey, J.L.; Ribeiro da Silva, T.R.; Suwa, U.F.; Vicente, A.C.P.; Fernandes de Medeiros, J.; Pessoa, F.A.C.; Luz, S.L.B. Molecular verification of new world *Mansonella perstans* parasitemias. *Emerg. Infect. Dis.* **2017**, *23*, 545–547. [CrossRef]
35. Orihel, T.C. *Brugia guyanensis* sp. n. (Nematoda: Filarioidea) from the Coatimundi (*Nasua nasua vittata*) in British Guiana. *J. Parasitol.* **1964**, *50*, 115–118. [CrossRef]
36. Moraes, M.F.D.; da Silva, M.X.; Magalhães-Matos, P.C.; de Albuquerque, A.C.A.; Tebaldi, J.H.; Mathias, L.A.; Lux Hoppe, E.G. Filarial nematodes with zoonotic potential in ring-tailed coatis (*Nasua nasua* Linnaeus, 1766, Carnivora: Procyonidae) and domestic dogs from Iguaçu National Park, Brazil. *Vet. Parasitol. Reg. Stud. Rep.* **2017**, *8*, 1–9. [CrossRef]
37. De Argôlo, E.G.G.; Reis, T.; Fontes, D.A.T.; Gonçalves, E.C.; Giese, E.G.; de Vasconcelos Melo, F.T.; dos Santos, J.N.; Furtado, A.P. Canine filariasis in the Amazon: Species diversity and epidemiology of these emergent and neglected zoonoses. *PLoS ONE* **2018**, *13*, 1–9. [CrossRef]
38. Eisenberg, J.F.; Redford, K.H. Alouatta. In *Mammals of the Neotropics. The Central Neotropics: Ecuador, Perú, Bolivia, Brazil*; University of Chicago Press: Chicago, IL, USA, 1999; Volume 3, pp. 265–266.
39. Stuart, M.; Pendergast, V.; Rumfelt, S.; Pierberg, S.; Greenspan, L.; Glander, K.; Clarke, M. Parasites of wild howlers (*Alouatta* spp.). *Int. J. Primatol.* **1998**, *19*, 493–512. [CrossRef]
40. Cortés-Ortiz, L.; Bermingham, E.; Rico, C.; Rodríguez-Luna, E.; Sampaio, I.; Ruiz-García, M. Molecular systematics and biogeography of the Neotropical monkey genus, *Alouatta*. *Mol. Phylogenet. Evol.* **2003**, *26*, 64–81. [CrossRef]
41. Groves, C. Primate taxonomy. In *The International Encyclopedia of Biological Anthropology*; John Wiley & Sons: Hoboken, NJ, USA, 2018; Volume 1. [CrossRef]
42. Stevenson, P.R. Potential determinants of the abundance of woolly monkeys in neotropical forests. In *The Woolly Monkey: Behavior, Ecology, Systematics, and Captive Research*; Pablo, R., Ed.; Springer: New York, NY, USA, 2014; ISBN 9781493906970.
43. Medkour, H.; Davoust, B.; Levasseur, A.; Mediannikov, O. Molecular evidence of *Leishmania infantum* and *Leishmania guyanensis* in red howler monkey (*Alouatta seniculus*) from French Guiana. *Vector Borne Zoonotic Dis.* **2019**, *19*, 896–900. [CrossRef] [PubMed]
44. Bain, O.; Mutafchiev, Y.; Junker, K.; Guerrero, R.; Martin, C.; Lefoulon, E.; Uni, S. Review of the genus *Mansonella* Faust, 1929 sensu lato (Nematoda: Onchocercidae), with descriptions of a new subgenus and a new subspecies. *Zootaxa* **2015**, *3918*, 151–193. [CrossRef] [PubMed]
45. Crainey, J.L.; Marín, M.A.; Da Silva, T.R.R.; De Medeiros, J.F.; Pessoa, F.A.C.; Santos, Y.V.; Vicente, A.C.P.; Luz, S.L.B. *Mansonella ozzardi* mitogenome and pseudogene characterisation provides new perspectives on filarial parasite systematics and CO-1 barcoding. *Sci. Rep.* **2018**, *8*, 6158. [CrossRef]
46. Ruiz-García, M.; Cerón, Á.; Sánchez-Castillo, S.; Rueda-Zozaya, P.; Pinedo-Castro, M.; Gutierrez-Espeleta, G.; Shostell, J.M. Phylogeography of the mantled howler monkey (*Alouatta palliata*; Atelidae, Primates) across its geographical range by means of mitochondrial genetic analyses and new insights about the phylogeny of Alouatta. *Folia Primatol.* **2018**, *88*, 421–454. [CrossRef]
47. De Thoisy, B.; Michel, J.C.; Vogel, I.; Vié, J.C. A survey of hemoparasite infections in free-ranging mammals and reptiles in French Guiana. *J. Parasitol.* **2000**, *86*, 1035–1040. [CrossRef]
48. Klein, A.; Strube, C.; Radespiel, U.; Springer, A.; Zimmermann, E. Differences in infection patterns of vector-borne blood-stage parasites of sympatric Malagasy primate species (*Microcebus murinus*, *M. ravelobensis*). *Int. J. Parasitol. Parasites Wildl.* **2019**, *10*, 59–70. [CrossRef]
49. Lainson, R.; Shaw, J.J.; Fraiha, H.; Miles, M.A.; Draper, C.C. Chagas's disease in the amazon basin: I. *Trypanosoma cruzi* infections in silvatic mammals, triatomine bugs and man in the state of North Brazil. *Trans. R. Soc. Trop. Med. Hyg.* **1979**, *73*, 193–204. [CrossRef]
50. Petit, G.; Bain, O.; Roussilhon, C. Deux nouvelles filaires chez un singe, *Saimiri sciureus*, au Guyana. *Ann. Parasitol. Hum. Comp.* **1985**, *60*, 65–81. [CrossRef]
51. De León, G.P.-P.; Nadler, S.A. What we don't recognize can hurt us: A plea for awareness about cryptic species. *J. Parasitol.* **2010**, *96*, 453–464. [CrossRef]
52. De Ley, P.; Tandingan De Ley, I.; Morris, K.; Abebe, E.; Mundo-Ocampo, M.; Yoder, M.; Heras, J.; Waumann, D.; Rocha-Olivares, A.; Burr, A.H.J.; et al. An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. *Philos. Trans. R. Soc. B Biol. Sci.* **2005**, *360*, 1945–1958. [CrossRef] [PubMed]

53. Blaxter, M.; Mann, J.; Chapman, T.; Thomas, F.; Whitton, C.; Floyd, R.; Abebe, E. Defining operational taxonomic units using DNA barcode data. *Philos. Trans. R. Soc. B Biol. Sci.* **2005**, *360*, 1935–1943. [CrossRef] [PubMed]
54. Meldal, B.H.M.; Debenham, N.J.; De Ley, P.; De Ley, I.T.; Vanfleteren, J.R.; Vierstraete, A.R.; Bert, W.; Borgonie, G.; Moens, T.; Tyler, P.A.; et al. An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. *Mol. Phylogenet. Evol.* **2007**, *42*, 622–636. [CrossRef] [PubMed]
55. Nuchprayoon, S.; Junpee, A.; Poovorawan, Y.; Scott, A.L. Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction-restriction fragment length polymorphism analysis. *Am. J. Trop. Med. Hyg.* **2005**, *73*, 895–900. [CrossRef]
56. Lefoulon, E.; Bain, O.; Bourret, J.; Junker, K.; Guerrero, R.; Cañizales, I.; Kuzmin, Y.; Satoto, T.B.T.; Cardenas-Callirgos, J.M.; de Souza Lima, S.; et al. Shaking the tree: Multi-locus sequence typing usurps current Onchocercid (Filarial Nematode) phylogeny. *PLoS Negl. Trop. Dis.* **2015**, *9*, 1–19. [CrossRef]
57. Gaillard, C.M.; Pion, S.D.; Hamou, H.; Sirima, C.; Bizet, C.; Lemarcis, T.; Rodrigues, J.; Esteban, A.; Peeters, M.; Mpoudi Ngole, E.; et al. Detection of DNA of filariae closely related to *Mansonella perstans* in faecal samples from wild non-human primates from Cameroon and Gabon. *Parasit. Vectors* **2020**, *13*, 313. [CrossRef]
58. Ferri, E.; Barbuto, M.; Bain, O.; Galimberti, A.; Uni, S.; Guerrero, R.; Ferté, H.; Bandi, C.; Martin, C.; Casiraghi, M. Integrated taxonomy: Traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Front. Zool.* **2009**, *6*, 1. [CrossRef]
59. Casiraghi, M.; Anderson, T.J.C.; Bandi, C.; Bazzocchi, C.; Genchi, C. A phylogenetic analysis of filarial nematodes: Comparison with the phylogeny of *Wolbachia* endosymbionts. *Parasitology* **2001**, *1*, 93–103. [CrossRef]
60. Taylor, M.J.; Hoerauf, A. *Wolbachia* bacteria of filarial nematodes. *Parasitol. Today* **1999**, *15*, 437–442. [CrossRef]
61. Laidoudi, Y.; Ringot, D.; Watier-Grillot, S.; Davoust, B.; Mediannikov, O. A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France. *Parasites* **2019**, *26*, 72. [CrossRef]
62. Rossi, M.I.D.; Aguiar-Alves, F.; Santos, S.; Paiva, J.; Bendas, A.; Fernandes, O.; Labarthe, N. Detection of *Wolbachia* DNA in blood from dogs infected with *Dirofilaria immitis*. *Exp. Parasitol.* **2010**, *126*, 270–272. [CrossRef] [PubMed]
63. Satjawongvanit, H.; Phumee, A.; Tiawsirisup, S.; Sungpradit, S.; Brownell, N.; Siriyasatien, P.; Preativatanyou, K. Molecular analysis of canine filaria and its *Wolbachia* endosymbionts in domestic dogs collected from two animal university hospitals in Bangkok metropolitan region, Thailand. *Pathogens* **2019**, *8*, 114. [CrossRef] [PubMed]
64. Monica, A.; Matei, I.A.; Amico, G.D.; Bel, L.V.; Dumitrache, M.O.; Modrý, D.; Mihalca, A.D. *Dirofilaria immitis* and *D. repens* show circadian co-periodicity in naturally co-infected dogs. *Parasit Vectors* **2017**, *10*, 116.
65. Mircean, M.; Ionică, A.M.; Mircean, V.; Györke, A.; Codea, A.R.; Tăbăran, F.A.; Taulescu, M.; Dumitrache, M.O. Clinical and pathological effects of *Dirofilaria repens* and *Dirofilaria immitis* in a dog with a natural co-infection. *Parasitol. Int.* **2017**, *66*, 331–334. [CrossRef] [PubMed]
66. Eberhard, M.L.; Orihel, T.C. The genus *Mansonella* (syn. Tetrapetalonema): A new classification. *Ann. Parasitol. Hum. Comp.* **1984**, *59*, 483–496. [CrossRef]
67. Carme, B.; de Thoisy, B.; Motard, A.; Aznar, C.; Vié, J.C. Parasitoses humaines et mammifères sauvages en Guyane Française. *Med. Trop.* **2000**, *60*, 223–231.
68. Vianna, L.M.M.; Martins, M.; Cohen, M.J.; Cohen, J.M.; Belfort, R. *Mansonella ozzardi* corneal lesions in the Amazon: A cross-sectional study. *BMJ Open* **2012**, *2*, e001266. [CrossRef]
69. Garrido, C.; Campos, M. First report of presumed parasitic keratitis in Indians from the Brazilian Amazon. *Cornea* **2000**, *19*, 817–819. [CrossRef]
70. Cohen, J.M.; Santana Ribeiro, J.A.; Martins, M. Ocular manifestations in mansonelliasis. *Arq. Bras. Oftalmol.* **2008**, *71*, 167–171.
71. Baird, J.K.; Neafie, R.C.; Connor, D.H. Nodules in the conjunctiva, bung-eye, and bulge-eye in Africa caused by *Mansonella perstans*. *Am. J. Trop. Med. Hyg.* **1988**, *38*, 553–557. [CrossRef]
72. Paniz-Mondolfi, A.E.; Gárate, T.; Stavropoulos, C.; Fan, W.; González, L.M.; Eberhard, M.; Kimmelstiel, F.; Sordillo, E.M. Zoonotic filariasis caused by novel *Brugia* sp. Nematode, United States, 2011. *Emerg. Infect. Dis.* **2014**, *20*, 1248–1250. [CrossRef] [PubMed]

73. Orihel, T.C.; Beaver, P.C. Zoonotic *Brugia* infections in North and South America. *Am. J. Trop. Med. Hyg.* **1989**, *40*, 638–647. [CrossRef] [PubMed]
74. Orihel, T.C.; Eberhard, M.L. Zoonotic filariasis. *Clin. Microbiol. Rev.* **1998**, *11*, 366–381. [CrossRef] [PubMed]
75. Chalifoux, L.V. Filariasis, New World Primates. In *Nonhuman Primates I. Monographs on Pathology of Laboratory Animals*; Jones, T.C., Mohr, U., Hunt, R.D., Eds.; Springer: Berlin/Heidelberg, Germany, 1993; pp. 206–214.
76. Notarnicola, J.; Jiménez, F.A.; Gardner, S.L. A new species of *Dipetalonema* (Filarioidea: Onchocercidae) from ateles chamek from the beni of Bolivia. *J. Parasitol.* **2007**, *93*, 661–667. [CrossRef]
77. Notarnicola, J.; Pinto, C.M.; Navone, G.T. Host occurrence and geographical distribution of *Dipetalonema* spp. (Nematoda: Onchocercidae) in neotropical monkeys and the first record of *Dipetalonema gracile* in Ecuador. *Comp. Parasitol.* **2008**, *75*, 61–68. [CrossRef]
78. Chabaud, A.G. Le genre *Dipetalonema* Diesing 1861; Essai de classification. *Ann. Parasitol. Hum. Comp.* **1952**, *27*, 250–285. [CrossRef]
79. Bain, O.; Petit, G.; Rosales-Loesener, L. Filaires de singes sud-américains. *Bull. Muséum Natl. Histoire Nat. Sect. A Zool. Biol. Ecologie Anim.* **1986**, *8*, 513–542.
80. Bain, O.; Diagne, M.; Muller, R. Une cinquième filaire du genre *Dipetalonema*, parasite de singes sud-américains. *Ann. Parasitol. Hum. Comp.* **1987**, *62*, 262–270. [CrossRef]
81. Esslinger, J.H. *Dipetalonema obtusa* (McCoy, 1936) comb. n. (Filarioidea: Onchocercidae) in colombian primates, with a description of the adult. *J. Parasitol.* **1966**, *52*, 498–502. [CrossRef]
82. Vanderhoeven, E.A.; Notarnicola, J.; Agostini, I. First record of *Dipetalonema robini* Petit, Bain & Roussilhon 1985 (Nematoda: Onchocercidae) parasitizing sapajus nigritus in northeastern argentina. *Mastozoologia Neotropical.* **2017**, *24*, 483–488.
83. UICN France. *La Liste rouge des espèces menacées en France*; Muséum National d'Histoire Naturelle: Paris, France, 2015; p. 12.
84. Boubli, J.P.; di Fiore, A.; Mittermeier, R.A.; dos Santos, M. *Alouatta macconnelli. The IUCN Red List of Threatened Species 2020*, e.T40642A17926817. Downloaded on 22 July 2020. [CrossRef]
85. Folmer, O.; Black, M.; Hoeh, W.; Lutz, R.; Vrijenhoek, R. DNA primers for amplification of mitochondrial Cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **1994**, *3*, 294–299. [PubMed]
86. Meyer, C.P. Molecular systematics of cowries (Gastropoda: Cypraeidae) and diversification patterns in the tropics. *Biol. J. Linn. Soc.* **2003**, *79*, 401–459. [CrossRef]
87. Hasegawa, M.; Kishino, H.; Yano, T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **1985**, *22*, 160–174. [CrossRef]
88. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef]
89. Fong, M.Y.; Noordin, R.; Lau, Y.L.; Cheong, F.W.; Yunus, M.H.; Idris, Z.M. Comparative analysis of *ITS1* nucleotide sequence reveals distinct genetic difference between *Brugia malayi* from Northeast Borneo and Thailand. *Parasitology* **2013**, *140*, 39–45. [CrossRef]
90. McNulty, S.N.; Mitreva, M.; Weil, G.J.; Fischer, P.U. Inter and intra-specific diversity of parasites that cause lymphatic filariasis. *Infect. Genet. Evol.* **2013**, *14*, 137–146. [CrossRef]
91. Burland, T.G. DNASTAR's Lasergene sequence analysis software. *Methods Mol. Biol.* **2000**, *132*, 71–91.
92. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **2012**, *13*, 134. [CrossRef]
93. Werren, J.H.; Windsor, D.M. *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? *Proc. R. Soc. B Biol. Sci.* **2000**, *267*, 1277–1285. [CrossRef]
94. Song, H.; Buhay, J.E.; Whiting, M.F.; Crandall, K.A. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13486–13491. [CrossRef]
95. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [CrossRef]
96. Hall, T.; Biosciences, I.; Carlsbad, C. BioEdit: An important software for molecular biology. *GERF Bull. Biosci.* **2011**, *2*, 60–61.
97. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]

98. Tamura, K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol. Biol. Evol.* **1992**, *9*, 678–687.
99. Tamura, K.; Nei, M.; Kumar, S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 11030–11035. [CrossRef]

Chapitre 3 :

Surveillance et contrôle de maladies vectorielles chez le réservoir animal

3.1 : Moyen de contrôle de maladies vectorielles canines

Préambule

Avec la propagation mondiale des arthropodes, vecteurs d'agents pathogènes, les maladies à transmission vectorielle n'ont désormais plus de frontières à travers la planète. De plus, l'association de plusieurs facteurs tels que les changements écologiques et climatiques, la dynamique des populations d'homme et d'animaux peuvent affecter, à des degrés divers, la survenue et la propagation de ces maladies. Les échanges internationaux (commerce, travail, tourisme, etc.) ont conduit à l'introduction (ou à la ré-introduction) et l'implantation de « nouvelles » espèces de vecteurs et d'agents pathogènes dans des zones auparavant indemnes de maladies (Reiter et Sprenger 1987). Tel est le cas pour le moustique tigre d'Asie, *Aedes albopictus*, qui est un vecteur compétent de *Dirofilaria* spp. pour les chiens (WHO 2009), du virus de West-Nile, de celui de l'encéphalite japonaise B et du virus chikungunya, pour l'homme (Abramides et al. 2011). Cette espèce de moustique s'est répandue, avec succès, dans de nombreuses régions du monde, y compris le Bassin méditerranéen, où elle est réputée active tout au long de l'année, en permettant la transmission de nombreuses maladies aux animaux et à l'homme (Abramides et al. 2011, Worobey et al. 2013).

Les maladies vectorielles du chien (*Canine Vector-Borne Diseases* : CVBD) représentent un groupe d'infections à distribution mondiale impliquant des virus, des bactéries et des parasites (protozoaires et helminthes) transmis par des arthropodes hématophages tels que les tiques, les puces, les poux, les triatomines, les moustiques et les phlébotomes (Gibson et Colwell 2007, Otranto et al. 2009). Tout comme les autres maladies vectorielles, les CVBD sont en augmentation continue en raison, entre autres, du changement climatique, de la propagation des vecteurs, et de l'absence ou des moyens limités de contrôle (Marié et al. 2009, Colwell et al. 2011, Haines et al. 2006). La mobilité accrue et la répartition mondiale des chiens et des chats domestiques ont également contribué à l'extension rapide de certaines CVBD (Shaw

et al. 2001). En outre, l'importation de chiens provenant de zones d'endémie a entraîné une augmentation globale du nombre de CVBD émergentes dans des zones auparavant non endémiques (Otranto et al. 2009). En plus de leur impact sur le bien-être canin, les CVBD suscitent un intérêt médical croissant en raison de la nature zoonotique de certains des agents pathogènes en cause (Otranto et al. 2009). Du fait de l'absence de vaccin et de traitement efficace contre la majorité des CVBD, la prévention contre les piqures d'arthropodes vecteurs ou contre les agents pathogènes eux-mêmes, par l'emploi de thérapies préventives, demeure la solution adéquate pour protéger les chiens, et, par conséquent, leurs propriétaires, quand il s'agit de zoonoses. De nombreux produits sont utilisés à cet effet. Malgré le fait, qu'ils sont sensés rompre le cycle de transmission des CVBD, de nombreuses études ont identifié les failles suivantes, auxquelles nous avons essayé de répondre au mieux :

I. Emergence des phénomènes de résistance à la fois chez les vecteurs et les agents pathogènes.
II. Apparition de nouvelles espèces de vecteurs rustiques et dotés d'une activité annuelle, tel que le moustique-tigre.
III. Absence d'adéquation entre les protocoles de prévention choisis et le risque épidémiologique de CVBD.

J'ai, dans un premier temps, testé l'effet de la combinaison de trois substances préventives des CVBD : dinotéfurane, perméthrine et pyriproxyfène (DPP) (Vectra® 3D, Ceva santé animale, Libourne, France) sur le comportement et les préférences trophiques d'*Aedes albopictus*, en utilisant un modèle de rongeurs de laboratoires (rats/souris). En effet, cette étude avait pour objectif principal d'affirmer ou d'infirmer l'hypothèse suggérant le fait qu'en traitant un chien on aide à protéger son propriétaire. L'exposition simultanée aux piqures de moustiques de deux hôtes différents (rats/souris) a révélé que lorsqu'on traite, uniquement, les rats par le DPP, cela permettait une protection directe de 82% et 61% le jour 1 et le jour 7, respectivement,

tandis que lorsqu'on traite uniquement les souris, les rats non traités et présents à côté (30 cm) bénéficiaient d'une protection indirecte de 21% et 10% le jour 1 et le jour 7, respectivement. De plus, les résultats de l'étude ont montré que le DPP n'influence pas les préférences trophiques d'*Aedes albopictus* (**Publication N°7**).

Dans un deuxième temps, nous avons orienté notre travail vers le terrain afin d'évaluer de nouvelles stratégies de prévention et de traitement des CVBD. Nous avons évalué une stratégie de Prophylaxie Mensuelle Multimodale (PMM) combinant l'administration concomitante d'un produit ectoparasiticide (Vectra® 3D) et d'un produit dérivé des lactones macrocycliques (Milbactor®, Ceva santé animale, Libourne, France) actif contre les nématodes. La stratégie PMM a été, initialement, proposée expérimentalement contre une souche résistance de *Dirofilaria immitis*. A l'occasion de mon travail, j'ai évalué la stratégie PMM contre les CVBD sévissant en Corse, zone endémique pour la leishmaniose (Davoust et al. 2013), les dirofilarioses (Tahir et al. 2017) et l'ehrlichiose (Dahmani et al. 2017). L'étude a été menée en tant qu'essai de supériorité impliquant une cohorte annuelle de 80 chiens répartis en deux groupes : (i) un groupe test composé de 25 chiens sous PMM, avec administration *per os* de 1,5 comprimé de milbémycine oxime-praziquantel (Milbactor®) et une application topique (pipette) de 3,6 mL d'une solution de dinotéfuran-perméthrine pyriproxyfène (Vectra® 3D), (ii) un groupe contrôle sous divers Traitements Prophylactiques en situation Réelle (TPR) basés sur l'utilisation de produits ectoparasiticides [différentes formulations : deltaméthrine, fluralaner, fipronil] et/ou de produits macrocycliques à base de lactone [milbémycine oxime/praziquantel, milbémycine oxime, moxidectine]. L'évaluation de l'efficacité des traitements a été faite par la comparaison de l'incidence semi-annuelle cumulée. De ce fait, tous les chiens ont fait l'objet d'une prise de sang à J 0, à J +6 mois et à J +12 mois. Par biologie moléculaire, nous avons recherché la présence de dirofilaires (*Dirofilaria immitis* et *D. repens*), tandis que la leishmaniose et l'ehrlichiose ont été dépistées sérologiquement en utilisant la

technique d'immunofluorescence indirecte. A l'issue de cette étude, aucun nouveau cas de CVBD n'a été enregistré dans le groupe test. En revanche, l'incidence cumulée des CVBD était à 20% (n= 11 ; p= 0,015) dans le groupe contrôle. Ceci concerne, principalement, la dirofilariose à *D. immitis* et/ou *D. repens* avec 16,4 % (n= 9 ; p=0,027) et seulement 5,5% (n= 3 ; p=0,241) pour l'ehrlichiose. Aucun nouveau cas de leishmaniose n'a été observé dans les deux groupes. L'étude montre donc l'intérêt de l'utilisation en synergie de produits efficaces à la fois contre les vecteurs et les parasites, tout en prenant en compte l'activité de transmission annuelle dans les zones à haut risque (**Publication N°8**).

Nous avons aussi porté une attention particulière à la leishmaniose canine, compte tenu du manque d'efficacité, des complications et des effets secondaires observés lors de son traitement avec les substances actuellement recommandées tel que l'allopurinol, les antimoines pentavalents et la miltéfosine (Miró et al. 2017, Mateo et al. 2009, Saridomichelakis et al. 2006). Nous avons évalué un traitement à base d'artésunate pour la leishmaniose canine clinique, une thérapie qui avait montré son efficacité contre la leishmaniose murine (Muzamil et al. 2015, Islamuddin et al. 2015, Muzamil et al. 2017). Pour ce faire, nous avons mené un essai terrain de non-infériorité entre le traitement par l'artésunate et l'antimoniate de méglumine/allopurinol (traitement actuel) pour la leishmaniose clinique. Un total de 42 chiens d'Algérie, cliniquement atteints de leishmaniose canine a été divisé en deux groupes : (i) groupe essai (n=16) traité par l'administration *per os* de 25 mg/kg/jour d'artésunate pendant six jours et (ii) groupe témoin (n=26) qui a reçu 100 mg/kg/jour d'antimoniate de méglumine et 30 mg/kg/jour d'allopurinol pendant 28 jours. L'efficacité des traitements a été évaluée par l'évolution de l'état clinique (scores), la charge parasitaire (par qPCR) et la réponse humorale (charge d'anticorps), à différents moments : J 0, 30, 90, 180 jours post-traitement. A l'issue de ce protocole thérapeutique, les chiens des deux groupes ont montré une amélioration significative sur tous les plans. Cependant, le groupe essai, traité par l'artésunate a montré une

réduction significative (p=0,0001) des scores cliniques, de la charge parasitaire et des titres des anticorps au sixième mois post-traitement en le comparant avec le groupe contrôle avec une rapidité significative d'amélioration (P < 0,012). On a enregistré une baisse de 3,04 *versus* 2,28 dans le titre en anticorps entre le groupe traitement et le groupe contrôle, respectivement, et ce au bout de 30 jours post-traitement. En se basant sur ces résultats, nous pensons que l'artésunate peut offrir une bonne alternative, à un moindre coût et avec plus d'efficacité que les traitements actuels. Cependant d'autres études de ce genre demeurent nécessaires pour confirmer nos résultats préliminaires (**Publication N°9**).

Avec l'émergence du phénomène de résistance aux insecticides chez les arthropodes vecteurs (Bouwman et al. 2011), le besoin de trouver de nouvelles molécules insecticides, devient urgent (Zaim et Guillet 2002, Raoult et Abat 2017). La lutte biologique a constitué, depuis longtemps, une bonne alternative pour agir contre les gites larvaires d'insectes nuisibles. Par exemple, *Bacillus thuringiensis*, une bactérie du sol représentant l'espèce de bactérie la plus largement utilisée pour la lutte biologique, avec au moins quatre sous-espèces utilisées contre les insectes ravageurs à savoir des lépidoptères (papillons), coléoptères et diptères (vraies mouches) (Zhang et al. 2015, O'Connor et al. 2012). Des gènes de *Bacillus thuringiensis* ont, également, été incorporés dans des cultures transgéniques, ce qui permet aux plantes d'exprimer certaines des toxines de la bactérie, qui sont des protéines (Favia et al. 2008). La bactérie *Paenibacillus popilliae*, constitue également un autre succès de la lutte biologique. Elle s'est avérée utile dans la lutte contre le scarabée japonais en tuant les larves sans effet néfaste sur les vertébrés (Shane et al. 2018). Nous avons mené, au laboratoire cette fois-ci, une étude destinée à la recherche et à la caractérisation préliminaire de molécules bactériennes à effet insecticide en utilisant les larves d'*Aedes albopictus*. Durant cette étude, on s'est focalisé sur la caractérisation des composés actifs qu'on a identifiés à l'issue du dépistage de métabolites secondaires des souches bactériennes de la collection de l'IHU. D'abord, la souche bactérienne

Serratia marcescens P400 a induit une mortalité de 78% des larves de moustiques, faisant d'elle la souche la plus efficace. Ensuite, les différents métabolites de cette souche ont été isolés et testés séparément. Nous avons caractérisé les molécules actives par chromatographie liquide HPLC, suivi de spectrométrie de masse. Les résultats de la formule brute montrent qu'il s'agit de trois amines différentes. Une parmi elles s'est avérée soluble dans l'eau, et une fois testé seule, elle a donné un effet insecticide intense en le comparant à celui induit par l'ivermectine. Cette étude montre que la substance que nous venons de décrire partiellement, constitue un candidat potentiel utilisable pour la lutte antivectorielle (**Publication N°10**).

Toujours dans le cadre de la lutte biologique contre les vecteurs, nous avons exploré moléculairement la présence de guêpes parasitoïdes chez un ensemble de 785 tiques dures collectées en Afrique de l'Ouest (Côte d'Ivoire et Sénégal) et en Russie. De nombreuses guêpes parasitoïdes du genre *Ixodiphagus* (Hymenoptera, Chalcidoidea: Encyrtidae) sont réputées pour être des tueuses de tiques en les utilisant comme niche pour pondre leurs œufs, qui une fois éclos, se servent des tiques comme source alimentaire. Les résultats de notre étude montrent que 3% (28/785) des tiques analysées sont parasitées par des guêpes. L'analyse moléculaire montre que, sur l'ensemble des tiques parasitées, *Ixodiphagus hookeri* était présente dans 86% (24/28) des tiques, tandis qu'une autre espèce de guêpes parasitoïdes (potentiellement nouvelle) a été détectée dans les 14% restantes. Cette étude montre que les guêpes parasitoïdes pourraient être utilisées pour une approche prometteuse dans la lutte biologique contre les tiques (**Publication N°11**).

Publication N°7

Effect of Dinotefuran, Permethrin, and Pyriproxyfen (Vectra®3D) on the Foraging and Blood-Feeding Behaviors of *Aedes albopictus* Using Laboratory Rodent Model

Younes Laidoudi[1,2], Djamel Tahir[1,2], Hacène Medkour[1,2], Marie Varloud[3], Oleg Mediannikov[1,2] and Bernard Davoust[1,2,*]

[1]AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, IRD,Aix Marseille Univ, 19-21, Bd Jean Moulin, 13005 Marseille, France
[2]IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France
[3]Ceva Santé Animale, 10, Av de la Ballastière, 33500 Libourne, France

ARTICLE DE RECHERCHE

Publié dans : Insects, 2020, 11(8), 507

(doi.10.3390/insects11080507)

insects

MDPI

Article

Effect of Dinotefuran, Permethrin, and Pyriproxyfen (Vectra® 3D) on the Foraging and Blood-Feeding Behaviors of *Aedes albopictus* Using Laboratory Rodent Model

Younes Laidoudi [1,2], Djamel Tahir [1,2], Hacène Medkour [1,2], Marie Varloud [3], Oleg Mediannikov [1,2] and Bernard Davoust [1,2,*]

1 AP-HM, Microbes, Evolution, Phylogeny and Infection (MEPHI), IHU Méditerranée Infection, IRD, Aix Marseille Univ, 19-21, Bd Jean Moulin, 13005 Marseille, France; younes.laidoudi@yahoo.com (Y.L.); djamel.tahir@yahoo.fr (D.T.); hacenevet1990@yahoo.fr (H.M.); olegusss1@gmail.com (O.M.)

2 IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13005 Marseille, France

3 Ceva Santé Animale, 10, Av de la Ballastière, 33500 Libourne, France; marie.varloud@ceva.com

* Correspondence: bernard.davoust@gmail.com

Received: 26 June 2020; Accepted: 4 August 2020; Published: 5 August 2020

Simple Summary: Tiger mosquito (*Aedes albopictus*) is a harmful vector involved in the transmission of several diseases to humans and their pets. Currently, several veterinary products are used to prevent pets against bites of arthropod vectors. However, there is no available information on the effect of these products on feeding and host choice behaviors of *Aedes albopictus* in the presence of treated and untreated hosts, as is the case of treated dogs present in close physical contact with their owners. The present study investigated the effect of a spot-on product (Vectra® 3D) on the feeding and host choice behaviours of *Aedes albopictus* when treated and untreated hosts are presents. Laboratory rodent model was performed to simulate the natural conditions. Rat and mouse hosts were alternately treated with Vectra® 3D and exposed simultaneously to starved mosquitoes. Results showed that Vectra® 3D-treated hosts are perfectly protected against up to 82% of mosquitoes. While up to 21% of mosquitoes were repelled from untreated hosts when these latter are present in close physical contact (30 cm) with treated ones suggesting an indirect protection that can allowed the protection of owners who treat their pets with Vectra® 3D.

Abstract: Dinotefuran-Permethrin-Pyriproxyfen (DPP) is used to kill and repel mosquitoes from dogs. However, the influence of the product on the host-seeking behavior of mosquitoes remains unknown. The interference of DPP with the host selection of unfed female *Aedes albopictus* was investigated. A total of 18 animals (9 mice and 9 rats) were divided into three groups of six animals each. DU: DPP treated rats ($n = 3$) with untreated mice ($n = 3$), UD: DPP treated mice ($n = 3$) with untreated rats ($n = 3$) and control UU: untreated mice ($n = 3$) and untreated rats ($n = 3$). In each group, the rats and mice were placed 30 cm apart. After sedation, the animals in each group were exposed twice (Day 1 and Day 7 post-treatment) for one hour to 71 ± 3 female mosquitoes. Mosquitoes were categorized after the 2-h post-exposure period as dead or alive. Blood-meal origin was determined from mosquitoes using a newly customized duplex qPCR. The highest values of forage ratio ($1.36 \geq wi \leq 1.88$) and selection index ($0.63 \geq Bi \leq 0.94$) for rat hosts indicates a preference of mosquitoes for this species as compared to mice when co-housed during the exposure. The mosquitoes only seldom fed on mice, even in the untreated group. The anti-feeding effect of DPP was therefore only assessed on rat's hosts. The results showed that DPP, when directly applied on rats, provided a direct protection of 82% and 61% on Day 1 and Day 7, respectively, while when applied on mice hosts (UD), the DPP provided an indirect protection of 21% and 10% on Day 1 and Day 7, respectively. The results showed also that DPP, when applied on rats, provided a direct protection against *Ae. albopictus* bites. This effect did not result in increased exposure of the untreated host placed in the same cage at a distance of 30 cm.

Keywords: *Aedes albopictus*; animal model; host selection; blood-meal identification; rodent-qPCR; Vectra® 3D

1. Introduction

The Asian tiger mosquito, *Aedes albopictus* (Skuse, 1894), is considered the most invasive mosquito species in the world [1]. *Ae. albopictus* is a forest species that has adapted to rural, suburban, and urban environments In recent decades, this species of mosquito has spread throughout the world, from Asia to the Middle East, Australia, Africa, Europe, and Americas, especially through the international trade of used tires [2–4].

Ae. albopictus is a day biter and unusually aggressive mosquito, deteriorating life quality and may induce hypersensitivity reaction [5,6]. This mosquito has an opportunistic feeding behavior on a wide range of hosts, such as domestic and wild animals, reptiles, birds, and amphibians as well as humans [7,8], with a preference for mammalian host, especially humans, cats, and dogs [9]. This is why it has become a major pest in many communities.

Ae. albopictus is a competent vector of the main deadly viruses such as chikungunya, dengue, Zika, Japanese encephalitis, West Nile, yellow fever, and Usutu [10], and acts as a competent vector for filarial parasites, such as *Dirofilaria* spp. in Asia, North America, and Europe [11]. *D. immitis* induces canine and feline heartworm disease (Syn. cardiopulmonary dirofilariasis) and human pulmonary dirofilariasis, while *D. repens* causes respectively subcutaneous and subcutaneous/ocular dirofilariosis in canine and human hosts [12].

Currently, the use of insecticidal repellents is the most effective measure to protect pets against hematophagous arthropods such as mosquitoes [13]. Among veterinarian products, Vectra® 3D (Ceva Santé animale SA, Libourne, France), a broad spectrum topical solution of dinotefuran, permethrin, and pyriproxyfen (DPP), has been experimentally proven to be effective in blocking the feed of *Aedes* mosquitoes on dogs [14,15] and on mice [16]. It is noteworthy that rodent model remain the easier to set and less expensive than the other ones, such as canine, chickens, pigeons, and goats [16].

DPP compounds are contact poisons and do not require ingestion by the insect to be effective [17]. Dinotefuran and Permethrin act as synergetic compounds inducing continuous nerve stimulation, incoordination, tremors, and death of the insect [17,18]. While the third compound (Pyriproxyfen) acts as a suppressor of embryogenesis of the insect [18]. Despite the absence of spatial repellency, pet owners often report that they had less mosquito bites when they stay close to their treated dogs with DPP. Until now, experimental studies demonstrated the effectiveness of these products by using test treatment and control groups exposed one by one to the mosquitoes [19,20]. However, such demonstration may not depict the host-seeking and feeding behaviors of mosquitoes in the presence of both treated and untreated hosts as is the case in the real life.

To determine whether the feeding behavior and host preference of mosquitoes (in this case *Ae. albopictus*) is influenced by the presence of a treated host with insecticidal repellent products, we conducted an experimental study in which two different rodent species were exposed simultaneously.

2. Materials and Methods

2.1. Laboratory Animals and Ethics Statement

A total of eighteen healthy animals were involved in this study: (i) Nine adults (aged 6 weeks) male Sprague-Dawley rats with a mean ± standard deviation (SD) weight of 160.7 ± 3.6 g and (ii) nine adults (6-week-old), female Swiss CD1 mice weighing a mean ± standard deviation (SD) of 20.16 ± 0.4 g (Charles River Laboratories, Écully, France). The animals were housed in groups of three individuals per cage of 58 × 36 × 20 cm for rats and 50 × 20 × 20 cm for mice, at a temperature of 22 °C under an LD 12: 12 h cycle. They were fed an appropriate maintenance ration of commercial food

pellets (SAFE D03 Rats Mice breeding diet, usine SAFE, Augy, France). Filtered tap water was available ad libitum. Animals were checked by the veterinarian once a day. They were handled according to French rules on the protection of animals used for scientific purposes (decree no. 2013-118; 1 February 2013, Paris) [21]. The experimental protocol was reviewed and approved by the Ethics Committee for Animal Experimentation at Aix-Marseille University (approval n° 201602071706710).

2.2. Treatment Administration

Dinotefuran, Pyriproxyfen and Permethrin (DPP) are the active ingredients of the insecticide Vectra® 3D (Ceva Santé animale SA, Libourne, France) product and are concentrated at 54.00 mg/mL of dinotefuran (CAS number: 165252-70-0), 4.84 mg/mL of pyriproxyfen (CAS number: 95737-68-1) and 397.00 mg per mL and permethrin (CAS number: 52645-53-1). The minimal recommended dose of Vectra® 3D is 0.12 mL/kg of body weight (BW) for dogs. This dose was adjusted for use on laboratory animals as described in anterior studies [22–24]. Briefly, the equivalent dose (Ed) for each animal unit was appropriately calculated as follow: the Ed expressed in mg/kg BW was obtained by multiplying the dog dose expressed in mg/kg BW by the appropriate conversion factor for each host, wherein the conversion factors reported in Reference [22]. We therefore transformed each dose from mg/kg BW to mg/m^2 body surface area (BSA) by multiplying the dose by the correction factor (K_m) as described in Reference [23]. Finally, the appropriate amount of each compound was obtained by multiplying by the BSA of each host as shown in Table 1.

Table 1. Dose calculation of Vectra® 3D applied on laboratory animals.

Species Description	Active Product	Conversion Factor from Dogs (dog dose mg/kg BW)	Dose mg/kg BW	Correction Factor (km)	Dose mg/m^2 BSA	Dose Per Animal, mg	Vectra® 3D (mL/animal unit)
Mouse (0.02 kg BW, 0.0066 m^2 BSA)	Dinotefuran	6 (6.4)	38.4	3	115.2	0.76	0.014
	Pyriproxyfen	6 (0.6)	3.6	3	10.8	0.071	
	Permethrin	6 (46.6)	279.6	3	838.8	5.54	
Rat (0.15 kg BW, 0.025 m^2 BSA)	Dinotefuran	4 (6.4)	25.6	6	153.6	3.84	0.070
	Pyriproxyfen	4 (0.6)	2.4	6	14.4	0.36	
	Permethrin	4 (46.6)	186.4	6	1118.4	27.96	

After an acclimation period, the animals were allocated into three groups of three rats and three mice each and were named TU, UT, and UU according to their treatment statues, where TU corresponds to DPP treated rats (n = 3) with untreated mice (n = 3), UT: untreated rats (n = 3) with DPP treated mice (n = 3) and UU: untreated rats (n = 3) and untreated mice (n = 3). This latter was used as control group. Once treatment has been applied, rats and mice from each group were housed in separate cages and maintained under the same conditions. The exposure to mosquito bites was performed twice at day 1 and day 7 post-treatment in order to determine the persistence of the repellent and killing effects.

2.3. Source of Ae. albopictus

The Marseille strain of *Ae. albopictus* mosquitoes was maintained in the insectary of our laboratory (Aix-Marseille University, Marseille, France) from 2012, without exposure to any insecticides. Eggs were obtained from adult females fed on defibrinated human blood (according to an agreement with the Etablissement Français du Sang) using a Hemotek (Discovery Workshops, Accrington, UK). Adult mosquitoes were maintained in a climate-controlled chamber (Sanyo incubator MIR-254-PE; Sanyo Electric Co. Ltd, Tokyo, Japan) adjusted at 27 ± 5 °C and 80 ± 15% of the relative humidity (RH). They were allowed to feed on a 10% sucrose-water solution. Adult females aged 4 to 5 days were starved for 24 h prior to each challenge exposure.

2.4. Animal Exposure to Ae. albopictus

At each exposure time, 71 (margin of error "me" = 3) adult females of *Ae. albopictus* mosquitoes were introduced in each of the 9 cages (W24.5 × D24.5 × H63.0 cm). After sedation by intraperitoneal injection of a combination of 90mg/kg BW ketamine (Imalgene® 500; Boehringher Ingelheim SA, Lyon, France) and 10 mg/kg BW xylazine (Rompun® 2%; Bayer Santé animale SA, Loos, France), the animals in each group were divided into three exposure subgroups. Each exposure subgroup was made of one rat and one mouse and was exposed for 1 h to mosquitoes on Day 1 and 7 post-treatment. A distance of 30 cm separated the sedated rats and mice in the exposure cages (Figure 1). The animals were carefully observed during and after the exposure to mosquito bites. The evaluation gird (Table S1) was adapted from the Reference [25] and was used to evaluate the tolerance degrees of the experiment by the animals in order to avoid any pain, distress, or discomfort throughout the whole experiment according to their signs. At the end of the exposure period, all animals were carefully reintroduced in their appropriate cages and surveilled until they woke up. Mosquitoes were then examined and classified as dead or alive at two hours after the exposure period. The engorgement determination was confirmed later by qPCR.

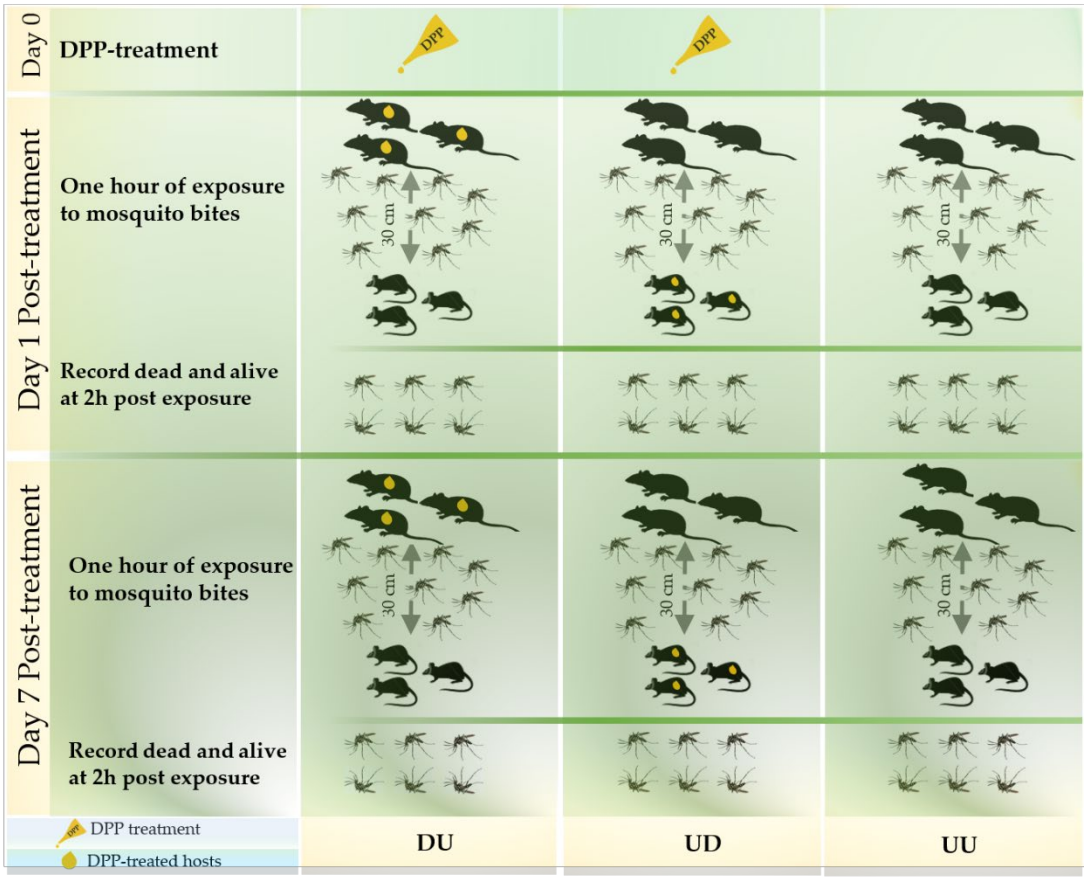


Figure 1. Overview of the study designs of the rat–mouse model.

2.5. Engorgement Determination and Blood-Meal Identification

We developed a duplex qPCR to detect and discriminate between mosquito blood-meals taken from rat and mouse. The gene encoding for the adrenergic receptor subtypes alpha 2b (ADRA2B) was selected for the development of a discriminatory duplex-qPCR. The ADRA2B is known to discriminate between rodent species [26–29].

The available sequences of the ADRA2B genes of *Mus musculus* (mouse) and *Rattus norvegicus* (rat) were retrieved from GenBank database, then aligned using ClustalW application with BioEdit v 7.0.5.3 software [30]. Designed primers (Fwd-mu-rat-1117: 5′-GAGATCCTCAGGTGGGGTG-3′ and Rwd-mu-rat-1397: 5′-AACCAGGAGGTAGAGACACC-3′) target a partial sequence (280 bp) of ADRA2B gene of both rat and mice. The specificity of the qPCR was confined to the TAMRA probes, namely: P-mu-1343: 6VIC-5′-TTACGAACCCTCATGGCGAT-3′ specific to *M. musculus* and P-rat-1354: 6FAM-5′- CACAGTGATATGGCTGTGCA-3′ specific to *R. norvegicus*. The assay specificity was validated using the single and mixed DNA of rat and mouse as well as a wide range of negative controls from hosts (e.g., human, rabbit, cat, dog, donkey) and microorganisms (e.g., *Wolbachia* spp. *Leishmania*, *Dirofilaria* spp.) as previously described [31]. Once validated, the duplex qPCR was used to detect and identify blood-meals from all *Ae. albopictus* mosquitoes used in this study. Briefly, the DNA was extracted individually from every *Ae. albopictus* mosquito used in this study using the QIAGEN DNA tissues extraction kit (QIAGEN, Hilden, Germany), following the manufacturer's recommendations. The qPCR reaction was carried out in a total volume of 20 µL, containing 3 µL of ultra-purified water DNAse-RNAse free, 5 µL of DNA template, 10 µL of Master Mix Roche (Eurogentec), 0.75 µL of each primer (at 50 µM concentration), and 0.5 µL of both UDG and each probe (at 20 µM concentration). The TaqMan qPCR run included two hold steps at 50 °C for 2 min followed by 15 min at 95 °C, and 39 cycles of two steps each (95 °C for 30 s and 60 °C for 30 s). qPCR reaction was performed in a thermal cycler CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France). Mixed DNA extracted from mice and rat bloods was used as positive control, while DNA extracted from unfed *Ae. albopictus* was used as negative control.

2.6. Data Analysis

For each exposure time, the Kruskal Wallis test with an exact computation of the *p*-value was used to compare between groups in terms of engorged, dead, and alive mosquitoes, as well as the blood-meal origin (rats, mice, or mixed hosts). The Conover-Iman test was used for pairwise comparisons between groups for each parameter. The difference was considered at p-value ≤ 0.05. The statistical analysis was performed using Addinsoft 2018 (XLSTAT 2018: Data Analysis and Statistical Solution for Microsoft Excel, Paris, France).

The total number of alive *Ae. albopictus* females was transformed to the natural logarithm of (count + 1) for calculation of geometric means. The Abbott's formula (Abbott, 1987) was applied to calculate the insecticidal effect induced by DPP treatment at each time of exposure: Insecticidal efficacy $(\%) = 100 \times \frac{NCi - \mathrm{NTi}}{NCi}$. Where NCi and NTi represent the geometric mean (GM) of alive *Ae. albopictus* in the control and treated groups, respectively.

The total number of engorged mosquitoes identified by qPCR for each group at each exposure time was transformed to the natural logarithm of (count + 1) for calculating geometric means (GM). The GM numbers of mixed (rat + mice) blood-meals were added to the GM number of single blood-meals upon each host. Two repellent effects were evaluated in each treated group at each time exposure: (i) the direct repellency against *Ae. albopictus* toward DPP-treated hosts (rats of DU and mice of UD) in comparison to the same hosts from UU, and (ii) the indirect repellency effect against *Ae. albopictus* toward DPP-untreated hosts (mice of DU and rats of UD) comparatively to the same hosts from UU. The direct and indirect repellency effects of DPP in treated groups (DU and UD) were calculated using Abbott's formula (Abbott, 1987) [32] as follows: Anti feeding efficacy for host *i* (%) = 100 × (NCei−NTei)/$\mathrm{NCe_i}$. Where $\mathrm{NCe_i}$ and $\mathrm{NTe_i}$ represent respectively the geometric mean (GM) numbers of engorged females of *Ae. albopictus* upon host *i* from the control and treated group according to the qPCR results.

In addition, the forage ratio (*wi*) [33–35] and selection index (*Bi*) of Manly et al. [36] were calculated to assess feeding preferences of *Ae. albopictus* toward rats and mouse hosts regarding their treatment (DPP) statues for each exposure time. The percent of feeding upon host *i* was first calculated as the rate of engorgement upon host *i* (*Ri*) (i.e., considering the total number of females of *Ae. albopictus* used) using the following formula: $\%Ri = \frac{(Fei + Fex)}{(N+Fex)}$, where *Fei* is the total number of blood taken

upon host *i*, *Fex* is the total number of mixed blood-meals, and *N* represents the total number of females of *Ae. albopictus* used in each group. In addition, in order to evaluate the feeding preference of *Ae. albopictus* toward hosts from each group, the rate of engorgement upon host *i* (*Oi*) was calculated by considering only the number of blood-meals taken by *Ae. albopictus* in each group, using the following formula: $\%Oi = \frac{(Fei + Fex)}{\sum_i^2 Fe}$. Where, *Fei* is the total number of blood taken upon host *i*, *Fex* is the total number of mixed blood-meals and Fe is the total number of blood-meals.

The forage ratio *wi* of *Ae. albopictus* for host *i* was calculated as: $wi = \frac{Oi}{Pi}$. Where *wi* is the forage ratio of *Ae. albopictus* for host *i*, *Oi* is the proportion of blood-meals upon host *i* and *Pi* is the proportion of host *i* available in each group. It was 50% at each time point.

The selection index of *Ae. albopictus* for hosts was calculated as follows: $Bi = \frac{wi}{\sum_{i=1}^{n} wi}$. Where *Bi* is the selection index of *Ae. albopictus* for host *i*, *wi* is the forage ratio of *Ae. albopictus* for host *i* and *n* is the number of available blood-meals. Two types ($n = 2$) of hosts were available at each time exposure, values of 0.5 ($1/n$) of the selection index (*Bi*) indicate no preference, those below 0.5 indicate relative avoidance, and values above 0.5 indicate relative preference of host species *i*.

3. Results

The animals tolerated the treatment well for the duration of the experiment. Mosquitoes began attacking the animals immediately after they were released. Visual observations indicated that in the control group, mosquitoes preferred to bite rats over mice and seldom fed on the latter. This observation was confirmed following the molecular identification of blood-meals of mosquitoes. The comparative analysis between groups are shown in Table 2. At Day 1, the DPP treatment decreased the engorgement of *Ae. albopictus* mosquitoes in both DU and UD compared to UU, wherein the number of engorged mosquitoes upon treated hosts was significantly decreased in comparison to untreated ones. At Day 7, the same effect was only observed in DU. DPP treatment significantly increased the mortality of *Ae. albopictus* mosquitoes in both DU and UD compared to UU at each exposure time (Day 1 and Day 7) and was particularly high when the treatment was applied on rats (DU).

Table 2. Statistical analysis of engorgement status, blood-meal origin, and mosquito viability between the three groups.

		Molecular Identification of Blood-Meals				**Viability at 2 h after Exposure**		**Total Used Per Group**
	Statistics	**Feed on Rats**	**Feed on Mice**	**Feed on Both**	**Engorged**	**Dead**	**Alive**	
Day 1	DU	15 (4.9)	8 (2.3)	1 (0.6)	24 (7.9)	162 (53.6)	53 (16.1)	215 (71.6)
	UD	74 (21.9)	2 (0.6)	3 (0.8)	79 (23.0)	41 (13.5)	172 (57.3)	213 (71.0)
	UU	85 (28.2)	10 (3.2)	2 (0.6)	97 (32.1)	8 (2.4)	201 (66.9)	209 (96.5)
		Kruskal Wallis test						
	***p*-value**	**0.049**	0.997	0.996	**0.043**	**0.004**	**0.004**	/
		Pairwise comparisons (*p*-value)						
	DU vs. UD	**0.015**	0.079	0.674	**0.025**	**0.010**	**0.009**	/
	DU vs. UU	**0.027**	0.430	1.000	**0.014**	**<0.0001**	**<0.0001**	/
	UD vs. UU	0.657	**0.025**	0.674	0.653	**0.010**	**0.009**	/
Day 7	DU	33 (10.9)	7 (2.1)	2 (0.6)	42 (13.7)	96 (31.3)	129 (42.6)	225 (75.0)
	UD	78 (26.0)	8 (2.4)	2 (0.6)	88 (29.3)	31 (10.0)	187 (62.3)	218 (72.6)
	UU	87 (28.6)	13 (3.9)	3 (0.8)	103 (33.6)	13 (4.2)	191 (63.6)	204 (68.0)
		Kruskal Wallis test						
	***p*-value**	**0.039**	0.561	0.996	**0.05**	**0.004**	**0.029**	/
		Pairwise comparisons (*p*-value)						
	DU vs. UD	**0.028**	0.833	1.000	**0.030**	**0.010**	**0.028**	/
	DU vs. UU	**0.012**	0.314	0.674	**0.012**	**<0.0001**	**0.012**	/
	UD vs. UU	0.500	0.413	0.674	0.506	**0.010**	0.50	/

Values in bold are significant at the level alpha = 0.05; DU: Dinotefuran-Pyriproxyfen-Permethrin (DPP) treated rats and untreated mice, UD: DPP treated mice and untreated rats, UU: untreated rats and mice.

3.1. Insecticidal Efficacy

A large majority of mosquitoes survived after exposure in the untreated group. In contrast, a significant mortality rate was recorded at each challenge time point within DU and UD in comparison to UU (Tables 2 and 3). The insecticidal effect of DPP was 75.9% (Day 1) and 33.0% (Day 7) in Group 1 and 14.3% (Day 1) and 2.0% (Day 7) in Group 2 (Table 3).

Table 3. Insecticidal efficacy of Vectra® 3D against *Ae. albopictus* in combined mouse–rat model at 2 h post-exposure.

Groups/Times		Mortality Statutes of *Ae. albopictus*		Insecticidal Effect (%)
		GM	Percentage	
Day 1	DU	53.6	74.9	75.9
	UD	13.5	19.1	14.3
	UU	2.4	3.5	/
Day 7	DU	31.3	41.7	33.0
	UD	10.0	13.8	2.0
	UU	4.2	6.2	/

3.2. Direct and Indirect Anti-Feeding Efficacy

The engorgement rate in the control group was 46.4% and 50.5% at Day 1 and Day 7, respectively. A significant reduction in the number of engorged *Ae. albopictus* females was observed in DU compared to UD and UU at each challenge time point, while no significant reduction was observed between UD and UU (Tables 2 and 4). In all groups, the blood-meals taken upon rat hosts were always higher than those taken upon mice. In contrast to rat-blood-meals from the control group, where they ranged between 41.2 and 43.5, the rate of mouse blood-meals in the control group (Table 4) did not reach the threshold of 25% set by the WHO for the calculation of the repellent efficacy [37].

DPP repellent effects were calculated only for rat hosts (Table 4). The direct repellency was calculated from DPP-treated rats (DU) and it was 81.9% and 61.0% at Day 1 and Day 7, respectively. While the indirect repellency was calculated from UD, wherein the untreated rats were exposed simultaneously with DPP-treated mice. The indirect repellency was 21.1 and 9.9% at Day 1 and Day 7, respectively.

Table 4. Results of the engorgement rates on rodents (rats and mice) and the direct and indirect repellent effect of Vectra® 3D against *Ae. albopictus* in the presence of both treated and untreated hosts respectively, at a distance of 30 cm between each.

Groups/Times		Engorged *Ae. albopictus*		Percent of Blood-Meals (% *Ri*)		Repellent Effect (%)
		GM	Percentage	Rats	Mice	Rat
Day 1	DU	7.9	11.2	7.4	4.2	81.9 [a]
	UD	23.0	37.1	35.7	2.3	21.1 [b]
	UU	32.1	46.4	41.2	5.7	/
Day 7	DU	13.7	18.7	15.4	4.0	61.0 [a]
	UD	29.3	40.4	36.4	4.6	9.9 [b]
	UU	33.6	50.5	43.5	7.7	/

[a] and [b] indicate respectively the direct and indirect repellencies.

3.3. Host Preference

Results of the forage ratio (*wi*) and the selection indexes (*Bi*) for each host are shown in Table 5 and Figure 2. Both *wi* and *Bi* indicated that rats were the preferred source of blood-feeding relative to mice in each challenge exposure irrespective to their treatment (Table 5, Figure 2). Data from UU indicates that *Ae. albopictus* had a tropism of 6–7 times higher for rat than for mice hosts.

Table 5. Feeding preference of *Ae. albopictus* in each group towards treated/untreated hosts expressed by forage ratio and selection index.

Groups		Percent of Blood-Meals (% *Oi*)		Forage Ratio for Host *i* (*wi*)		Selection Index for Host *i* (*Bi*)	
		Rat	Mouse	Rat	Mouse	Rat	Mouse
Day 1	DU	64.0	36.0	1.28	0.72	0.64	0.36
	UD	93.9	6.1	1.88	0.12	0.94	0.06
	UU	87.9	12.1	1.76	0.24	0.88	0.12
Day 7	DU	79.6	20.4	1.59	0.41	0.80	0.20
	UD	88.9	11.1	1.78	0.22	0.89	0.11
	UU	84.9	15.1	1.70	0.30	0.85	0.15

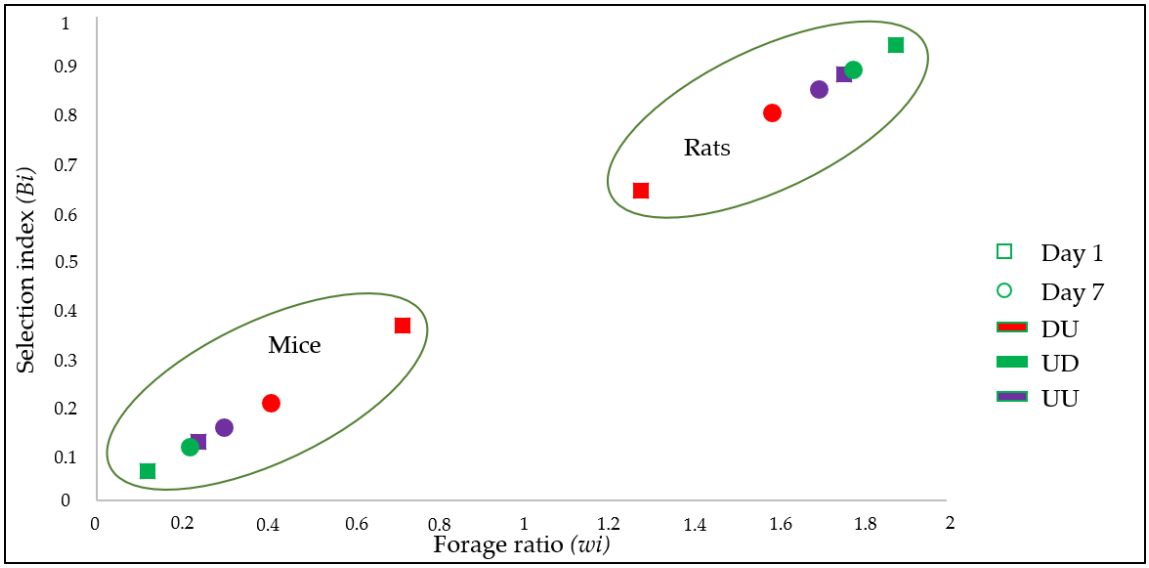


Figure 2. Scatter chart showing feeding preferences of *Ae. albopictus* mosquitoes in term of hosts and treatments according to the forage ratio (*wi*) and selection index (*Bi*). DU: DPP-treated rats vs. untreated mice, UD: DPP-treated mice vs. untreated rats, UU: Control group. Day 1 and 7: Time of post-treatment exposure.

4. Discussion

The present study focused on how the treatment by DPP influences the behavioral responses (blood-feeding and host choice) of *Ae. albopictus* mosquitoes toward both treated and untreated hosts available at the same time. The animal model was performed in order to reduce the potential confounders (e.g., rearing conditions of both animals and mosquitoes, treatment doses, experiment procedures, and the host defensive behaviors against mosquitoes) that can impair the results [38]. As expected, our data from the control group (UU) indicated that both the engorgement (mean = 48.5%) and the mortality rates (mean = 5.0%) of mosquitoes are in compliance with those recommended by the WHO, where the minimum (25%) and the maximum (20%) thresholds of, respectively, the engorgement and the mortality rates are necessary to validate the experiment [37,38].

As expected, DPP treatment induced a significant mortality of *Ae. albopictus* mosquitoes in both DU and UD compared to UU. The insecticidal effect was higher in DU (33–75.9%) than in UD (14.3–2%), thus highlighting the correlation between the insecticidal effect and the amount of DDP. The total amount of DPP used for the treatment of rats in DU was 5 times greater than that used to treat mice in UD. However, the insecticidal effect decreased rapidly (Table 1). Our data showed that the insecticidal effect had a rapid decrease from 75.9 to 33% and from 14.3 to 2% in DU and UD, respectively, at 7 days

post-treatment. These results contrast with the previous reports where the single administration of DPP maintained a high insecticidal effect against mosquitoes for 30 days post-treatment [16,39,40]. The availability of untreated hosts at the same time with treated ones could limit contact between the mosquitoes and the DPP-treated hosts. DPP formulation was designed to repel and kill mosquitoes and other arthropods once the contact between the arthropod and the treated hosts is established [16,39,40].

Surprisingly, our data demonstrated that, despite the aggressive biting behavior of *Ae. albopictus* [40], the rate of blood-meals taken upon mice was lower than that taken on rats in UU and that reported by Tahir et al., 2017, using the same *Ae. albopictus* strain on mouse model [16]. This could be the results of body weight differences between rats and mice. The body size is positively correlated to the mosquito attractiveness [41]. Another hypothesis could be a change in trophic preference of *Ae. albopictus* when the simultaneous availability of both hosts given the opportunity to choose between them according to the feeding preference of the mosquito. Under natural conditions, *Ae. albopictus* preferred human blood-meals rather than the other vertebrates (e.g., chicken, dog, and calf) if the choice is offered [9,42]. Further studies are needed to elucidate these transient trophic behaviors of *Ae. albopictus* that can be affected in experimental as in natural conditions by availability and abundance of hosts [9].

In the present study, we demonstrated that the use of DPP treatment did not affect the feeding preference of *Ae. albopictus*, where blood-meals were primarily taken upon rats in all groups, regardless of the treatment statutes (Table 5, Figure 2). A few data are currently available on the feeding behavioral changes that might be induced by the insecticidal and repellent products [43]. A previous study demonstrated that despite the strong excito-repellency of pyrethroids, there was no diversion of host-seeking mosquitoes from treated cattle to nearby humans [44,45].

Another surprising finding in this study was the additional protection offered to untreated hosts when present near DPP-treated ones. This statement corroborates with the previous studies on malaria, where the community with pyrethroid-treated cattle showed a strong reduction in malaria transmission [44,45]. This is the result of the strong repellency induced by these pyrethroids [46,47]. Once contact is established between mosquito and repellent product-treated area [48], intense disorder of the mosquito's sensory functions occurs, making it unable to detect the host and causing the mosquito to move in a disorganized manner away from a host to which it would otherwise be attracted [43].

In natural conditions, *Ae. albopictus* is one of the few is one of the few, if not the only mosquito species that showed the ability to feed upon rodent hosts such as Gray squirrel (*Sciurus carolinensis*) and White-footed mouse (*Peromyscus leucopus*) with a weaker engorgement rat compared to the other mammalian hosts [49]. However, the combined rat–mouse model used in the present study to assess host feeding patterns of *Ae albopictus* regarding to the type of hosts as well as the effect of the insecticide repellent (Vectra® 3D) are limited by the absence of robust data on the feeding behaviors of *Ae. albopictus* towards rodent hosts. The hypothesis that rodents constitute a suitable host for this mosquito species cannot be ruled in the absence of further data.

5. Conclusions

Ae. albopictus mosquitoes are very selective in terms of feeding preference when the opportunity of host choice is offered. DPP treatment does not alters the feeding behavior of *Ae. albopictus* and provides a partial protection to hosts in close physical proximity to treated animals. We encourage the use of this product on dogs to protect them and partially their owners against *Ae. albopictus* borne diseases such as dirofilariosis. However, further studies are needed to reinforce the usefulness of the combined models to simulate the real-life conditions that can occur between treated dogs and untreated owners.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/8/507/s1, Table S1: Relationship between signs and degree of pain, stress, and discomfort.

Author Contributions: Y.L., D.T. designed the study. Y.L., D.T., H.M. did the laboratory work. Y.L., D.T., B.D., M.V. carried out the data analysis. Y.L., D.T., M.V., O.M. and B.D. drafted the manuscript. All authors read and approved the final version of the manuscript.

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program « Investissements d'avenir », reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: The authors thank Lionel Alméras who participated in the success of the study.

Conflicts of Interest: Marie Varloud is currently an employee of Ceva Santé animale. All the other authors declare no conflicts of interest related to this article.

Availability of Data and Material: The data supporting the conclusions of this article are included within the article.

Ethics Approval and Consent to Participate: The experimental protocol and procedures were approved by the Ethics Committee for Animal Experimentation at Aix-Marseille University (approval n° 201602071706710). Laboratory animals were handled according to the French legislation for the protection of animals used for scientific purposes (decree no. 2013-118; 1 February 2013, Paris).

References

1. Benedict, M.Q.; Levine, R.S.; Hawley, W.A.; Lounibos, L.P. Spread of the tiger: Global risk of invasion by the mosquito *Aedes albopictus*. *Vector Borne Zoonotic Dis.* **2007**, *7*, 76–85. [CrossRef] [PubMed]
2. European Centre for Disease Prevention and Control. Aedes albopictus—current known distribution. Available online: https://www.ecdc.europa.eu/en/publications-data/aedes-albopictus-current-known-distribution-may-2020 (accessed on 15 May 2015).
3. Reiter, P.; Sprenger, D. The used tire trade: A mechanism for the worldwide dispersal of container breeding mosquitoes. *J. Am. Mosq. Control Assoc.* **1987**, *3*, 494. [PubMed]
4. World Health Organisation (WHO). *WHO Guidelines on Hand Hygiene in Health Care: First Global Patient Safety Challenge Clean Care Is Safer Care*; World Health Organisation (WHO): Geneva, Switzerland, 2009.
5. Abramides, G.C.; Roiz, D.; Guitart, R.; Quintana, S.; Guerrero, I.; Giménez, N. Effectiveness of a multiple intervention strategy for the control of the tiger mosquito (*Aedes albopictus*) in Spain. *Trans. R. Soc. Trop. Med. Hyg.* **2011**, *105*, 281–288. [CrossRef] [PubMed]
6. Worobey, J.; Fonseca, D.M.; Espinosa, C.; Healy, S.; Gaugler, R. Child outdoor physical activity is reduced by prevalence of the Asian tiger mosquito, *Aedes albopictus*. *J. Am. Mosq. Control Assoc.* **2013**, *29*, 78–80. [CrossRef]
7. Eritja, R.; Escosa, R.; Lucientes, J.; Marquès, E.; Molina, R.; Roiz, D.; Ruiz, S. Worldwide invasion of vector mosquitoes: Present European distribution and challenges for Spain. *Biol. Invasions* **2005**, *7*, 87–97. [CrossRef]
8. Bonizzoni, M.; Gasperi, G.; Anthony, A.J. The invasive mosquito species *Aedes albopictus*: Current knowledge and future perspectives. *Trends Parasitol.* **2014**, *29*, 460–468. [CrossRef]
9. Richards, S.L.; Ponnusamy, L.; Unnasch, T.R.; Hassan, H.K.; Apperson, C.S. Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in relation to availability of human and domestic animals in suburban landscapes of central North Carolina. *J. Med. Entomol.* **2006**, *43*, 543–551. [CrossRef]
10. Pereira-Dos-Santos, T.; Roiz, D.; Lourenço-De-Oliveira, R.; Paupy, C. A systematic review: Is *Aedes albopictus* an efficient bridge vector for zoonotic arboviruses? *Pathogens* **2020**, *9*, 1–24. [CrossRef]
11. Paupy, C.; Delatte, H.; Bagny, L.; Corbel, V.; Fontenille, D. *Aedes albopictus*, an arbovirus vector: From the darkness to the light. *Microbes Infect.* **2009**, *11*, 1177–1185. [CrossRef]
12. Simón, F.; Siles-lucas, M.; Morchón, R.; González-miguel, J.; Mellado, I.; Carretón, E.; Montoya-alonso, J.A. Human and animal dirofilariasis: The emergence of a zoonotic mosaic. *Clin. Microbiol. Rev.* **2012**, *25*, 507–544. [CrossRef]
13. Dantas-Torres, F.; Otranto, D. Best practices for preventing vector-borne diseases in dogs and humans. *Trends Parasitol.* **2016**, *32*, 43–55. [CrossRef] [PubMed]
14. Mccall, J.W.; Varloud, M.; Hodgkins, E.; Mansour, A.; Dicosty, U.; Mccall, S.; Carmichael, J.; Carson, B.; Carter, J. Shifting the paradigm in *Dirofilaria immitis* prevention: Blocking transmission from mosquitoes to dogs using repellents/insecticides and macrocyclic lactone prevention as part of a multimodal approach. *Parasites Vectors* **2017**, *10*, 525. [CrossRef] [PubMed]

15. McCall, J.W.; Hodgkins, E.; Varloud, M.; Mansour, A.; Dicosty, U. Blocking the transmission of heartworm (*Dirofilaria immitis*) to mosquitoes (*Aedes aegypti*) by weekly exposure for one month to microfilaremic dogs treated once topically with dinotefuran-permethrin-pyriproxyfen. *Parasites Vectors* **2017**, *10*, 511. [CrossRef] [PubMed]
16. Tahir, D.; Davoust, B.; Almeras, L.; Berenger, J.M.; Varloud, M.; Parola, P. Anti-feeding and insecticidal efficacy of a topical administration of dinotefuran–pyriproxyfen–permethrin spot-on (Vectra® 3D) on mice against *Stegomyia albopicta* (= *Aedes albopictus*). *Med. Vet. Entomol.* **2017**, *31*, 351–357. [CrossRef] [PubMed]
17. Cartereau, A.; Houchat, J.N.; Mannai, S.; Varloud, M.; Karembé, H.; Graton, J.; Le Questel, J.Y.; Thany, S.H. Permethrin enhances the agonist activity of dinotefuran on insect cholinergic synaptic transmission and isolated neurons. *Neurotoxicology* **2018**, *67*, 206–214. [CrossRef]
18. Gwaltney-Brant, S.M. *Atypical Topical Spot-On Products*, 3rd ed.; WB Saunders: Philadelphia, PA, USA, 2013; pp. 741–754. ISBN 9781455707171.
19. Agency, E.M. Guideline on data requirements for veterinary medicinal products for the prevention of transmission of vector- borne diseases in dogs and cats. *Eur. Med. Agency* **2018**, *44*, 1–11.
20. U.S. Department of Health and Human Services Food and Drug Administration. *E 10 Choice of Control Group and Related Issues in Clinical Trials*; U.S. Department of Health and Human Services Food And Drug Administration: Maryland, DC, USA, 2009; Volume 2012.
21. Legifrance Decree No. 2013–118 of 1 February 2013 Concerning the Protection of Animals Used for Scientific Purposes. 2013. Available online: https://www.legifrance.gouv.fr/eli/decret/2013/2/1/AGRG1231951D/jo/texte (accessed on 15 August 2015).
22. Freireich, E.J.; Gehan, E.A.; Rall, D.P.; Schmidt, L.H.; Skipper, H.E. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother. Rep.* **1966**, *50*, 219.
23. Reagan-Shaw, S.; Nihal, M.; Ahmad, N. Dose translation from animal to human studies revisited. *FASEB J.* **2008**, *22*, 659–661. [CrossRef]
24. Nair, A.; Jacob, S. A simple practice guide for dose conversion between animals and human. *J. Basic Clin. Pharm.* **2016**, *7*, 27. [CrossRef]
25. Morton, D.B.; Griffiths, P.H. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet. Rec.* **1985**, *116*, 431–436. [CrossRef]
26. Blanga-Kanfi, S.; Miranda, H.; Penn, O.; Pupko, T.; Debry, R.W.; Huchon, D. Rodent phylogeny revised: Analysis of six nuclear genes from all major rodent clades. *BMC Evol. Biol.* **2009**, *9*, 71. [CrossRef] [PubMed]
27. Céspedes, H.A.; Zavala, K.; Vandewege, M.W.; Opazo, J.C. Evolution of the α2-adrenoreceptors in vertebrates: ADRA2D is absent in mammals and crocodiles. *Gen. Comp. Endocrinol.* **2017**, *250*, 85–94. [CrossRef] [PubMed]
28. Douzery, E.J.P.; Delsuc, F.; Stanhope, M.J.; Huchon, D. Local molecular clocks in three nuclear genes: Divergence times for rodents and other mammals and incompatibility among fossil calibrations. *J. Mol. Evol.* **2003**, *57*, 201–213. [CrossRef] [PubMed]
29. Gibbs, R.A.; Weinstock, G.M.; Metzker, M.L.; Muzny, D.M.; Sodergren, E.J.; Scherer, S.; Scott, G.; Steffen, D.; Worley, K.C.; Burch, P.E.; et al. Genome sequence of the Brown Norway rat yields insights into mammalian evolution. *Nature* **2004**, *428*, 493–520. [PubMed]
30. Hall, T.; Biosciences, I.; Carlsbad, C. BioEdit: An important software for molecular biology. *GERF Bull. Biosci.* **2011**, *2*, 60–61.
31. Younes, L.; Bernard, D.; Marie, V.; Niang, E.H.A.; Fenollar, F.; Mediannikov, O. Development of a multiplexed qPCRs-based approach for the diagnosis of *Dirofilaria immitis*, *D. repens*, *Acanthocheilonema reconditum* and the others filariosis. *BioRxiv* **2019**, *24*, 5–27.
32. Abbott, W.S. A method of computing the effectiveness of an insecticide. 1925. *J. Am. Mosq. Control Assoc.* **1987**, *3*, 302–303.
33. Bashar, K.; Tuno, N.; Ahmed, T.; Howlader, A. Blood-feeding patterns of *Anopheles mosquitoes* in a malaria-endemic area of Bangladesh. *Parasites Vectors* **2012**, *5*, 39. [CrossRef]
34. Silver, J.B. *Mosquito Ecology-Field Sampling Methods*, 3rd ed.; Springer science & business media: Berlin, Germany, 2008; ISBN 978-1-4020-6666-5.
35. Hess, A.D.; Richard, O.; Hayes, C.H.T. The use of the forage ratio technique in mosquito host preference studies. *Mosq. News* **1968**, *23*, 386–388.

36. Manly, B.F.L.; McDonald, L.; Thomas, D.L.; McDonald, T.L.; Erickson, W.P. *Resource Selection by Animals. Statistical Design and Analysis for Field Studies*; Springer: London, UK, 1993; Volume 53, ISBN 9781402006777.
37. World Health Organization. *WHO Pesticide Evaluation Scheme Guidelines for Efficacy Testing of Spatial Repellents*; World Health Organization: Geneva, Switzerland, 2013.
38. World Health Organization. *Guidelines for Testing Mosquito Adulticides for Indoor Residual Spraying and Treatment of Mosquito Nets (No. Who/Cds/Ntd/Whopes/Gcdpp/2006.3)*; World Health Organization: Geneva, Switzerland, 2006.
39. Franc, M.; Genchi, C.; Bouhsira, E.; Warin, S.; Kaltsatos, V.; Baduel, L.; Genchi, M. Efficacy of dinotefuran, permethrin and pyriproxyfen combination spot-on against *Aedes aegypti* mosquitoes on dogs. *Vet. Parasitol.* **2012**, *189*, 333–337. [CrossRef]
40. Bouhsira, E.; Liénard, E.; Lyazrhi, F.; Jacquiet, P.; Varloud, M.; Deflandre, A.; Franc, M. Repellent and insecticidal efficacy of a combination of dinotefuran, pyriproxyfen and permethrin (Vectra® 3D) against *Culex pipiens* in dogs. *Parasite Epidemiol. Control* **2016**, *1*, 233–238. [CrossRef] [PubMed]
41. Takken, W.; Verhulst, N.O. Host preferences of blood-feeding mosquitoes. *Annu. Rev. Entomol.* **2013**, *58*, 433–453. [CrossRef] [PubMed]
42. Delatte, H.; Desvars, A.; Bouétard, A.; Bord, S.; Gimonneau, G.; Vourc'h, G.; Fontenille, D. Blood-feeding behavior of *Aedes albopictus*, a vector of chikungunya on la réunion. *Vector-Borne Zoonotic Dis.* **2010**, *10*, 249–258. [CrossRef] [PubMed]
43. Ogoma, S.B.; Moore, S.J.; Maia, M.F. A systematic review of mosquito coils and passive emanators: Defining recommendations for spatial repellency testing methodologies. *Parasites Vectors* **2012**, *5*, 287. [CrossRef]
44. Hewitt, S.; Rowland, M. Control of zoophilic malaria vectors by applying pyrethroid insecticides to cattle. *Trop. Med. Int. Heal.* **1999**, *4*, 481–486. [CrossRef]
45. Habtewold, T.; Prior, A.; Torr, S.J.; Gibson, G. Could insecticide-treated cattle reduce Afrotropical malaria transmission? Effects of deltamethrin-treated Zebu on *Anopheles arabiensis* behaviour and survival in Ethiopia. *Med. Vet. Entomol.* **2004**, *18*, 408–417. [CrossRef]
46. Kawada, H.; Ohashi, K.; Dida, G.O.; Sonye, G.; Njenga, S.M.; Mwandawiro, C.; Minakawa, N. Insecticidal and repellent activities of pyrethroids to the three major pyrethroid-resistant malaria vectors in western Kenya. *Parasites Vectors* **2014**, *7*, 1–9. [CrossRef]
47. Koama, B.; Namountougou, M.; Sanou, R.; Ndo, S.; Ouattara, A.; Dabiré, R.K.; Malone, D.; Diabaté, A. The sterilizing effect of pyriproxyfen on the malaria vector *Anopheles gambiae*: Physiological impact on ovaries development. *Malar. J.* **2015**, *14*, 101. [CrossRef]
48. Grieco, J.P.; Achee, N.L.; Chareonviriyaphap, T.; Suwonkerd, W.; Chauhan, K.; Sardelis, M.R.; Roberts, D.R. A new classification system for the actions of IRS chemicals traditionally used for malaria control. *PLoS ONE* **2007**, *2*, e716. [CrossRef]
49. Faraji, A.; Egizi, A.; Fonseca, D.M.; Unlu, I.; Crepeau, T.; Healy, S.P.; Gaugler, R. Comparative host feeding patterns of the asian tiger mosquito, *Aedes albopictus*, in urban and suburban northeastern USA and implications for disease transmission. *PLoS Negl. Trop. Dis.* **2014**, *8*, e3037. [CrossRef]

Publication N°8

Field Implementation of a Multi-Modal Prophylactic Strategy (Dinotefuran-Permethrin-Pyriproxyfen and Milbemycine Oxime-Praziquantel) Against Canine Vector-Borne Diseases in Corsica

Younes Laidoudi[1,2], Hacène Medkour[1,2], Djamel Tahir[1,2], Handi Dahmana[1,2], Jean-Lou Marié[3], Marie Varloud[4], Oleg Mediannikov[1,2] and Bernard Davoust[1,2,3*]

[1]Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France
[2]IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France
[3]Animal Epidemiology Working Group of the Military Health Service, Marseille, France
[4]Ceva Santé Animale, 10, Av de la Ballastière, 33500 Libourne, France

ARTICLE DE RECHERCHE

Publié en Preprint, Juillet 2020

(doi.org/10.20944/preprints202007.0156.v1)

Soumis à : Parasite

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 July 2020 doi:10.20944/preprints202007.0156.v1

Article

Field Implementation of a Multi-Modal Prophylactic Strategy (Dinotefuran-Permethrin-Pyriproxyfen and Milbemycine Oxime-Praziquantel) Against Canine Vector-Borne Diseases in Corsica

Younes Laidoudi[1,2], Hacène Medkour[1,2], Djamel Tahir[1,2], Handi Dahmana[1,2], Jean-Lou Marié[3], Marie Varloud[4], Oleg Mediannikov[1,2] and Bernard Davoust[1,2,3*]

[1]Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France; younes.laidoudi@yahoo.com (Y.L.); hacenevet1990@yahoo.fr (H. M.); handy92@hotmail.fr (H.D.); olegusss1@gmail.com (O. M.); bernard.davoust@gmail.com (B. D.)
[2]IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France; djamel.tahir@yahoo.fr (D.T.); handy92@hotmail.fr (H.D.)
[3]Animal Epidemiology Working Group of the Military Health Service, Marseille, France; jean-lou.marie@intradef.gouv.fr (J.L.M.)
[4]Ceva Santé Animale, 10, Av de la Ballastière, 33500 Libourne, France; marie.varloud@ceva.com (M.V.)
Correspondence: bernard.davoust@gmail.com; Tel.: +33-0413732401.

Running title: Prevention against canine vector-borne diseases in Corsica

Abstract: With a mild Mediterranean climate, Corsica is endemic with canine vector-borne diseases (CVBDs) such as dirofilariosis, leishmaniosis and ehrlichiosis. The aim of the present study was to evaluate a monthly multi-modal prophylactic (MMP) strategy against CVBDs occurring on the island. The study was conducted as a comparative field trial in which eighty dogs were allocated into two groups: (i) a test group consisted of 25 dogs under the MMP [*per-os* administration of 1.5 tablet of milbemycine oxime-praziquantel and a topical line-on application of a 3.6 mL solution of dinotefuran-permethrin-pyriproxyfen] and (ii) a control group under various real-life prophylactic treatments (RLP) based on the use of ectoparasiticide products [different formulations: deltamethrin, fluralaner, fipronil] and/or macrocyclic lactone based-products [milbemycin oxime/praziquantel, milbemycin oxime, moxidectin] during the period ranging from June to October 2017. All animals were followed for one year and had blood drawn at day 0, with follow-ups at 6 and 12 months. Samples were screened for filariosis using molecular tools, as well as for leishmaniosis and ehrlichiosis, using indirect immunofluorescence assay (IFA). At the end of the study, no new cases of CVBDs were recorded within the test group. In the control group, the cumulative incidence of CVBDs was 20.0% (n= 11; p= 0.015), including dirofilariosis due to *Dirofilaria immitis* and/or *D. repens*, with 16.4% (n= 9; p=0.027). Ehrlichiosis was 5.5% (n= 3; p=0.241). No new cases of leishmaniosis were detected in the control group. The data illustrated that, unlike the RLP treatment which failed to protect at least 20% of dogs, the MMP based on the concurrent administration of milbemycine oxime-praziquantel and dinotefuran-permethrin-pyriproxyfen is efficient in protecting dogs against CVBDs in a high-risk area.

Keywords: canine vector-borne diseases; mosquito-borne helminths; dirofilariosis; ehrlichiosis; leishmaniosis; dogs; multi-modal prophylaxis; Corsica

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 July 2020 doi:10.20944/preprints202007.0156.v1

1. Introduction

Canine vector-borne diseases (CVBDs) include a group of rapidly spreading and globally distributed infections that are caused by viruses, bacteria, protozoa and helminths [1]. Their transmission requires hematophagous arthropods such as mosquitoes, ticks, fleas, sandflies, tsetse flies, triatomine bugs, blackflies, mites or lice [1]. The geographical expansion and spread of CVBDs is directly related to the climate changes, vector propagation, human and animal travels across the world as well as the lack of field chemoprophylaxis and prevention in the exposed area [2]. The Mediterranean region is known for the emergence, spread and endemicity of several vector-borne diseases. Among CVBD-causing pathogens, *Dirofilaria immitis* and *Dirofilaria repens* are the most relevant mosquito-borne helminths (MBH) that threaten humans and pets in the Mediterranean basin. *D. immitis* induces the heartworm disease (Syn. cardiopulmonary dirofilariosis) in dogs and the pulmonary dirofilariosis in humans, while *D. repens* causes respectively subcutaneous and subcutaneous/ocular dirofilariosis in canine and human hosts [3]. *Ehrlichia canis*, an obligatory intracellular bacterium transmitted by ticks, is responsible for both canine and human monocytic ehrlichiosis [1]. *Leishmania infantum*, a sandfly-borne protozoan causes canine leishmaniosis and human visceral leishmaniosis, both are often responsible for considerable morbidity if left untreated [4].

Corsica is endemic for a wide range of CVBDs. The prevalence rates in dogs were recorded at 21.3% [5], for *D. immitis*. The presence of canine ehrlichiosis was confirmed on the island [6,7]. The cumulative incidence of canine leishmaniosis throughout the past 20 years (from 1993 to 2012) was reported at 42.5% [8]. Furthermore, several studies reported that Corsica is an epidemiological focus from which the main cases of human and canine vector-borne diseases are imported, such as human leishmaniosis by tourists [9], *D. immitis* in imported dogs from Corsica [10,11] and human dirofilariosis due to *D. repens* in visitors to the island [12]. Consequently, CVBDs in Corsica need to be further investigated and cost-effective control programs need to be put in place.

Recently, a multi-modal prophylactic (MMP) strategy targeting simultaneously the pathogens and their vectors provided promising results against heartworm. This MMP was implemented by combining the administration to dogs of a macrocyclic lactone with larvicidal activity (Interceptor®, Elanco, Neuilly-sur-Seine, France) and a repellent insecticide active against mosquitoes (dinotefuran, pyriproxyfen and permethrin (DPP), Vectra® 3D, Ceva Santé Animale, Libourne, France) [13,14]. The MMP were more effective (100%) than topical Vectra® 3D (96%) or Interceptor® (58%) in preventing the lactone macrocyclic-resistant strain of *D. immitis* under experimental conditions [13,14].

In addition, DPP was designed to treat and prevent a wide range of external parasitic infestations (ticks, mosquitoes, sandflies, stable flies, fleas and triatomes) [15–17]. However, no data were available on the effectiveness of the MMP strategy against CVBDs in the field. In this field study, we tested the MMP protocol based on the concurrent treatment with a broad-spectrum insecticides (DPP) and a larvicidal products (milbemycine oxime-praziquantel) against the major CVBDs occurring in Corsica, an area well known for its endemicity [1,6–8].

2. Methods

2.1. Study Area

The study was carried out in Ventiseri (41°55'52.5"N; 9°23'58.3"E), Solaro Marine (41°50.1114′N; 9°22.482′E) and Aleria (42°6'15.293"N; 9°30'44.744"E) on the island. Corsica is some 400 km from mainland France in the Mediterranean Sea between the European and the African continents. The island is inhabited by some 320,000 people and is visited by more than three million tourists a year [18]. The ecological conditions are characterized by a forest environment (more than 46% of the total area of the island) and a clement temperature (annual mean of 17°C) with a moderate precipitation (600 - 800 millimeters per annum), which offers a suitable environment for the development, spread and expansion of vector-borne diseases [18,19].

2.2. Animal Enrollment

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 July 2020 doi:10.20944/preprints202007.0156.v1

From March 2017 to April 2018, a comparative field trial was carried out. The study was conducted according to the guidelines on data requirements for veterinary medicinal products for the prevention of transmission of vector-borne diseases in dogs and cats [20]. Dogs were enrolled and analyzed according to the following criteria: (i) the consent of dog owners was confirmed at the enrollment at Day (D) 0. (ii) The dogs were negative for at least one of the selected CVBDs, (iii) dogs did not present clinical signs compatible with heartworm, canine leishmaniosis or ehrlichiosis threatening the dog's life during the follow-up, (iii) the dogs were at least one year old at the time of registration and were receiving regular prophylactic treatment based on ectoparasiticide and/or macrocyclic lactone based-products. However, the exclusion criteria were: animals not remaining permanently in their residence throughout the study period or refusal by owners to participate. The trial evaluation was based on the incidence of CVBDs at the middle and at the end of the trial. Once the laboratory diagnosis had been established after each visit, the results of the screening were communicated to the owners of the animals or their veterinarians in order to implement the appropriate treatment; the preventive treatment was maintained for these dogs, and they were not excluded from the study.

2.3. Canine Population, Treatment Protocol and Blood Collection

In March 2017 (D0), 114 dogs were enrolled and allocated into two groups:

i) The test group (test treatment group, n=36) consisted of military working dogs (MWD) housed individually in wire cages in a kennel located in Ventiseri (Figure 1a). Dogs received monthly multi-modal prophylactic treatment (MMP) [*per os* administration of 1.5 tablet of milbemycine oxime-praziquantel (Milbactor®, Ceva Santé Animale, Libourne, France) and a topical line-on application of a 3.6 mL solution of dinotefuran-permethrin-pyriproxyfen (Vectra® 3D, Ceva Santé Animale)].

ii) The control group (active control, n=78) consisted of civilian dogs of Solaro Marine and those from the kennel of Aleria. All dogs had an outdoor lifestyle in wire cages or free in gardens (Figure 1b). These dogs received various real-life prophylactic treatments (RLP).

Figure 1. Photo shoot showing house conditions of (A) military working dogs and (B) civilian dogs.

Three and ten dogs were lost for follow-up at D0 + 6 months, 8 and 13 other dogs were lost for follow-up at D0+ 12 months from the first and the second group, respectively (Figure 2). At the end of the study, a cohort of 80 dogs was reconstituted for a one-year follow-up, consisting of 25 MWD (test

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 July 2020 doi:10.20944/preprints202007.0156.v1

group), represented by 23 males and 2 females, aged 2 to 11 years old, with a median of 4 years, and 55 civilian dogs (control group), consisting of 31 males and 24 females aged from 1 to 17 years old, with a median age of 5 years. The 55 civilian dogs included 5 sheepdogs, 25 hunting dogs and 25 pets. Blood on citrate and plain tubes for serum harvesting were collected by a veterinarian from the dogs' cephalic veins. Each dog was sampled and tested 3 times at 6-month intervals (D0, D0+6 and D0+12 months) for the presence of heartworm (*D. immitis* and its endosymbiotic *Wolbachia*), *D. repens*, *Acanthocheilonema reconditum*, *L. infantum* and *E. canis* infections.

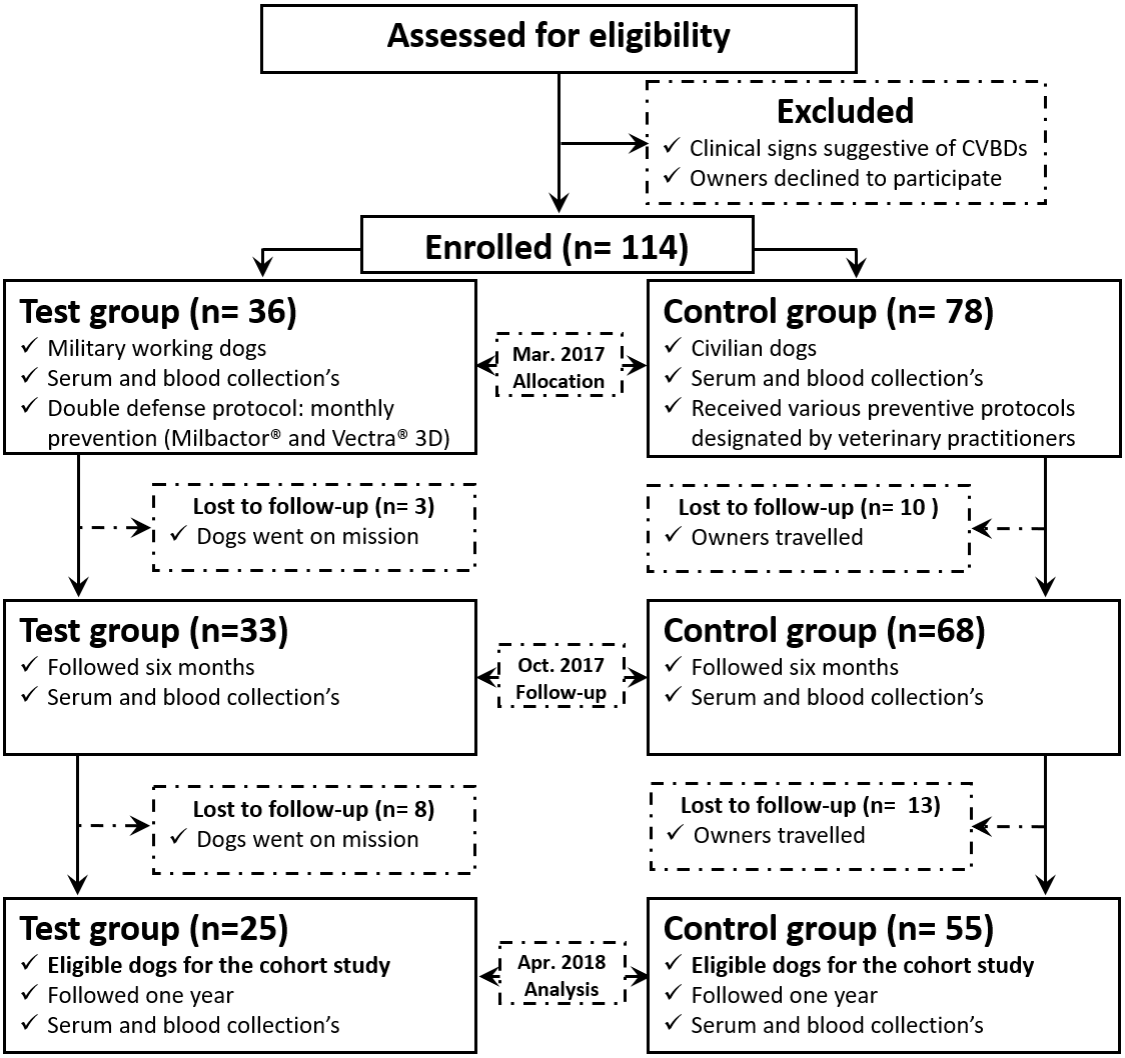


Figure 2. Flow diagram showing the semi-annual evolution of the cohort.

2.4. Laboratory Analysis

2.4.1. Molecular Detection of Filarial Infections

Genomic DNA was extracted from the citrate blood using the Biorobot EZ1 System with the EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Dogs were screened at each examination for the presence of *D. immitis*, *D. repens* and *A. reconditum* DNA using a triplex qPCR assay (Triplex TaqMan COI qPCR) [21]. This molecular assay is able to detect up to 5×10^{-1} microfilaria per milliliter of blood (corresponding to 2.5×10^{-3} mfs per qPCR reaction) of each species simultaneously. A second qPCR (*fts*Z-WD.immitis- qPCR) targeting the specific *Wolbachia* endosymbiont of *D. immitis* was used to complete the molecular diagnosis of heartworm infection [22]. The detection of the specific genotype of *Wolbachia* in filarial infections improved the diagnosis value

and resolved the occult infections [21][22], as the endosymbiont are released by the filarial worms irrespective of their gender [23,24]. The detection of *Wolbachia* DNA together with that of its filarial host (e.g. *D. immitis*) had 100 and 99.3% of sensitivity and specificity respectively [21].

qPCRs reactions were carried out in total volume of 20 µL, containing 5 µL of DNA template, 10 µL of Master Mix Roche (Eurogentec), 0.75 µL of each primer (20 µM concentration) per reaction for the triplex and 0.5 µL for simplex qPCR, 0.5 µL of both UDG and each probe (5 µM concentration). Finally, the volume was made up to 20 µL using ultra-purified water free of DNAse-RNAse, i.e. 1.5 µL for the triplex and 3 µL for the simplex qPCR. The qPCR run protocol included two hold steps at 50°C for 2 minutes, followed by a cycle at 95°C for 15 minutes, and 40 cycles of two steps each (95°C for 30 s and 60°C for 30 s). These reactions were performed in a thermal cycler CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France) after activating the readers of the dyes used in each qPCR system.

2.4.2. Serological Analysis

Sera were individually screened for canine leishmaniosis and monocytic ehrlichiosis antibodies using the quantitative indirect immunofluorescence test. The assay was performed using *L. infantum* and *E. canis* commercialized antigens (Zoetis, Lyon, France) according to the manufacturer's instructions. A two-fold dilutions (1:50 and 1:100) of sera (including positive and negative sera) were prepared in phosphate buffered saline (PBS), and a dilution of 1:100 was selected as the cut-off value. Slide wells were sensitized by 20 µL or 10 µL of *L. infantum* or *E. canis* commercial antigens (Zoetis) respectively. Later, 20 µL of each serum dilution was applied per well and slides were incubated for 30 min at 37 °C. Sensitized slides were washed twice with PBS for 5 minutes and once with distilled water. 20 µL of rabbit anti-dog IgG conjugated with fluorescein isothiacyanate (FITC) (Sigma-Aldrich, St Louis, MO, USA) were added into each well. Slides were immediately incubated at 37° C for 30 minutes. The washing procedure was repeated as described above, and cover slips were mounted in mounting medium. Slides were examined under a UV light microscope (Leica® DM LB2, Gx40).

2.5. Incidence Estimation and Data Analysis

Positive dogs are defined here as dogs positive for one or more selected CVBDs. Chi-Square (x^2) and exact Fisher (F) tests were applied to compare the proportion of infected dogs in test and control groups at Day 0 (baseline). The incidence was first determined using the incidence density rate (IDR) represented by the rate of positive dogs (i.e., new infections) observed at every 6th month of follow-up for each pathogen or group of pathogens. IDRs were calculated for each CVBD every 6 months over the follow-up (i.e., considering only results of Day +6 and Day +12 months in comparison to Day 0 and Day+6 months respectively). In addition, the cumulative incidence (CI) rates [25] for each pathogen or group of pathogens were estimated from the half-year-crude incidence (i.e., considering only results of the new infections after the sixth and twelfth month, regardless of what happened in between). Finally, Gray's test [26] was applied to compare cumulative incidence rates in test and control groups. Significance was defined at alpha ≤0.05. Statistical analysis was performed using Addinsoft. 2018. XLSTAT 2018: Data Analysis and Statistical Solution for Microsoft Excel. Paris, France (2018). The protection level of MMP was expressed in terms of the percentage of success in blocking the transmission of the pathogen or group of pathogens, calculated at the end of the follow-up using the following formula: Protection (%) = $100 \times [(\mathrm{CIt} - \mathrm{CIc})/\mathrm{CIc}]$, where the CIt and CIc represent the cumulative incidence rate of CVBDs in test and control group respectively [20].

3. Results

At Day 0 (baseline), the infectious status of test and control groups were homogeneous (p>0.05) in terms of dogs positive for pathogens or groups of pathogens [i.e., canine vector-borne diseases (CVBDs) and mosquito-borne helminths (MBHs)]. Prior to the trial, 20 and 21.8% of dogs were positive for at least one of the selected CVBD, in test and control group respectively (Table 1). Only *E. canis* was

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 July 2020 doi:10.20944/preprints202007.0156.v1

detected in five dogs (20%) from the test group, while heartworm, *L. infantum* and *E. canis* were respectively detected in 4 (7.2%), 5 (9.1%) and 4 (7.2%) dogs in the control group (Table 1). All dogs were negative for *D. repens* at the enrollment.

Table 1. Number and prevalence of infections by pathogens or groups of pathogens [i.e., canine vector-borne diseases (CVBDs) and mosquito-borne helminths (MBHs)] detected at baseline (March 2017).

Pathogens	Positive dogs (%)		Significance (*p*-value)
	Test group (N=25)	Control group (N=55)	
CVBDs	5 (20)	12 (21.8)	0.85
MBHs	0 (0)	4 (7.2)	0.304
Wolbachia of *D. immitis*	0 (0)	3 (5.4)	0.548
D. immitis	0 (0)	3 (5.4)	0.548
Heartworm (combined detection)	0 (0)	4 (7.2)	0.304
D. repens	0 (0)	0 (0)	//
L. infantum	0 (0)	5 (9.1)	0.317
E. canis	5 (20%)	4 (7.2)	0.129

At the end of the follow-up, 80 dogs (25 from the test group and 55 from the control group) were included in the cohort study. Unlike the test group, dogs from the control group were vulnerable to at least one CVBD irrespective of their treatment. However, dogs receiving the concurrent treatment with a repellent insecticide and macrocyclic lactone-based products remained the most protected (Table 2).

No new cases of CVBDs were recorded within the test group under the MMP which corresponds to a protection of 100%. However, 2 (3.6%) and 9 (16.4%) new cases of CVBDs were recorded in control group at 6 and 12 months of follow-up respectively. The incidence density rates and the cumulative incidences of CVBDs are detailed in Table 3. The highest IDR was observed for MBHs at Day +12 months in control group, wherein 14.6% (n=8) and 1.8% (n=1) of dogs were positive for heartworm and *D. repens* respectively. No new cases of *L. infantum* were reported in either group throughout the follow-up. The IDRs of ehrlichiosis were 3.6% (n=2) and 1.8% (n=1) at Day +6 months and Day +12 months respectively, in the control group. The CI for all CVBDs, grouped MBHs and *D. immitis* were significantly higher in the control than in the test group. Finally, no difference was observed between the groups for *D. repens*, *L. infantum* and *E. canis.*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 July 2020 doi:10.20944/preprints202007.0156.v1

Table 2. Treatment and cumulative number of newly infected dogs in terms of CVBDs-causing pathogens or groups of pathogens [i.e., canine vector-borne diseases (CVBDs) and mosquito-borne helminths (MBHs)] detected by serology or qPCR in dogs at Day 0, 6 and 12-months of follow-up.

Group	Treatment	Treatment period	Dogs (N)	*D. immitis* and/or *W.Dimm*	*D. repens*	*L. infantum*	*E. canis*	MBHs	CVBDs
Test group	a. Milbemycine oxime/praziquantel (Milbactor®) b. Dinotefuran/Permethrin/Pyriproxyfen (Vectra® 3D)	Once a month throughout the year*	25	0	0	0	0	0	0
Control group (RLP)	a. Fipronil (Flivox®) b. Milbemycin oxime/praziquantel (Milbemax®) c. Imidacloprid/permethrin (Advantix®)	a and b: Once a month from June to October* c: Two times per year (March and October)	38	1	1	1**	1	2	3
	Imidacloprid and permethrin (Advantix®)	Once every two months from June to September	2	1	0	0	0	1	1
	a. Fluralaner (Bravecto®) b. Milbemycin oxime/praziquantel (Milpro®)	Once every 6 months*	2	0	0	0	1	0	1
	Deltamethrin (Scalibor®)	Once every 6 months	3	2	0	0	0	2	2
	Fipronil (Frontline®)	Occasionally in summer	2	1	0	0	0	1	1
	Moxidectin (Guardian®)	Occasionally in summer	3	1	0	0	0	1	1
	Milbemycin oxime (Interceptor®)	Once per year (between June to September)	3	1	0	0	1	1	1*
	Milbemycin oxime/praziquantel (Milbemax®)	Once every 6 months	2	1	0	0	0	1	1
	Total	//	55	8	1	0	3	9	11

* Samples positive for at least 2 CVBDs, which were considered only once in the calculation of grouped CVBDs.
W.Dimm: *Wolbachia* endosymbiont of *D. immitis*.
RLP: real-life prophylactic treatments.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 July 2020 doi:10.20944/preprints202007.0156.v1

Table 3. Incidence density rates (IDR) and cumulative incidences (CI) of canine vector-borne diseases (CVBDs), mosquito-borne helminths (MBHs) or for each single pathogen. *P*-values compare the CI rates in both test and control groups.

	IDRs (%)		Cumulative incidences (%)		Pairwise comparison (Gray's test)	
Pathogens/time	**Test group**	**Control group**	**Test group**	**Control group**	**Statistic**	***p*-value**
CVBDs (positive for at least one CVBD-causing pathogens)						
Day +6 months	0	3.6	0	20	6.06	0.014
Day +12 months	0	16.4				
MBHs (dirofilariosis)						
Day +6 months	0	0	0	16.4	4.55	0.033
Day +12 months	0	16.4				
Heartworm (combined detection of *D. immitis* and its endosymbiotic *Wolbachia*)						
Day +6 months	0	0	0	14.6	4.0	0.046
Day +12 months	0	14.6				
D. repens						
Day +6 months	0	0	0	1.8	0.46	0.500
Day +12 months	0	1.8				
L. infantum						
Day +6 months	0	0	0	0	-	-
Day +12 months	0	0				
E. canis						
Day +6 months	0	3.6	0	5.5	1.37	0.241
Day +12 months	0	1.8				

 doi:10.20944/preprints202007.0156.v1

4. Discussion

To the best of our knowledge, this is the first reported field study demonstrating the efficacy of the consistent administration of larvicidal (macrocyclic lactones) + vector prevention products (repellents) as a MMP against CVBDs in endemic areas, and the first European field study confirming the year-round efficacy of the MMP on a monthly schedule against CVBDs. The MMP was first designed to protect against vector bites and the development of larval stages in both the resistant and the non-resistant strains of *D. immitis* and should be efficient in blocking the transmission of dirofilariosis despite the exposure of dogs to infected mosquitoes [13]. In the field, Labarthe et al. (2015), demonstrated that the monthly administration of topical moxidectin + imidacloprid to dogs was highly effective (100%) in preventing the transmission of *D. immitis* in a high risk area [27].

Because dogs should be shown to be exposed to transmission risk in a given area, we proceeded to demonstrate the effectiveness of preventive treatment in a zone in which the certainty that transmission exists. The percentage of positive tests for grouped pathogens [i.e. *D. immitis, D. repens. L. infantum* and *E. canis*] was above 20% in both groups thereby demonstrating how intense the challenge was and confirming the very high risk of CVBD previously reported in the site study [5–9]. Therefore, the severe life-threatening condition of these infections as well as their zoonotic potential highlights the real need for a cost-effective chemoprophylaxis easy to implement in order to control transmission.

For obvious ethical reasons, the demonstration of MMP efficacy was based on a comparison with real-life preventive strategies (treated with several existing approved products irrespective of the treatment period or the number of dogs in the subgroups) as an active control instead of an untreated control. The active control groups are often used to demonstrate the efficacy of new treatments on the field [28–30].

Our findings indicate that MMP treatment using Milbactor® and Vectra® 3D is efficient under field conditions to block the transmission of single and multiple infections with CVBD caused by *D. immitis, D. repens, L. infantum* and *E. canis*. A significantly higher level of protection against all these CVBDs was observed in our study, in contrast to the control group, in which dogs were vulnerable to CVBDs (n=11, CI=20%). The protection success in the test group compared to the control group is expected to be the result of different elements. Firstly, the compliance in military working dogs is expected to be higher than in civilian dogs. As far as civilian dogs are concerned, surveys conducted in France among dog owners indicate that the compliance rate of dogs is low, with an average of 6% [31]. Owners' poor or noncompliance due to incorrect or inconsistent administration of preventive treatments was the main reason for prevention failures [32]. In addition, the synergistic effect due to the simultaneous combination of a macrocyclic lactone and a parasiticide-repellent product provides better protection against CVBDs and in particular against MBHs, with respect to which both products are active. The combination milbemycin oxime/praziquantel, like other macrocyclic lactones, was designed to prevent dirofilarial infections in dogs [33]. DPP has a proven record of effectiveness in preventing a broad spectrum of vectors such as ticks, mosquitoes, sandflies, stable flies, fleas and triatomes [15–17], and this partly explains the successful prevention of the other CVBDs in the present study. Another key success factor is the year-round chemoprophylaxis which appears to be more adapted to the active vectors in the area compared to the 5-month seasonal protection in the control group, despite the use of both a macrocyclic lactone and a repellent insecticide in some of the dogs in that group (Table 2). In southern Europe as well as in Corsica, a year-round active vector such as *Stegomyia albopicta* (a main vector of *Dirofilaria* spp.) has recently been identified [5,34]. The year-round chemoprophylaxis against this vector is recommended in Southern Europe [35]. Indeed, the protection level depends on several factors related to epidemiological conditions, host, vectors and the pathogen itself [36]. The European Scientific Counsel Companion Animal Parasites (ESCCAP), the European Society for Dirofilariosis and Angiostrongylosis (ESDA), and the American Heartworm Society (AHS) recommend the preventive strategies combining the administration of macrocyclic lactones with vector prevention products (repellents) [37–38]. This study illustrates the usefulness of

this strategy to protect dogs from dirofilariosis and from other vector-borne diseases occurring at the same time and throughout the year; chemoprophylaxis should be implemented in high-risk areas such as the Mediterranean region [12].

The treatment of the vector-borne diseases such as heartworm can be life-threatening, expensive and complex [3,39]. Canine leishmaniosis is potentially fatal if left untreated, besides being a major zoonosis. Current treatments for leishmaniosis are very expensive and difficult to implement with limitations such as adverse reactions and relapses [35]. The concept of multimodal prevention can be complemented by other products, such as vaccines against leishmaniosis, which are available in Europe [35] and can improve protection for dogs when combined with vector-borne protection at a lower cost compared to the complicated treatment of visceral leishmaniosis.

5. Conclusion

Besides the direct impact upon mosquito-borne helminths, the purposed multimodal prophylactic strategy based on year-round administration of Vectra® 3D and Milbactor® on a monthly schedule, provides reliable control against other major canine vector-borne diseases (i.e. *L. infantum* and *E. canis*) in high-endemic areas. Multi-modal prophylactic treatment should be promoted on a broader scale, especially in and around the endemic areas, such as southern Europe, in order to stop canine vector-borne diseases spreading to dogs and humans.

Abbreviations

AHS: American Heartworm Society;
CVBDs: canine vector-borne diseases;
ESCCAP: European Scientific Counsel Companion Animal Parasites;
ESDA: European Society for Dirofilariosis and Angiostrongylosis;
IP: intermittent prophylactic treatment;
MMP: multi-modal prophylactic treatment;
MBHs: mosquito-borne helminths.

Acknowledgments: We thank Mahdi Toumert, Massinissa Hachemi, Sandrine Ferrandi, Marie-Hélène Filippi, Mickaël Boni, Laetitia Guidicelli and all the dog owners for their help.

Conflicts of Interest statement: All the authors declare no conflicts of interest related to this article. One author (Marie Varloud) is currently employee of Ceva Santé Animale. However, there were no conflicts of interest that could have biased the work presented in this paper.

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program « Investissements d'avenir », reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI. This work was also funded by Ceva Santé Animale (supply of pharmaceutical products).

Availability of data and material: The data supporting the conclusions of this article are included within the article.

Authors' contributions: YL, BD, MV, J-LM and OM designed the study. YL, BD, DT, HD and J-LM intervened in the field study. YL and HM performed the laboratory analyzes. YL, BD, OM designed, and YL carried out the data analysis. YL, BD, MV and OM drafted the manuscript. All authors read and approved the final version of the manuscript.

Consent for publication: Not applicable.

Ethics approval and consent to participate: All owners of dogs gave their informed consent to participate in the study.

References

1. Otranto, D.; Dantas-Torres, F.; Breitschwerdt, E.B. Managing canine vector-borne diseases of zoonotic concern: part one. *Trends Parasitol.* **2009**, *25(4)*, 157-163.
2. Simón, F.; González-Miguel, J.; Diosdado, A.; Gómez, P.J.; Morchón, R.; Kartashev, V. The complexity of zoonotic filariasis episystem and its consequences: A multidisciplinary view. *Biomed Res. Int.* **2017**, 6436130.
3. Simón, F.; Siles-Lucas, M.; Morchón, R.; González-Miguel, J.; Mellado, I.; Carretón, E.; Montoya-Alonso, J.A. Human and animal dirofilariasis: The emergence of a zoonotic mosaic. *Clin. Microbiol. Rev.* **2012**, *25(3)*, 507-544.
4. Velez, R.; Ballart, C.; Domenech, E.; Abras, A.; Fernández-Arévalo, A.; Gómez, S.A.; Tebar, S.; Muñoz, C.; Cairó, J.; Gállego, M. Seroprevalence of canine *Leishmania infantum* infection in the Mediterranean region and identification of risk factors: The example of North-Eastern and Pyrenean areas of Spain. *Prev. Vet. Med.* **2019**, *162*, 67-75.
5. Tahir, D.; Bittar, F.; Barré-Cardi, H.; Sow, D.; Dahmani, M.; Mediannikov, O.; Raoult, D.; Davoust, B.; Parola, P. Molecular survey of *Dirofilaria immitis* and *Dirofilaria repens* by new real-time TaqMan®PCR assay in dogs and mosquitoes (Diptera: Culicidae) in Corsica (France). *Vet. Parasitol.* **2017**, *235*, 1–7.
6. Parzy, D.; Baudu, P.; Normand, T.; Audonnet, J.; Davoust, B.; Re, D. *Ehrlichia canis* phylogenetic analysis of the Borgo (Corsica) strain. *Clin. Microbiol. Infect.* **2009**, *15*, 76–78.
7. Dahmani, M.; Davoust, B.; Tahir, D.; Raoult, D.; Fenollar, F.; Mediannikov, O. Molecular investigation and phylogeny of Anaplasmataceae species infecting domestic animals and ticks in Corsica, France. *Parasit. Vectors* **2017**, *10(1)*, 302.
8. Davoust, B.; Roqueplo, C.; Parzy, D.; Watier-grillot, S.; Marié, J. A twenty-year follow-up of canine leishmaniosis in three military kennels in southeastern France. *Parasit. Vectors* **2013**, *6(1)*, 323.
9. di Muccio, T.; Scalone, A.; Bruno, A.; Marangi, M.; Grande, R.; Armignacco, O.; Gradoni, L.; Gramiccia, M. Epidemiology of imported leishmaniasis in Italy: Implications for a European endemic country. *PLoS One* **2015**, *10(6)*, e0129418.
10. Zahler, M.; Glaser, B.; Gothe, R. [Imported parasites in dogs: *Dirofilaria repens* and *Dipetalonema reconditum*]. *Tierarztl. Prax.* **1997**, *25(4)*, 388-392.
11. Pantchev, N.; Etzold, M.; Daugschies, A.; Dyachenko, V. Diagnosis of imported canine filarial infections in Germany 2008 - 2010. *Parasitol. Res.* **2011**, *109 Suppl 1*, S61-76.
12. Berger, S.M. *Infectious Diseases of France*; Gideon Informatics, I., Ed.; 2019th ed.; Gideon Informatics, Inc.: Los angeles, California, USA, 2019; ISBN 978-1-4988-2193-3.
13. McCall, J.W.; Varloud, M.; Hodgkins, E.; Mansour, A.; Dicosty, U.; McCall, S.; Carmichael, J.; Carson, B.; Carter, J. Shifting the paradigm in *Dirofilaria immitis* prevention: Blocking transmission from mosquitoes to dogs using repellents/insecticides and macrocyclic lactone prevention as part of a multimodal approach. *Parasit. Vectors* **2017**, *10(Suppl 2)*, 525.
14. McCall, J.W.; Hodgkins, E.; Varloud, M.; Mansour, A.; Dicosty, U. Blocking the transmission of heartworm (Dirofilaria immitis) to mosquitoes (Aedes aegypti) by weekly exposure for one month to microfilaremic dogs treated once topically with dinotefuran-permethrin-pyriproxyfen. *Parasites and Vectors* **2017**, *10*.

15. European Medicines Agency. Vectra® 3D Product information EPAR **2015**. http://www.ema.europa.eu/docs/en_GB/document_libra.
16. Tahir, D.; Davoust, B.; Almeras, L.; Berenger, J.M.; Varloud, M.; Parola, P. Anti-feeding and insecticidal efficacy of a topical administration of dinotefuran–pyriproxyfen–permethrin spot-on (Vectra® 3D) on mice against *Stegomyia albopicta* (= *Aedes albopictus*). *Med. Vet. Entomol.* **2017**, *31*, 351–357.
17. Tahir, D.; Davoust, B.; Varloud, M.; Berenger, J.M.; Raoult, D.; Almeras, L.; Parola, P. Assessment of the anti-feeding and insecticidal effects of the combination of dinotefuran, permethrin and pyriproxyfen (Vectra® 3D) against *Triatoma infestans* on rats. *Med. Vet. Entomol.* **2017**, *31*, 132–139.
18. Grech-Angelini, S.; Stachurski, F.; Lancelot, R.; Boissier, J.; Allienne, J.F.; Marco, S.; Maestrini, O.; Uilenberg, G. Ticks (Acari: Ixodidae) infesting cattle and some other domestic and wild hosts on the French Mediterranean island of Corsica. *Parasit. Vectors* **2016**, *9(1)*, 582.
19. Toty, C.; Barré, H.; Le Goff, G.; Larget-Thiéry, I.; Rahola, N.; Couret, D.; Fontenille, D. Malaria risk in Corsica, former hot spot of malaria in France. *Malar. J.* **2010**, *9*, 231.
20. European Medicines Agency. Guideline on data requirements for veterinary medicinal products for the prevention of transmission of vector- borne diseases in dogs and cats. **2018**, *44*, 1–11. https://www.ema.europa.eu/en/documents/scientific-guideline/draft-guideline-data-requirements-veterinary-medicinal-products-prevention-transmission-vector-borne_en.pdf.
21. Laidoudi, Y.; Davoust, B.; Varloud, M.; Niang, E.H.A.; Fenollar, F.; Mediannikov, O. Development of a multiplex qPCR-based approach for the diagnosis of Dirofilaria immitis, D. repens and Acanthocheilonema reconditum. *Parasit. Vectors* **2020**, *13*.
22. Medkour, H.; Laidoudi, Y.; Marié, J.L.: Fenollar, F.; Davoust, B.; Mediannikov, O. Molecular investigation of vector-borne pathogens in red foxes (*Vulpes vulpes*) from southern France. *J. Wildl. Dis.* **2020,** *56(4)*.
23. Taylor, MJ.; Hoerauf, A. *Wolbachia* bacteria of filarial nematodes. *Parasitol Today*. **1999**, 15, 437–442.
24. Taylor MJ.; Bandi C, Hoerauf A. *Wolbachia* bacterial endosymbionts of filarial nematodes. *Adv Parasitol*. **2005**, *60,* 245–284.
25. Pepe, M.S.; Mori, M. Kaplan—meier, marginal or conditional probability curves in summarizing competing risks failure time data? *Stat. Med.* **1993**, *12(8)*, 737-751.
26. Dignam, J.J.; Kocherginsky, M.N. Choice and interpretation of statistical tests used when competing risks are present. *J. Clin. Oncol.* **2008**, *26(24)*, 4027-4034.
27. Labarthe, N.V.; Willi, L.M.V.; Paiva, J.P.; De Miranda, M.G.N.; Zoreck, K.; Mendes De Almeida, F. Chemoprophylaxis of Dirofilaria immitis (Leidy 1856) infection at a high challenge environment. *Parasites and Vectors* **2015**, *8*, 6–9.
28. U.S. Department of Health and Human Services, F.; Administration, D. E 10 Choice of Control Group and Related Issues in Clinical Trials. **2009**, *2012*.
29. Moraes-Da-Silva, M.D.F.C.V.; Mendes-De-Almeida, F.; Abdalla, L.; Merlo, A.; Paiva, J.P.; Labarthe, N.V. Selamectin for the prevention of canine Dirofilaria immitis infection: Field efficacy in client-owned dogs in a high risk area. *Parasites and Vectors* **2016**, *9*, 1–5.
30. Six, R. H., Clemence, R. G., Thomas, C. A., et al. Efficacy and safety of selamectin against

Sarcoptes scabiei on dogs and *Otodectes cynotis* on dogs and cats presented as veterinary patients. *Vet. Parasitol.* **2000**, *91(3-4)*, 291-309.

31. Roussel, C.; Drake, J.; Ariza, J.M. French national survey of dog and cat owners on the deworming behaviour and lifestyle of pets associated with the risk of endoparasites. *Parasit. Vectors* **2019**, *12(1)*, 480.
32. McTier, T.L.; Kryda, K.; Wachowski, M.; Mahabir, S.; Ramsey, D.; Rugg, D.; Mazaleski, M.; Therrien, C.; Adams, E.; Wolff, T.; et al. ProHeart® 12, a moxidectin extended-release injectable formulation for prevention of heartworm (*Dirofilaria immitis*) disease in dogs in the USA for 12 months. *Parasit. Vectors* **2019**, *12*, 369.
33. di Cesare, A. ; Braun, G. ; di Giulio, E. ; et al. Field clinical study evaluating the efficacy and safety of an oral formulation containing milbemycin oxime/praziquantel (Milbemax®, Novartis Animal Health) in the chemoprevention of the zoonotic canine infection by *Dirofilaria repens*. *Parasit Vectors* **2014**, *7*, 347.
34. Otranto, D.; Dantas-Torres, F.; Brianti, E.; Traversa, D.; Petrić, D.; Genchi, C.; Capelli, G. Vector-borne helminths of dogs and humans in Europe. *Parasit. Vectors* **2013**, *6*, 16.
35. Dantas-Torres, F.; Otranto, D. Best practices for preventing vector-borne diseases in dogs and humans. *Trends Parasitol.* **2016**, *32*, 43–55.
36. Schorderet-Weber, S.; Noack, S.; Selzer, P.M.; Kaminsky, R. Blocking transmission of vector-borne diseases. *Int. J. Parasitol. Drugs Drug Resist.* **2017**, *7(1)*, 90-109.
37. Genchi, C.; Kramer, L. ESCCAP: European Scientific Counsel Companion Animal Parasites. **2015**, *21*, 25-54. https://gallery.mailchimp.com/2dfcfcceccf8b9e02ac641a61/files/Parassiti21.pdf.
38. Nelson, C.T.; McCall, J.W.; Jones, S.; Moorhead, A. Current Canine Guidelines for the Prevention, Diagnosis, and Management of Heartworm (Dirofilaria immitis) Infection in Dogs. *Am. Hear. Soc.* **2018**, 1–35.
39. Bowman, D.D.; Atkins, C.E. Heartworm Biology, Treatment, and Control. *Vet. Clin. North Am. - Small Anim. Pract.* **2009**, *39*, 1127–1158.

Publication N°9

Potential of Artesunate in the treatment of visceral leishmaniasis in dogs naturally infected by *Leishmania infantum*: Efficacy evidence from a randomized field trial

Hacène Medkour[1,2,3], Idir Bitam[4,5], **Younes Laidoudi**[1,2,3], Ismail Lafri[4,6,7], Abdelaziz Lounas[6,7], Hamidat Hamza Karim[8], Mekroud Abdeslam[3], Marie Varloud[9], Bernard Davoust[1,2], and Oleg Mediannikov[1,2*]

[1]IHU-Méditerranée Infection, Marseille, France
[2]Aix Marseille Univ., IRD, AP-HM, MEPHI, Marseille, France
[3]PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, 25100, El Khroub, Algeria
[4]IHU Méditerranée Infection - Vectors-Tropical and Mediterranean Infections (VITROME), UMR Aix-Marseille Université, IRD, SSA, APHM -19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France
[5]Superior School of Food Sciences and Food Industries of Algiers, Algeria
[6]Institute of Veterinary Sciences, University of Blida 1, Algeria
[7]Laboratory of Biotechnology related to Animal Reproduction (LBRA), University of Blida, Blida, Algeria
[8]Department of Biology, Faculty of Sciences, University of Boumerdes, Algeria
[9]Ceva Santé Animale, 33500 Libourne, France

ARTICLE DE RECHERCHE

Soumis à : PLOS Neglected Tropical Diseases

PLOS Neglected Tropical Diseases

Research Article

Potential of Artesunate in the treatment of visceral leishmaniasis in dogs naturally infected by *Leishmania infantum:* Efficacy evidence from a randomized field trial.

Hacène Medkour[1,2,3], Idir Bitam[4,5], Younes Laidoudi[1,2,3], Ismail Lafri[4,6,7], Abdelaziz Lounas[6,7], Hamidat Hamza Karim[8], Mekroud Abdeslam[3], Marie Varloud[9], Bernard Davoust[1,2], and Oleg Mediannikov[1,2*]

[1]IHU-Méditerranée Infection, Marseille, France

[2]Aix Marseille Univ., IRD, AP-HM, MEPHI, Marseille, France

[3]PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, 25100, El Khroub, Algeria

[4]IHU Méditerranée Infection - Vectors-Tropical and Mediterranean Infections (VITROME), UMR Aix-Marseille Université, IRD, SSA, APHM -19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France

[5]Superior School of Food Sciences and Food Industries of Algiers, Algeria

[6]Institute of Veterinary Sciences, University of Blida 1, Algeria

[7]Laboratory of Biotechnology related to Animal Reproduction (LBRA), University of Blida, Blida, Algeria

[8]Department of Biology, Faculty of Sciences, University of Boumerdes, Algeria

[9]Ceva Santé Animale, 33500 Libourne, France

[*] **Correspondence**: olegusss1@gmail.com

Address: IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France

Abstract

Leishmaniosis is among the world's most neglected diseases. Dogs are the main reservoir/host of *Leishmania infantum*, causative agent of both canine and human visceral leishmaniosis. Canine leishmaniosis (CanL) represents a public health problem as one of the most prevalent zoonotic diseases worldwide. Current therapeutics present drawbacks; thus, there is a need for more effective, safer, and cheaper drugs. The aim of this study was to evaluate and to compare the efficacy of oral administration of artesunate or meglumine antimoniate/allopurinol in dogs with clinical leishmaniosis.

Forty-two dogs with naturally occurring clinical leishmaniosis were included in this open-label randomized positive-control clinical field trial with 6 months of follow-up. Dogs received meglumine antimoniate 100 mg/kg/day and allopurinol 30 mg/kg/day for 28 days (control group, n=26) or artesunate 25 mg/kg/day for 6 days (test group, n=16). The animals were evaluated for their clinical evolution, parasite load (by qPCR) and humoral response at different time points: 0, 30, 90, and 180 days after treatment.

Data analyses showed a significant improvement in both groups in clinical scores, parasitemia and antibody titers after treatment. Compared to the control group, the artesunate group showed significantly lower clinical score ($P = 0.0001$), lower parasitemia ($P = 0.0001$) and antibody titers after 6 months of follow-up. Compared to baseline values, a rapid, significant reduction ($P < 0.012$) in antibody levels, 2.28- *versus* 3.04-fold for the control *versus* artesunate groups, respectively, was observed 30 days after treatment. Antibody levels continued to decrease further in the artesunate group, where 58% of cases became seronegative at the 6-month follow-up. All PCR-positive dogs were negative after treatment with artesunate, while 14.3% remained positive with the appearance of two new cases in the control group. Artesunate was well tolerated, and no side effects were recorded. Treatment failures were similar in both groups with 27.27% (6/22), including 18.18% (4/22) mortality in the control group, *versus* 26.66% (4/15), including 13.33% (2/15) mortality in the artesunate group.

This is the first report showing the potential of artesunate in the treatment of dogs with clinical leishmaniosis. Artesunate showed higher efficacy than the current first-line treatment for CanL without any adverse effects, could be a good alternative chemotherapy for CanL, and may be considered for further studies in human leishmaniases. Further clinical trials are needed to confirm these findings, to determine if dogs remain infective to sandflies, and to define the ideal therapeutic dosage and duration of treatment with artesunate.

Key word: CanL; canine; disease control; artesunate; meglumine antimoniate/allopurinol; clinical trial.

Authors' summary:

Canine leishmaniosis (CanL) is a fatal, zoonotic vector-borne disease caused by *Leishmania infantum*, a common pathogen for both humans and dogs. Most CanL therapeutics are toxic, expensive, or ineffective. Artemisinin and derivatives have recently demonstrated potent antileishmanial activity in vitro and in experimental models. In this study, dogs with clinical leishmaniosis were randomly included in one of the treatment groups: meglumine antimoniate/allopurinol (control) or artesunate (alternative). Dogs were followed up for 6 months for their clinical score, parasitemia and *Leishmania* antibody levels. Both groups showed improved clinical scores, parasitemia and antibody titers after treatment. After six months of follow-up, treatment success was very similar in both groups, and 72.73% (16/22) of the controls *versus* 73.34% (11/15) in the artesunate group had clinical improvement. All dogs initially seropositive by PCR became negative after artesunate treatment, while 14.3% remained positive with the appearance of new cases in the control group. Antibody titers decreased rapidly (from day 30) from baseline especially in the artesunate group, where 58% of the dogs converted to seronegative after 6 months. Artesunate could be a good alternative for treatment of leishmaniosis. Additional clinical trials are needed to obtain more data on this drug.

Introduction

Visceral leishmaniosis (VL) is an infectious, noncontagious, severe, chronic systemic disease. It is a major zoonosis with significant clinical and epidemiological control priority in the world. The domestic dog is the main animal reservoir, while other wild animals, such as foxes, play a role in sylvatic transmission. *Leishmania infantum* (syn. *L. chagasi*) is the causative agent of both human VL and canine leishmaniosis (CanL) [1]. In the last few decades, epidemiological changes in VL, including increases in incidence and mortality rate and its spread to new and even urban areas, have been observed [2][3][4][5][6][7]. The number of leishmaniosis cases has been increasing worldwide. Among the reasons are the lack of vaccines, failures in controlling vectors and the increasing selection of drug-resistant parasites [8].

CanL is a severe disease that affects several million domestic dogs in countries on both sides of the Atlantic Ocean (mainly Europe and South America, but spreading in Africa and Asia as well) and may kill infected dogs when left untreated [9][10]. In Mediterranean countries, where infection rates are up to 63% including at least 2.5 million seropositive dogs [11][12][13], CanL represents one of the leading causes of death in dogs [14,15]. It is a growing disease that has become one of the most important canine diseases imported to Central Europe [16].

The range of clinical presentations and immune responses developed by *L. infantum*-infected dogs is very wide and variable. Consequently, several CanL forms can be typified by their numerous clinical signs, including cutaneous, mucocutaneous and visceral forms [17]. In addition, *L. infantum* infection in dogs can manifest as subclinical infection, a self-limiting illness or a serious life-threatening disease [18].

Treatment of CanL is a challenge because of the intracellular localization of the parasite [19]. Moreover, the World Health Organization (WHO) has suggested reserving antileishmanial drugs used for human VL for exclusive use in human leishmaniosis and not for veterinary medicine because of suspected drug-resistance development from use in animals [20]. Despite this, the first-line treatment for CanL is currently the combined use of leishmanicidal agents used as second-line drugs for humans (e.g., pentavalent antimonials, miltefosine) and allopurinol [21][22][23][17]. Some of the chemotherapeutic compounds used for CanL are included within the 19th edition of WHO Model List of Essential Medicines (April 2015) against leishmaniosis: pentavalent antimonials, miltefosine, amphotericin B deoxycolate or liposomal formulations, and paromomycin. However, some other drugs are also effective, such as allopurinol, pentamidine, enrofloxacin, marbofloxacine, metronidazole, spiramycin, and ketoconazole [24]. Determination of the best drug is based on clinical examination and staging of CanL, according to immunodiagnostic test results, clinical signs, and clinicopathological abnormalities and parasite load [21][17]. Most of these treatments are expensive and do not achieve complete cure of the disease, and some of them can cause important side effects[25][23]. Vomiting, lethargy, diarrhea and nephrotoxicity are common during the current treatments with pentavalent antimonials and miltefosine [19][25][26][27][28][23]. Prolonged administration of allopurinol frequently induces xanthine

urolithiasis [29]. Thus, there is an urgent need for new, efficient, safe, and affordable drugs for the treatment of canine leishmaniosis.

Artemisinin and derivatives (ARTs) have demonstrated efficacy against protozoan parasites, such as *Plasmodium* [30] and *Perkinsus* species [31]. The antiparasitic activity of ARTs against other human and animal protozoans, namely, *Leishmania* spp., has scarcely been explored. Chollet et al. [32] reported on the activity of fluoroartemisinins against promastigote forms of *L. donovani* (at micromolar concentrations). Other compounds, such as artesunate, deoxygenated artesunate, dihydroartemisinin, and deoxygenated dihydroartemisinin, have shown in vitro activity against *L. infantum* life stage forms (promastigote and amastigote) [33]. In addition, ARTs showed efficacy for the treatment of murine visceral leishmaniosis [34][35][36][37].

In this study, we explored for the first time the efficacy of artesunate (an artemisinin derivative) in the treatment of visceral leishmaniosis in *Leishmania infantum* naturally infected dogs. Our study is an open-label randomized comparative field trial in two groups of sick dogs: i) control: dogs treated with the current chemotherapy (antimoniate of meglumine/ allopurinol); and ii) test, alternative chemotherapy: dogs treated with artesunate. Dogs were followed up for their clinical, parasitological and serological status.

Materials and Methods

Ethics statement

The study protocol was also approved by the scientific college (Procès-Verbal du CSI N°6, 2018) at the Veterinary Science Institute, University Constantine 1, Algeria. To facilitate field work, collaborations were established with veterinary doctors and their assistants working in these establishments. All dog owners gave their verbal informed consent, and samples were collected by veterinarians. Risk assessment was also submitted to and approved by the decision board of the veterinary practitioners from the wilayas of northern Algeria affiliated with the Algerian Ministry of Agriculture and Rural Development (Directions des Services Vétérinaires). No infected untreated dogs, as control, were involved in this study for ethical reasons.

Study design

This was an open-label randomized efficacy evidence clinical field trial conducted in the North of Algeria. The prevalence observed in this area was 36% [38]. Client-owned dogs of any age, breed, or gender were enrolled, and 11 veterinary practitioners were responsible for the monitoring. The dogs were fully monitored, and treatment could be switched to antimoniate meglumine/allopurinol at the owner's request if any clinical impairment was noticed. A total of 187 dogs were screened for compliance with the inclusion criteria. Among them, 42 sick adult dogs (30 males and 12 females), average age 3.8 years (min=1, max=11 years), weighing between 3-37 kg and of different breeds (8 German shepherd, 1 Belgian Malinois shepherd, 2 Sloughi, 5 pointer, 6 Braques, 1 Bagnole, 1 American Staffordshire terrier, 1 beagle and 17 mixed breeds) were selected for this study.

Inclusion, exclusion and efficacy criteria

The main inclusion criteria were as follows: i) dogs with at least two clinical manifestations related to CanL (Table 1) and ii) dogs with positive serology for *Leishmania* by immunochromatographic-based qualitative rapid WITNESS® *Leishmania* test (Zoetis, France) and/or quantitative indirect immunofluorescence antibody test (IFAT≥100) or positive PCR result obtained from a blood sample.

The dogs with significant alterations in renal and/or hepatic function or with other clinically evident infectious diseases were excluded. The dogs were excluded if they had been vaccinated against or had been treated with leishmanicidal agents less than 3 months before preinclusion in the present study or with a leishmanistatic 1 month before preinclusion. Finally, no previous treatment with systemic antibiotics or antifungals within 7 days or with systemic long-acting corticosteroids within 1 month prior to preinclusion was allowed. Pregnant and lactating dogs were excluded. Dogs could be withdrawn from the study at any time if they showed intolerance to the treatment or if requested by the owner.

The efficacy criteria were determined by reduction in clinical scores (i.e., the sum of clinical scores was lower than before treatment) [28][39][14], which was a decrease in parasite

DNA and *Leishmania*-specific antibody titers. The score was defined according to the severity of each clinical sign, and the final value was obtained from the sum of all the values (Table 1). Quantitative parasite load was estimated through qPCR and antibody titers through quantitative IFAT.

Each manifestation was assigned a severity score ranging from 0 to 3, where 0 = none, 1 = mild, 2 = moderate, and 3 = severe (maximum total clinical score = 21). Mortality related to CanL was indicated by the highest score (22).

Experimental design

Selected dogs were randomly included in one of the two treatment arms (Table 2). Dogs (N=26) in **Group 1** (positive control) received 100 mg/kg antimoniate of meglumine (Glucantime®, Merial, France) daily for 28 days, subcutaneously, and 30 mg/kg allopurinol (Allopurinol Arraw® 300 mg, France) daily for one month, orally. Dogs (N=16) in **Group 2** (test group) received 25 mg/kg artesunate (Asu-Denk®; Denk Pharma, Germany) daily for 6 days, orally. After each administration, the dogs were observed for 30 min to monitor for reactivity, vomiting and/or regurgitation to ensure complete absorption of the drug. The dogs were followed up for 180 days (Fig 1).

Visit schedule and sample collection

The animals were checked on days D0, D+30, D+90 and D+180 after the treatment had started, and blood samples were collected at each time point. Clinical signs and lesions associated with leishmaniosis, infection status (parasite load-qPCR and serological) were evaluated and monitored at these points.

Clinical evaluation

The efficacy of treatments was assessed at each time point based on the clinical response to treatment (evolution of clinical scores over time and the percentage reduction of the total clinical score). For each dog, the same veterinarian conducted all clinical evaluations to maintain consistency. Clinical scores [39][25] were classified according to the severity of clinical signs listed in Table 1. Mortality due to CanL was indicated by the highest score (22). The reduction percentage was calculated according to the formula

Percentage reduction of CS %= (CS at D+xx- CS at D+0)/CS at D+0) x 100.

Parasite load-qPCR

Blood samples collected at each time point were analyzed for quantification of parasitic load by qPCR using primers and probe for detection and quantification of *L. infantum* DNA, targeting a conserved region of the kinetoplast minicircle DNA (kDNA) (several 1000-fold repeated sequence) [40]. Prior to DNA extraction, 200 µL of blood was digested with proteinase K and incubated at +56°C overnight. Extraction was performed using a commercial DNA extraction kit (QIAamp DNA Mini Kit®, [Qiagen, Courtaboeuf, France]) and was performed

on a BIOROBOT EZ1 (Qiagen, Qiagen, Courtaboeuf, France) per the manufacturer's instructions. DNA was eluted in 200 µl of distilled water and stored at -20°C until analysis. The qPCR was prepared and performed as described in [6]. Briefly, 20 µL final volume containing 10 µL of Eurogentec Master Mix Roche (Eurogentec, Liège, Belgium), 0.5 mM of each primer and the probe, 0.5 µL UDG, 3 µL of distilled DNAse- and RNAse-free water, and 5 µl of the DNA sample was amplified in a CFX96 Real-Time system (BioRad Laboratories, Foster City, CA, USA) using the following thermal profile: one incubation step at 50°C for two minutes and an initial denaturation step at 95°C for three minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 30 seconds. Quantification was based on a standard curve, which was an 8-fold serial dilution of 10^8 copies of plasmid DNA/mL from the kDNA region, equivalent to 10000 parasites/mL, and 5 µl of serial dilutions ranging from 10000 to 0.001 parasites/mL was introduced into reaction tubes. The results were expressed as the number of *Leishmania* parasites present in 1 ml of blood, taking into account the volume (200 µl of blood) and the elution (200 µl) introduced during the extraction process.

Serological monitoring

At day 0, the immunochromatographic-based qualitative rapid WITNESS® *Leishmania* test (Zoetis, France) was performed according to the manufacturer's instructions using one drop (10 µL) of whole blood from each dog directly after sampling. Quantitative IFAT for the titration of anti-*L. infantum*-specific immunoglobulins G (IgG) was performed on sera for monitoring as described previously [38]. Briefly, plate wells were coated with 20 µL of *L. infantum* commercial antigens (Zoetis, France). After dilution to 1:50, 20 µl of every serum dilution was applied per well, and plates were incubated for 30 min at 37 °C. Plates were washed twice with PBS for 5 min and once with distilled water. Then, 20 µL of anti-dog IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich, St Louis, MO, USA) were added into each well. The plates were incubated for 30 min at 37 °C in the dark. After another washing step, drops of mounting medium were added to the cover slips, and reading was performed. To avoid observation errors, all samples were examined by two different investigators, and positive and negative controls were added to each plate. All samples negative at 1:50 were considered negative. Positive results were further investigated using a five-fold serial dilution IFAT at 1:100 to 1:1600. At the greatest dilution, the samples were classified as high positive for *L. infantum* (> 1:1600).

Data analysis

XLSTAT Addinsoft version 2018.7 was used for the statistical analysis. A descriptive analysis of the data was performed according to the nature of the variables for each follow-up visit and assigned treatment. The nonparametric Kruskal-Wallis/bilateral test was used for comparison of averages taken at evaluation time points for both groups, Group 1 alone, and Group 2 alone. Multiple pairwise comparisons following the Dunn/bilateral test procedure was used for the CS, parasitemia and antibody titers. Treatment effects were compared between the two groups by analysis of percentage reduction of clinical signs, parasite load and *Leishmania* antibodies. The Mann-Whitney test was used to compare the efficacy of treatments using the difference between D0 and D+30 days, D0 and D+90 days and D0 and D+180 days. The

252 significance level used for the tests was 5% (Bonferroni Correction level of significance was
253 0.0083).
254

Results

Included dogs and monitoring

No significant difference was found for age, gender, breed (mongrel or purebred), clinical status of dogs, and mean parasitemia and *Leishmania* antibody titers between the two groups at the inclusion day (P-value> 0.05) (Table 2).

Clinical examinations of the selected dogs revealed the presence of general signs in 92.85% (39/42), cutaneous in 83.33% (35/42), visceral in 38.1% (16/42), ophthalmic in 23.8% (10/42), and musculo-skeletal in 4.76% (2/42). One dog (2.30%) showed oral erosion, another presented epistaxis, and two dogs (4.76%) had neurological disorders.

Statistical analysis was performed on 42 dogs, as one dog from Group 2 was lost to follow-up before the D+30 time point. During the 6-month follow-up, four dogs from Group 1 were lost to follow-up two months after the beginning of treatment, and two of these were due to failure of clients to return for the final revisits on D+90 and D+180. Two others were because of death not related to CanL. In addition, six dogs died in the period between D+60 after the beginning of treatment and before the D+90 visit with increase in clinical signs related to CanL. Four of these were in Group 1, and two were in Group 2 (Fig 1).

Clinical outcomes

Compared to the initial clinical score, improvement in total clinical scores in both treatment groups was observed throughout the study. Given the mortality outcome of the higher score (22), mean clinical scores decreased over time from 7.16± 3.47 at D0 to 4.24± 4.37 at visit D+ 30, increased again slightly at D+ 90 (5.75± 8.25), and then decreased below the level at D0 and remained constant (5.60± 8.35) until D+ 180. The same clinical score kinetics were observed in the two groups, separately (Fig 2).

Taking the CS at D0 and D+30 as the starting and end points, respectively, the mean percentage of reduction in clinical score was 43.3±47.8%. At each time point, D+30, D+90 and D+180, a statistically significant difference ($p<0.0001$) in mean percentage of reduction in clinical score was observed between the two groups. More improvement was noted in Group 2 than in Group 1 (Fig 3). At the level of individual response, approximately 26.66% (4/15) of Group 2 dogs complete recovered (CS of 0) *versus* 18.18% (4/22) in Group 1, while approximately half of the dogs showed partial amelioration at 6 months of follow-up in both groups with 54.54% (12/22) and 46.66% (7/15) in the control and test groups, respectively. At D+180, clinical worsening rates were similar in both groups at 27.27% (6/22), including 18.18% (4/22) mortality in the control group, *versus* 26.66% (4/15), including 13.33% (2/15) mortality, in the test group. Details on clinical changes over time for the dogs followed are described in Table 3.

Adverse events

No major side effects related to these treatments were reported in any dog. Both study compounds were well tolerated in all dogs, except for two dogs that had slight local pains and pruritus with 15 days of treatment with Glucantime®/allopurinol.

Quantitative real-time PCR kinetic parasitemia

At D0, 28.5% (12/42) dogs were positive by qPCR from a blood sample. Parasitic clearance was observed over time after treatment with artesunate, and all initially positive dogs (5/5) turned out to be *Leishmania*-negative. In contrast, 6/7 PCR-positive dogs treated with Glucantime®/allopurinol turned out to be negative, and 1/7 dogs remained positive. In addition, two dogs treated with Glucantime®/allopurinol were found to be positive, whereas they had been negative before. Individual evolution of parasitemia over the follow-up is shown in Table 4.

Serological status

Initially, all dogs were positive by both Witness® test and IFAT, and no significant difference ($P > 0.05$) was observed between groups. The mean *Leishmania* antibody titers decreased over time (mean ± SD, 444.05 ± 467.50 at D0, 181.25 ± 234.71 at D+ 30, 120.69 ± 298.36 at D+ 90 and 118.97 ± 297.73 at D+ 180) after treatment (Fig 4). Compared to baseline values, significant reductions ($P < 0.012$, IFAT serology) were observed in both groups at D+30 days (mean ± SD, artesunate 128,57 ± 212,78; Glucantime®/allopurinol 209,61 ± 244,96). These levels continued to decrease (mean ± SD, artesunate 37,50 ± 60,77; Glucantime®/allopurinol 176,471 ± 379,604) at D+ 180 (Fig 3). When groups were compared, the artesunate group showed a higher percent reduction in antibody titers at the end of the study ($P <0.0001$) (Fig S1). At the individual level, 78.57% (11/14) of dogs treated with artesunate showed a decrease in antibody titers at D+30, and 50% (7/14) of them became absolutely seronegative. In contrast, in the Glucantime®/allopurinol group, 57.7% (15/26) showed a decrease in serology at D+30, and only 15.38% (4/26) became seronegative. At the end of the follow-up, 41.37% (12/29) of dogs became seronegative, including 29.41% (5/17) in Group 1 *versus* 58.33% (7/12) in Group 2 (P-value, Z test/bilateral= 0.227).

Discussion

Today, there is lack of an effective and safe therapy against canine visceral leishmaniosis. New drugs, delivery systems and treatment strategies are necessary to achieve a cure in infected dogs [41]. This is important not only for dogs but also as a part of the fight against human leishmaniosis (reservoir sanction). Here, we compared the efficacy of artesunate as an alternative chemotherapy and Glucantime®/allopurinol, which is currently the most effective treatment against CanL [42]. The treatment responses were determined using a comprehensive clinical score, which was based on the main clinical signs associated with CanL, i.e., parasite load evolution using qPCR and serological monitoring by measurement of antibody titers through quantitative IFAT [17][43][44]. This is the first report on the efficacy of artesunate (an artemisinin derivative) in the treatment of visceral leishmaniosis in dogs under field conditions.

Both treatments led to clinical improvement and reduced parasite burden and *Leishmania* antibody titers, but a greater improvement in clinical signs was observed with artesunate. Artemisinin and its derivatives have proven therapeutic potential against malaria and have also demonstrated effectiveness in experimental models of leishmaniosis [45][34]. Artemisinin was active against six strains of *Leishmania* responsible for diverse forms of leishmaniosis [34]. In this study, artesunate was well tolerated without any adverse effects. In one study, artesunate was used in dogs as an anti-cancer treatment; dogs exhibited no adverse effects at all [46]. Two dogs in the control group showed local pain at the site of injection. Treatment in the control group was by injection+ oral administration, while artesunate was given by oral administration. Side effects after treatment with meglumine antimoniate/allopurinol that have been previously demonstrated were mainly as follows: local pain, pancreatitis, panniculitis and nephrotoxicity for meglumine antimoniate, and xanthinuria, urolithiasis and renal mineralization for allopurinol [23][25], where it has been observed that 16% of dogs developed adverse events after treatment with Glucantime®.

A rapid improvement in general conditions was observed a few days after treatment with artesunate. At the D+30 visit, 80% of dogs in the test group *versus* 73% in the control group showed clinical amelioration. Clinical improvement occurred rapidly in dogs 30 days after meglumine antimoniate/allopurinol therapy in several studies [47][19][48][42]. Treatments were successful in almost ¾ of cases and failed in almost ¼ of the cases after six months follow-up in both groups. Worsening was observed in 6 dogs *versus* 4 dogs (including the deaths of 4 dogs *versus* 2 dogs) in the control *versus* test groups. In one study, 10 months after the end of combined meglumine and allopurinol treatment in 6 dogs, 4 dogs had no clinical signs, 2 had relapsed, and the amastigote forms of *Leishmania* were present in the spleen of 5 dogs [49] . In addition, we estimate that Glucantime®/allopurinol treatment is at least 10-fold more expensive than artesunate. In this study, artesunate showed a greater reduction in clinical scores than Glucantime®/allopurinol. Compared to the current treatment, which is very burdensome and complicated in practice, artesunate treatment could be a good alternative chemotherapy, especially because it is cheap and easy to use (*per os*) for a short period (6 days of treatment in the present study) [23]. Furthermore, its efficacy can be improved by adjusting the dose or period of treatment, and further investigations can be used to determine the best regimen.

The blood PCR technique used had less diagnostic value than quantitative serology, and only 12/42 of sick seropositive dogs were found to be PCR-positive. This finding is in agreement with other authors [18][50] as well as with the fact that it is well known that blood parasitemia might be intermittent [51][52][22], and the blood *Leishmania* parasite load is much lower than the load found in other tissues, such as bone marrow in dogs with clinical leishmaniosis [50][53][54].

Parasitemia decreased continuously after treatment with artesunate until its total disappearance (100% parasite clearance, 180 days post-treatment), suggesting a leishmanicidal activity for this compound. The leishmanicidal activity of artemisinin and derivatives against *Leishmania* spp., causative agents of New World and Old World leishmaniases (including *L. infantum*), have been demonstrated in vitro [33] and in a mouse experimental model [34]. In this study, Glucantime®/allopurinol greatly reduced the parasite load at the end of treatment (4/7 initially positive dogs became PCR negative at D+ 30). A significant decline in the blood parasite load during the first 30 days using this combination therapy has been reported [18][55]. In addition, all PCR-negative dogs remained negative after treatment with artesunate; in contrast, two dogs in the control group became PCR-positive with worsening of clinical signs at D+ 90 follow-up. Failures of the combination Glucantime®/allopurinol therapy have been reported, mainly due to the drug-resistance of *Leishmania,* but time to relapse in treated dogs has not been documented [23]. In our case, relapse started the second month posttreatment.

The present study showed that 30 days following the initiation of treatment, there was an important significant decline (2.28- *versus* 3.04-fold, for control *versus* test group) in *L. infantum*-specific antibody titers corresponding with clinical improvement as has been reported in previous studies [56][18]. Antibody levels continued to decrease more in the test group, where 58% of cases became seronegative 6 months posttreatment (Fig 3). This indicates a regression in antigenic stimulation [41][57]. A positive association has been found between antibody levels and parasitic dissemination to different tissues [58][59]; therefore, it might be hypothesized that the decrease in antibody levels implies that there is no parasitic dissemination. Compared to other studies, the antibody level continued to decrease progressively but slowly during the treatment period [48][60][61]. As has been shown in previous studies, when compared to the baselines, only a minority of dogs became seronegative during the first year of treatment, but almost all of them reached much lower antibody levels [62][63]. Our finding showed that artesunate induces a rapid and greater reduction in antibody levels than the combination therapy Glucantime®/allopurinol. Moreover, compared to day 0, clinical worsening and death in all cases were associated with increasing or at least stable antibody levels without any serological resolve. Therefore, we corroborate that serological monitoring is very useful for the detection of clinical relapse and prognosis after treatment since it correlates with an increased antibody level in blood [21][17][48][18].

Conclusions

These findings indicate that artesunate as well as combination meglumine antimoniate/allopurinol are effective treatments against canine visceral leishmaniosis. Artesunate was safer and more effective in controlling and reducing the clinical signs of leishmaniosis, parasite load and antibodies than meglumine antimoniate/allopurinol. Furthermore, it is cheaper and easier to administrate (orally) with a short treatment period.

Now, it is important to obtain more data on oral artesunate treatment in other trials with a larger number of dogs and long-term follow-up. Further clinical trials are needed to determine if dogs remain infective to sandflies and to define the ideal therapeutic dosage and duration of treatment. Finally, because the dog is an ideal model for human visceral leishmaniosis (LV) studies, the efficacy of artesunate in the treatment of LV should be evaluated.

Supporting Information

Fig S1. Comparison of mean percent reduction in IFAT antibody titers in dogs with leishmaniosis treated with artesunate or Glucantime®/allopurinol. Data is reported as the mean ± SD, artesunate *versus* Glucantime®/allopurinol (Mann-Whitney test) 30-, 90- and 180-days posttreatment

Acknowledgments

We are grateful for all veterinarians and dog owners for their kind help in the monitoring of dogs. We thank C. Sokhna, B. Pradine and E.H. Amadou Niang for their help to get medicaments.

Funding

This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program « Investissements d'avenir », reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI,

References

1. Caixeta I, Helena L, Coura-vital W, Macedo G, Magalhães C, Almeida T, et al. Effectiveness of the Brazilian Visceral Leishmaniasis Surveillance and Control Programme in reducing the prevalence and incidence of Leishmania infantum infection. Parasites & Vectors; 2018; 1–12.

2. Dantas-Torres F, Brandão-Filho SP. Visceral leishmaniasis in Brazil: revisiting paradigms of epidemiology and control. Rev Inst Med Trop Sao Paulo. 2006;48(3):151-156. doi:10.1590/s0036-46652006000300007

3. Desjeux P. Leishmaniasis : current situation and new perspectives. 2004;27: 305–318. doi:10.1016/j.cimid.2004.03.004

4. Salomón OD, Feliciangeli MD, Quintana MG, Martins M, Rangel EF. Lutzomyia longipalpis urbanisation and control. 2015;110: 831–846. doi:10.1590/0074-02760150207

5. Romero GAS, Boelaert M. Control of Visceral Leishmaniasis in Latin America — A Systematic Review. 2010;4. doi:10.1371/journal.pntd.0000584

6. Medkour H, Davoust B, Dulieu F, Maurizi L, Lamour T, Marié J-L, et al. Potential animal reservoirs (dogs and bats) of human visceral leishmaniasis due to *Leishmania infantum* in French Guiana. PLoS Negl Trop Dis. 2019;13: e0007456. doi:10.1371/journal.pntd.0007456

7. Courtenay O, Quinnell RJ, Garcez LM, Dye C. Low infectiousness of a wildlife host of *Leishmania infantum*: the crab-eating fox is not important for transmission. Parasitology. 2002;125(Pt 5):407-414. doi:10.1017/s0031182002002238

8. Freitas-junior LH, Chatelain E, Andrade H, Siqueira-neto JL. International Journal for Parasitology : Drugs and Drug Resistance Visceral leishmaniasis treatment : What do we have , what do we need and how to deliver it ? Int J Parasitol Drugs Drug Resist. Australian Society for Parasitology; 2012;2: 11–19. doi:10.1016/j.ijpddr.2012.01.003

9. Baneth G, Aroch I. Canine leishmaniasis: A diagnostic and clinical challenge. Vet J. 2008;175: 14–15. doi:10.1016/j.tvjl.2006.11.011

10. Ribeiro RR, Suzan M, Michalick M, Da Silva ME, Peixoto CC, Santos D, et al. Canine Leishmaniasis: An Overview of the Current Status and Strategies for Control. Biomed Res Int. 2018;2018. doi:10.1155/2018/3296893

11. Moreno J, Alvar J. Canine leishmaniasis: Epidemiological risk and the experimental model. Trends Parasitol. 2002;18: 399–405. doi:10.1016/S1471-4922(02)02347-4

12. Leontides LS, Saridomichelakis MN, Billinis C, Kontos V, Koutinas AF, Galatos AD, et al. A cross-sectional study of *Leishmania* spp . infection in clinically healthy dogs with polymerase chain reaction and serology in Greece. 2002;109: 19–27.

13. Miró G, López-Vélez R. Clinical management of canine leishmaniosis versus human leishmaniasis due to *Leishmania infantum*: Putting “One Health” principles into practice. Vet Parasitol. Elsevier; 2018;254: 151–159. doi:10.1016/j.vetpar.2018.03.002

14. Nogueira S, Avino VC, Galvis-ovallos F, Pereira-chioccola VL, Antonio M, Moreira B, et al. Use of miltefosine to treat canine visceral leishmaniasis caused by *Leishmania infantum* in Brazil. Parasites & Vectors; 2019;0: 1–11.

15. Dujardin JC, Campino L, Cañavate C, Dedet JP, Gradoni L, Soteriadou K, et al. Spread of vector-borne diseases and neglect of leishmaniasis, Europe. Emerg Infect Dis. 2008;14: 1013–1018. doi:10.3201/eid1407.071589

16. Pineda C, Aguilera-tejero E, Morales MC, Belinchon- S, Gomez-nieto LC, Garcia P, et al. Treatment of canine leishmaniasis with marbofloxacin in dogs with renal disease. 2017; 1–17.

17. Cardoso L. Parasites & Vectors LeishVet guidelines for the practical management of canine leishmaniosis. 2011; 1–16. doi:10.1186/1756-3305-4-86

18. Solano-Gallego L, Di Filippo L, Ordeix L, Planellas M, Roura X, Altet L, et al. Early reduction of *Leishmania infantum*-specific antibodies and blood parasitemia during treatment in dogs with moderate or severe disease. Parasites and Vectors. Parasites & Vectors; 2016;9: 1–9. doi:10.1186/s13071-016-1519-0

19. Oliva G, Cruz I, Veterinaria M, Ii F, Animal P. Multicentric , controlled clinical study to evaluate effectiveness and safety of miltefosine and allopurinol for canine leishmaniosis. 2009; 397–404. doi:10.1111/j.1365-3164.2009.00824.x

20. Committee WHOE. Control of the leishmaniases. 2010; 22–26.

21. Cardoso L, Pennisi MG, Solano-gallego L, Koutinas A, Miro G, Ferrer L, et al. Veterinary Parasitology Directions for the diagnosis , clinical staging , treatment and prevention of canine leishmaniosis. 2009;165: 1–18. doi:10.1016/j.vetpar.2009.05.022

22. Travi BL, Cordeiro-da-Silva A, Dantas-Torres F, Miró G. Canine visceral leishmaniasis: Diagnosis and management of the reservoir living among us. PLoS Negl Trop Dis. 2018;12(1):e0006082. Published 2018 Jan 11. doi:10.1371/journal.pntd.0006082

23. Miró G, Petersen C, Cardoso L, Bourdeau P, Baneth G, Solano-gallego L, et al. Novel Areas for Prevention and Control of Canine Leishmaniosis. Trends Parasitol. Elsevier Ltd; 2017;xx: 1–13. doi:10.1016/j.pt.2017.05.005

24. Noli C, Auxilia ST. Treatment of canine Old World visceral leishmaniasis: a systematic review. Vet Dermatol. 2005;16(4):213-232. doi:10.1111/j.1365-3164.2005.00460.x

25. Mateo M, Maynard L, Vischer C, Bianciardi P, Miró G. Comparative study on the short term efficacy and adverse effects of miltefosine and meglumine antimoniate in dogs with natural leishmaniosis. 2009; 155–162. doi:10.1007/s00436-009-1375-3

26. Bianciardi P, Brovida C, Valente M, Aresu L, Cavicchioli L, Vischer C, et al. Toxicologic Pathology. 2009; doi:10.1177/0192623309344088

27. Manna L, Vitale F, Reale S, Picillo E, Neglia G, Vescio F, et al. Study of efficacy of miltefosine and allopurinol in dogs with leishmaniosis. Vet J. Elsevier Ltd; 2009;182:

441–445. doi:10.1016/j.tvjl.2008.08.009

28. Woerly V, Maynard L, Sanquer A, Eun H. Clinical efficacy and tolerance of miltefosine in the treatment of canine leishmaniosis. 2009; 463–469. doi:10.1007/s00436-009-1404-2

29. Saridomichelakis MN, Savvas I, Leondides L. Effects of Allopurinol Treatment on the Progression of Chronic Nephritis in Canine Leishmaniosis (Leishmania infantum). 2006; 228–233.

30. Loo CS, Lam NS, Yu D, Su XZ, Lu F. Artemisinin and its derivatives in treating protozoan infections beyond malaria. Pharmacol Res. 2017;117:192-217. doi:10.1016/j.phrs.2016.11.012

31. Araujo NCP, Afonso R, Bringela A, Cancela ML, Cristiano MLS, Leite RB. Parasitology International Peroxides with antiplasmodial activity inhibit proliferation of *Perkinsus olseni*, the causative agent of Perkinsosis in bivalves. Parasitol Int.; 2013;62: 575–582. doi:10.1016/j.parint.2013.06.010

32. Bories C, Chollet C. In vitro antileishmanial activity of fluoro-artemisinin derivatives against *Leishmania donovani*. 2008;62: 462–465. doi:10.1016/j.biopha.2008.04.003

33. Cortes S, Albuquerque A, Cabral LIL, Lopes L, Campino L, Cristiano LS. In Vitro Susceptibility of *Leishmania infantum* to Artemisinin Derivatives and Selected Trioxolanes. 2015;59: 5032–5035. doi:10.1128/AAC.00298-15

34. Sen R, Ganguly S, Saha P, Chatterjee M. Efficacy of artemisinin in experimental visceral leishmaniasis. Int J Antimicrob Agents. Elsevier B.V.; 2010;36: 43–49. doi:10.1016/j.ijantimicag.2010.03.008

35. Want MY, Islamuddin M, Chouhan G, Ozbak HA, Hemeg HA, Dasgupta AK, et al. Therapeutic efficacy of artemisinin-loaded nanoparticles in experimental visceral leishmaniasis. Colloids Surfaces B Biointerfaces. Elsevier B.V.; 2015;130: 215–221. doi:10.1016/j.colsurfb.2015.04.013

36. Islamuddin M, Chouhan G, Farooque A, Dwarakanath BS, Sahal D, Afrin F. Th1-Biased Immunomodulation and Therapeutic Potential of *Artemisia annua* in Murine Visceral Leishmaniasis. PLoS Negl Trop Dis. 2015;9. doi:10.1371/journal.pntd.0003321

37. Want MY, Islammudin M, Chouhan G, Ozbak HA, Hemeg HA, Chattopadhyay AP, et al. Nanoliposomal artemisinin for the treatment of murine visceral leishmaniasis. Int J Nanomedicine. 2017;12: 2189–2204. doi:10.2147/IJN.S106548

38. Medkour H, Laidoudi Y, Lafri I, Davoust B, Mekroud A, Bitam I, et al. Canine vector-borne protozoa: Molecular and serological investigation for *Leishmania* spp., *Trypanosoma* spp., *Babesia* spp., and *Hepatozoon* spp. in dogs from Northern Algeria. Vet Parasitol Reg Stud Reports. Elsevier; 2020;19: 100353. doi:10.1016/j.vprsr.2019.100353

39. Manna L, Reale S, Vitale F, Elio A. Research in Veterinary Science Evidence for a relationship between Leishmania load and clinical manifestations. Res Vet Sci.

544 Elsevier Ltd; 2009;87: 76–78. doi:10.1016/j.rvsc.2008.12.009

545 40. Mary C, Faraut F, Lascombe L, Dumon H. Quantification of *Leishmania infantum*
546 DNA by a Real-Time PCR Assay with High Sensitivity. J Clin Microbiol. 2004;42:
547 5249–5255. doi:10.1128/JCM.42.11.5249

548 41. Baneth G, Shaw SE. Chemotherapy of canine leishmaniosis. Vet Parasitol.
549 2002;106(4):315-324. doi:10.1016/s0304-4017(02)00115-2

550 42. Manna L, Corso R, Galiero G, Cerrone A, Muzj P, Gravino AE. Long-term follow-up
551 of dogs with leishmaniosis treated with meglumine antimoniate plus allopurinol versus
552 miltefosine plus allopurinol. Parasites & Vectors; 2015; 1–9. doi:10.1186/s13071-015-
553 0896-0

554 43. Roura X, Fondati A, Lubas G, Gradoni L, Maroli M, Oliva G, et al. Prognosis and
555 monitoring of leishmaniasis in dogs : A working group report. Vet J. Elsevier Ltd;
556 2013;198: 43–47. doi:10.1016/j.tvjl.2013.04.001

557 44. Segarra S, Miró G, Montoya A, Pardo-marín L, Boqué N, Ferrer L, et al. Randomized,
558 allopurinol-controlled trial of the effects of dietary nucleotides and active hexose
559 correlated compound in the treatment of canine leishmaniosis. Vet Parasitol. Elsevier;
560 2017;239: 50–56. doi:10.1016/j.vetpar.2017.04.014

561 45. Yang DM, Liew FY. Effects of qinghaosu (artemisinin) and its derivatives on
562 experimental cutaneous leishmaniasis. Parasitology. 1993;106 (Pt 1):7-11.
563 doi:10.1017/s0031182000074758.

564 46. Rutteman GR, Erich SA, Mol JA, et al. Safety and efficacy field study of artesunate for
565 dogs with non-resectable tumours. Anticancer Res. 2013;33(5):1819-1827..

566 47. Denerolle P, Bourdoiseau G. Combination allopurinol and antimony treatment versus
567 antimony alone and allopurinol alone in the treatment of canine leishmaniasis (96
568 cases). J Vet Intern Med. 1999;13(5):413-415. doi:10.1892/0891-
569 6640(1999)013<0413:caaatv>2.3.co;2.

570 48. Torres M, Bardagí M, Roura X, Zanna G, Ravera I, Ferrer L. Long term follow-up of
571 dogs diagnosed with leishmaniosis (clinical stage II) and treated with meglumine
572 antimoniate and allopurinol. Vet J. Elsevier Ltd; 2011;188: 346–351.
573 doi:10.1016/j.tvjl.2010.05.025

574 49. Alvar J, Molina R, San Andrés M, et al. Canine leishmaniasis: clinical, parasitological
575 and entomological follow-up after chemotherapy. Ann Trop Med Parasitol.
576 1994;88(4):371-378. doi:10.1080/00034983.1994.11812879

577 50. Rodríguez-cortés A, Ojeda A, Francino O, López-fuertes L, Timón M, Alberola J.
578 *Leishmania* Infection : Laboratory Diagnosing in the Absence of a " Gold Standard ."
579 2010;82: 251–256. doi:10.4269/ajtmh.2010.09-0366

580 51. Manna L, Gravino E, Picillo E, Decaro N, Buonavoglia C. Leishmania DNA
581 Quantification by Real-time PCR in Naturally Infected Dogs Treated with Miltefosine.
582 2008;360: 358–360. doi:10.1196/annals.1428.018

583 52. Paltrinieri S, Gradoni L, Roura X, Zatelli A, Zini E. Laboratory tests for diagnosing
584 and monitoring canine leishmaniasis. Vet Clin Pathol. 2016;45: 552–578.
585 doi:10.1111/vcp.12413

586 53. Solano-gallego L, Rodriguez-cortes A, Trotta M, Zampieron C, Razia L, Furlanello T,
587 et al. Detection of *Leishmania infantum* DNA by fret-based real-time PCR in urine
588 from dogs with natural clinical leishmaniosis. Elsevier B.V.; 2007;147: 315–319.
589 doi:10.1016/j.vetpar.2007.04.013

590 54. Maia C, Ramada J, Cristóvão JM, Gonçalves L, Campino L. Diagnosis of canine
591 leishmaniasis: Conventional and molecular techniques using different tissues. Vet J.
592 2009;179: 142–144. doi:10.1016/j.tvjl.2007.08.009

593 55. Martínez V, Quilez J, Sanchez A, Roura X, Francino O, Altet L. Canine leishmaniasis:
594 The key points for qPCR result interpretation. Parasites and Vectors. 2011;4: 1–5.
595 doi:10.1186/1756-3305-4-57

596 56. Oliva BYG, Gradon LI, Cortese L, Ella PC, Medica C, Ii ÁF, et al. Comparative
597 efficacy of meglumine antimoniate and aminosidine sulphate , alone or in combination
598 , in canine leishmaniasis. Annals of Tropical Medicine and Parasitology, Volume 92,
599 Number 2, 1 March 1998, pp. 165-171(7). https://doi.org/10.1080/00034989860003.

600 57. Altet L, Solano-gallego L, Sa E, Timo M, Francino O, Alberola J. A long term
601 experimental study of canine visceral leishmaniasis. 2007;37: 683–693.
602 doi:10.1016/j.ijpara.2006.11.007

603 58. Martínez-Moreno A, Moreno T, Martínez-Moreno FJ, Acosta I, Hernández S. Humoral
604 and cell-mediated immunity in natural and experimental canine leishmaniasis. Vet
605 Immunol Immunopathol. 1995;48(3-4):209-220. doi:10.1016/0165-2427(95)05434-8

606 59. Solano-gallego L, Morell P, Arboix M, Alberola J, Farmacologia D De. Prevalence of
607 Leishmania infantum Infection in Dogs Living in an Area of Canine Leishmaniasis
608 Endemicity Using PCR on Several Tissues and Serology. 2006;39: 560–563.
609 doi:10.1128/JCM.39.2.560

610 60. Solano-gallego L, Riera C, Roura X, Iniesta L, Enric J. *Leishmania infantum* -specific
611 IgG , IgG1 and IgG2 antibody responses in healthy and ill dogs from endemic areas
612 Evolution in the course of infection and after treatment. 2001;96: 265–276.

613 61. Pasa S, Ozensoy S, Voyvoda H, Ozbel Y. Clinical and serological follow-up in dogs
614 with visceral leishmaniosis treated with allopurinol and sodium stibogluconate.
615 2005;128: 243–249. doi:10.1016/j.vetpar.2004.12.002

616 62. Vercammen F, DeDeken R. Antibody kinetics during allopurinol treatment in canine
617 leishmaniasis. Vet Rec. 1996;139(11):264. doi:10.1136/vr.139.11.264-a

618 63. Rodríguez A, Solano-Gallego L, Ojeda A, et al. Dynamics of *Leishmania*-specific
619 immunoglobulin isotypes in dogs with clinical leishmaniasis before and after treatment.
620 J Vet Intern Med. 2006;20(3):495-498. doi:10.1892/0891-
621 6640(2006)20[495:doliii]2.0.co;2

622

623 **Figure legends**

624 **Fig. 1:** Consort flow diagram showing the therapeutic protocols and dogs with leishmaniosis in
625 each stage of the study.

626 **Fig 2. Time course of total clinical score** (Kruskal-Wallis test). Clinical scores (mean ± SD)
627 of the dogs (n=42) were assessed by veterinarians at the time of scheduled visits at D0, D+30,
628 D+90 and D+180. A. All treated groups. B. Glucantime®/Allopurinol-treated group. C.
629 Artesunate-treated group.

630 **Fig 3. Comparison of the mean percent reduction in clinical score between groups** (Mann-
631 Whitney test); **Green**. Day 30 posttreatment; **Blue**. Day 90 posttreatment; **Red**. Day 180
632 posttreatment. Mortality was taken into consideration (mortality score=22). The mean percent
633 reduction in clinical score is higher in the artesunate-treated group than in the
634 Glucantime/allopurinol-treated group at all time points.

635 **Fig 4. Serology monitoring.** Antibody titers (mean ± SD) of the dogs (n=42) were assessed by
636 quantitative IFAT at the time of scheduled visits at D0, D+30, D+90 and D+180 (Kruskal-
637 Wallis test/bilateral). **A.** All dogs. **B.** Glucantime/allopurinol group. **C.** Artesunate group.
638

639 **Table 1.** Clinical signs related to CanL defined to evaluate the clinical scores of dogs.

Symptoms	Number of signs	Clinical manifestations
General conditions	5	Emaciation, anorexia, abbatement, anemia, hyperthermia
Cutaneous signs	6	Localized alopecia, pruritus, squamose, hyperkeratosis, onychogriffose, ulcers
Visceral lesions	4	Lymphadenopathy, splenomegaly, hepathomegaly, renal disorders
Ophthalmic level	4	Depilation around the eyes "in glasses", mucopurulent conjunctivitis, keratitis and/or uveitis
Oral level	1	Oral erosions
Skeletal level	3	Arthrites, synovitis, diffuse pain of the posterior train
Others	3	Epistaxis, chronic diarrhea, neurological signs

640

641 **Table 2.** Characteristics of dogs assigned to each treatment arm and homogeneity
642 analysis data, expressed as the mean ± standard deviation and frequencies (%).

Variable	Control treatment (Glucantime+ Allopurinol) (n=26)	Test treatment (Artesunate) (n=16)
Age, year	3.71 ± 2.11	3.94 ± 1.45
(min- max)	(1- 11)	(2.5- 8)
Gender, male (%)	17 (65.39)	13 (81.25)
Breed		
Breeder (%)	17 (65.39)	8 (50)
Mongrel (%)	9 (34.61)	8 (50)
Clinical score: points	7.46 ± 4.04	6.67 ± 2.30
(min- max)	(3- 20)	(3- 11)
No. of positive by PCR (%)	7 (26.92)	5 (31.25)
qPCR-Parasitemia (Leish/mL)	1108.72 ± 4154.5	78.04 ± 98.98
(min- max)	(0- 21000)	(0- 233)
No. Positive PCR (%*)	7 (26.92)	5 (31.25)
Leishmania Antibody titers	476.92 ± 448.38	390.62 ± (507.35)
(min- max)	(100- 1600)	(50- 1600)

643 **%*:** (Number of positive by PCR/Number of total positive) x 100.
644

Table 3. Individual clinical changes in dogs over time

Groups	Visit	Complete amelioration (%)	Partial amelioration (%)	No change (%)	Aggravation (%)
Artesunate treated dogs	D+30 (n=15)	-	12 (80)	-	3 (20)
	D+90 (n=15)	3 (20)	8 (53.33)	1 (6.66)	3 (20)
	D+180 (n=15)	4 (26.66)	7 (46.66)	-	4 (26.66)
Glucantime/allopurinol treated dogs	D+30 (n=26)	2 (7.69)	17 (65.38)	1 (3.84)	6 (23.07)
	D+90 (n=23)	3 (13.04)	13 (56.52)	1 (4.34)	6 (26.08)
	D+180 (n=22)	4 (18.18)	12 (54.54)	-	6 (27.27)
Total dogs	D+30 (N=41)	2 (4.87)	29 (70.73)	1 (2.44)	9 (21.91)
	D+90 (N=38)	6 (15.79)	21 (55.26)	2 (5.26)	9 (23.68)
	D+180 (N=37)	8 (21.62)	19 (51.35)	-	10 (27.02)

Mortality related to CanL had been included.

Table 4. Parasite load, Number of parasites per mL of blood and percentage reduction over time.

Dog's ID	Treatment	Parasitemia No. Leish/mL of blood (% of reduction)			
		D0	**D+ 30**	**D+ 90**	**D+ 180**
13	Glucantime/ Allopurinol	1500	288,7 (80,75)	30,28 (97,98)	357 (76,2)
5		0	0 (0,00)	176 (-100) *	177 (-100) *
A11N12		0	46,6 (-100) *	N/A*	N/A*
S26		2400	19,8 (99,18)	2 (99,92)	0 (100)
S27		3800	0 (100)	0 (100)	0 (100)
S33		21000	0 (100)	0 (100)	N/A
S54		7,7	0 (100)	0 (100)	N/A
C4P2G2		101	16,2 (83,96)	0 (100)	0 (100)
C1G2P4		18	0 (100)	0 (100)	0 (100)
10	Artesunate	6,8	0 (100)	0 (100)	0 (100)
S50		17,4	14 (19,54)	29 (-66,67)	0 (100)
A9N9		122	44 (63,93)	N/A	N/A
T11C2P1		233	1,7 (99,27)	0 (100)	0 (100)
A8 N8		11	0,17 (98,45)	0 (100)	0 (100)

All the other dogs were negative and remained negative during all of the follow-up.

N/A*: Mortality related to CanL

N/A: Lost to follow-up

Figure 1

Click here to access/download;Figure;Fig 1.tif

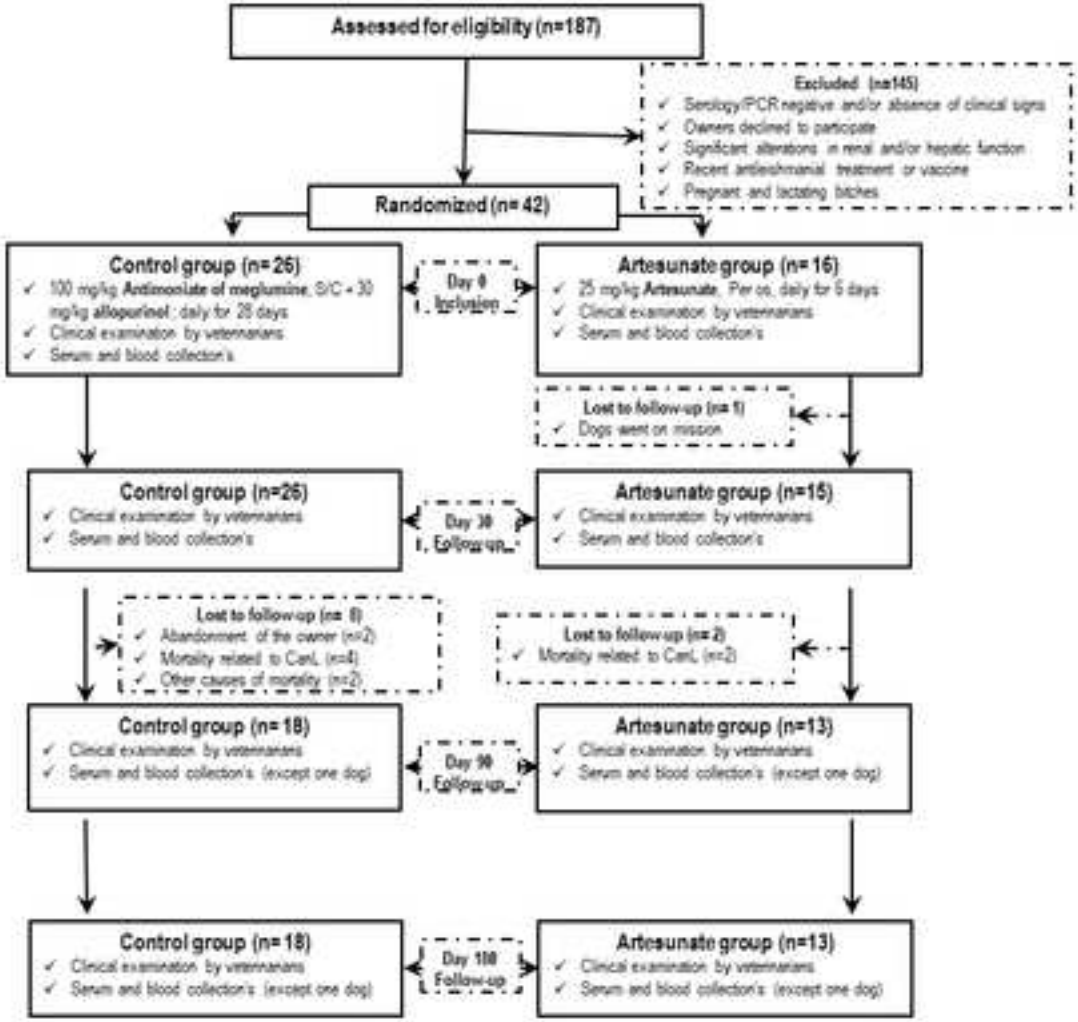


Figure 2

Click here to access/download;Figure;Fig 2..tif

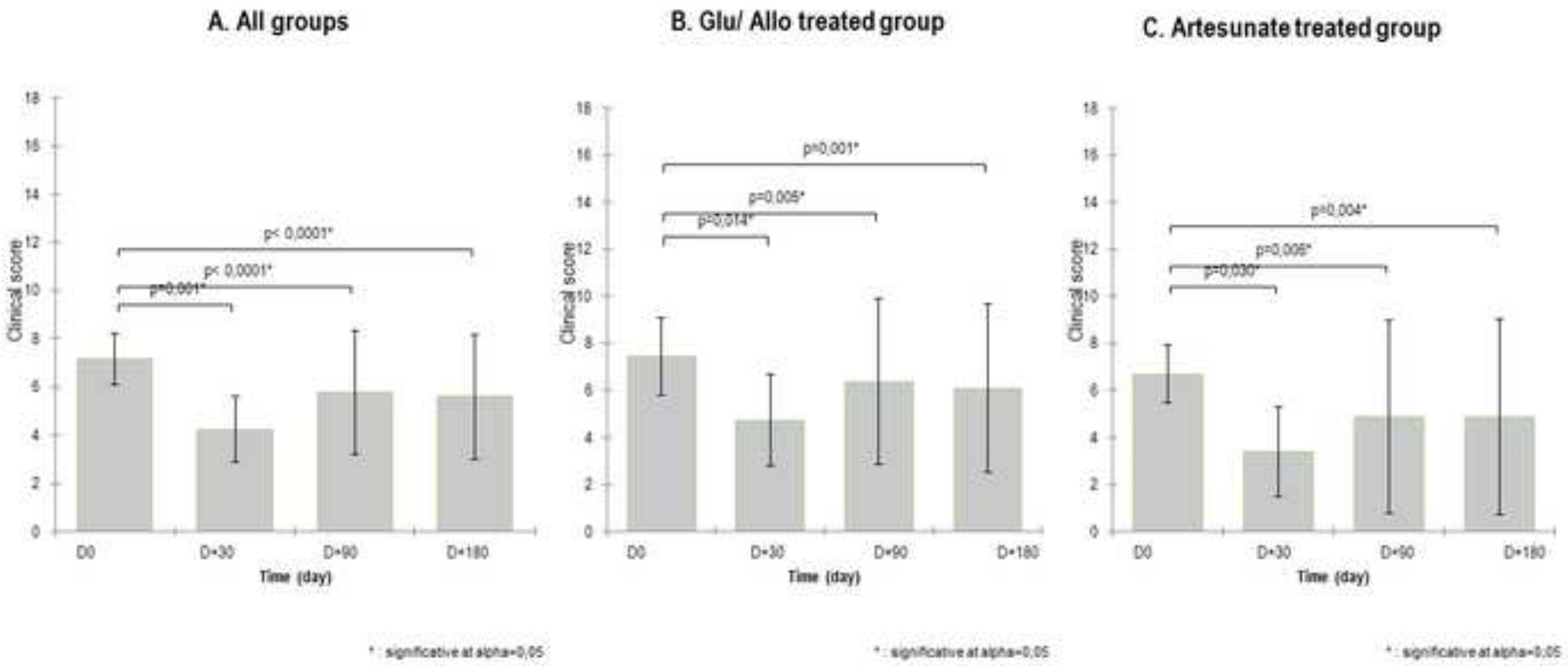


Figure 3

Click here to access/download;Figure;Fig 3.tif

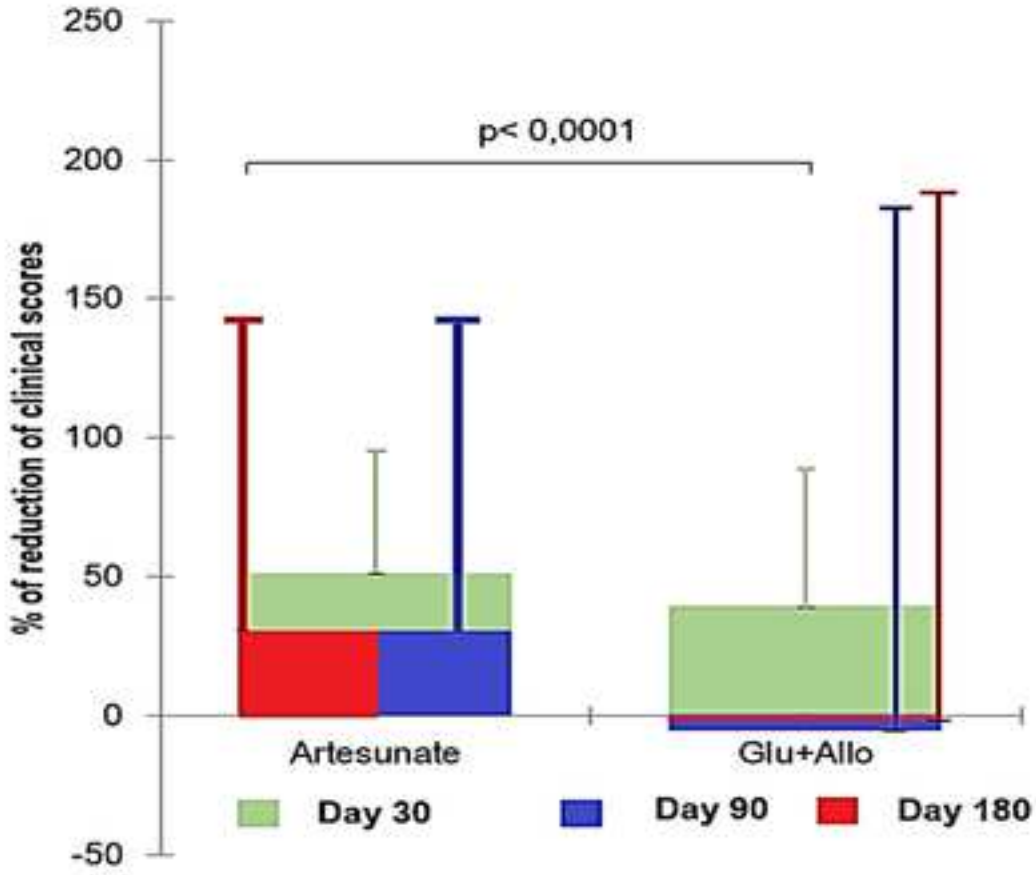
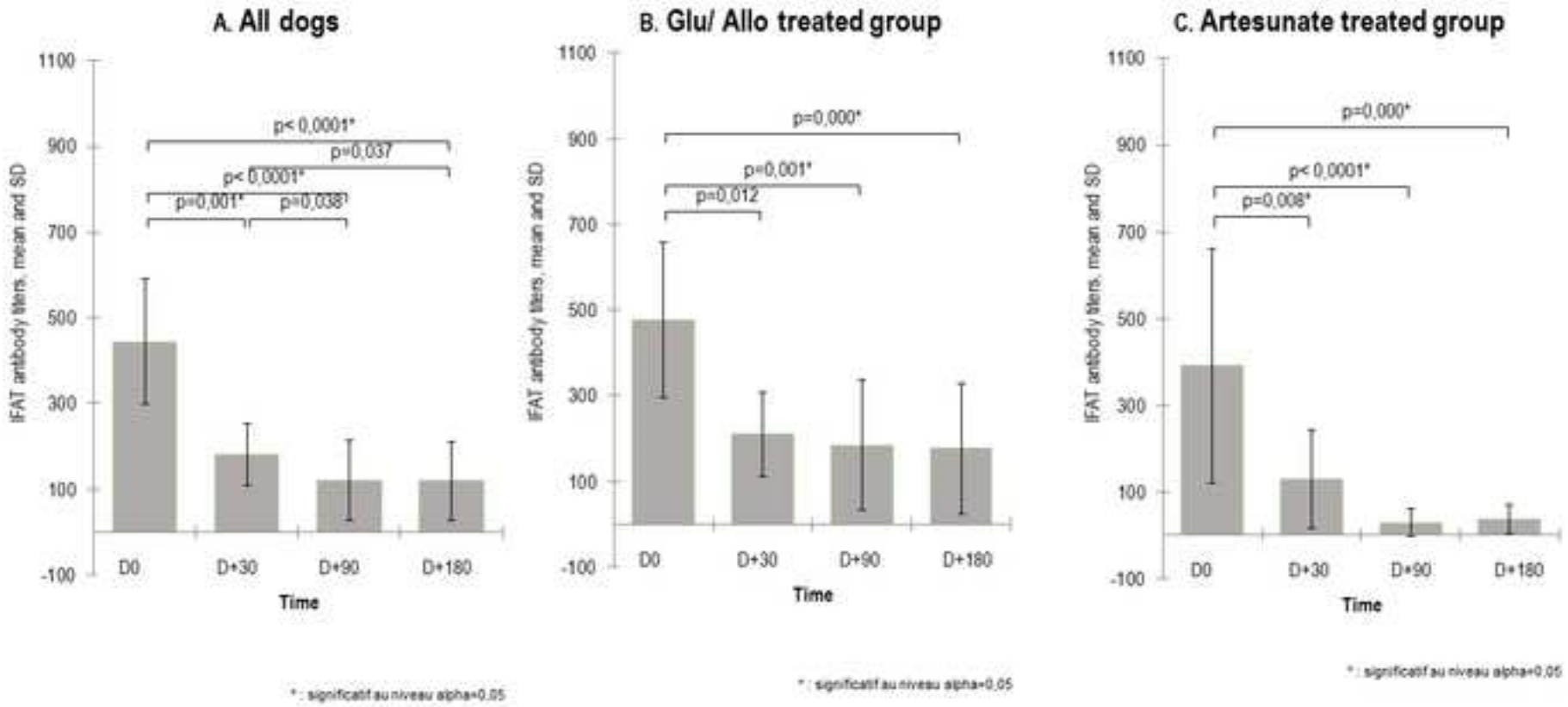


Figure 4

Click here to access/download;Figure;Fig 4..tif



Publication N°10

New insight in insect biocontrol: Isolation and characterization of active compounds from the clinical strain *Serratia marcescens* against *Aedes albopictus* larvae

Handi Dahmana[1,2], Nicholas Armstrong[1,2], **Younes Laidoudi**[1,2], Florence Fenollar[2,3], Didier Raoult[1,2], Eric Chabrière[1,2] and Oleg Mediannikov[1,2*]

[1]Aix Marseille Univ., IRD, AP-HM, MEPHI, Marseille, France
[2]IHU-Méditerranée Infection, Marseille, France
[3]Aix Marseille Univ., IRD, AP-HM, SSA, VITROME, Marseille, France

ARTICLE DE RECHERCHE

En préparation

Research article

New insight in insect biocontrol: Isolation and characterization of active compounds from the clinical strain *Serratia marcescens* against *Aedes albopictus* larvae

Handi Dahmana[1,2], Nicholas Armstrong[1,2], Younes Laidoudi[1,2], Florence Fenollar[2,3], Didier Raoult[1,2], Eric Chabrière[1,2], Oleg Mediannikov[1,2*]

[1] Aix-Marseille University, IRD, AP-HM, MEPHI, Marseille 13005, France

[2] IHU-Méditerranée Infection, Marseille 13005, France

[3] Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille 13005, France

Corresponding author: Oleg MEDIANNIKOV

Address: MEPHI, IRD, APHM, IHU-Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13385 Marseille Cedex 05

Tel: +33 (0)4 13 73 24 01, Fax: +33 (0)4 13 73 24 02, E-mail: olegusss1@gmail.com.

Abstract

Mosquitoes (*Diptera*: *Culicidae*) are vectors of devastating human and animal pathogens. The resistance against the current insecticide products has been identified in wild and laboratory insects. Consequently, their management and control are incomplete and require further development. Biological control seems to be a promising solution since that may assist in the control of resistant vectors such as dengue vectors. Here we assessed the insecticidal effect of bacterial metabolites, potentially active against *Aedes albopictus* larvae. A total of thirteen bacterial strains, initially isolated from clinical samples were retrieved from the strain collection CSUR (Collection de Souches de l'Unité des Rickettsies WDCM 875). First, culture supernatant from each bacterium was assessed at two different concentrations (i.e. 2 and 6mg/L of proteins) against *Aedes albopictus* larva. The trial was positively controlled by comparing to the commercialized *B. thuringiensis* subspecies *israelensis* (*Bti*). Nine strains (69.23%) among them showed a potential larvicidal activity. The mortality rates were ranged from 2% to 70% and from 2% to 78% at 2 and 6mg/L protein concentration respectively, which is almost higher than that induced by *Bti*, where the mortality ranged between 15% to 33%. Using 6mg/L protein concentration, the non-pigmented strain *Serratia marcescens* P400 exhibited the highest insecticidal activity of 78%. The culture supernatant from the P400 strain was subjected to the HPLC separation followed by mass spectrometry to identify the active compound. The crud formula indicated the presence of fatty amines. In order to confirm the insecticidal activity of fatty amines, we investigated the insecticidal activity of a commercialized fatty amine (Dibutyltetradecylamine) against *A. albopictus* larvae comparatively to ivermectin. Results showed that Dibutyltetradecylamine was more active than ivermectin. These findings emphasis the effectiveness of fatty amines against mosquitoes' larvae and reveal their possible use in pest biocontrol.

Keys words: Pest biocontrol, fatty amins, clinical bacteria, larvicidal, mosquitoes.

1. Introduction

Mosquitoes (Diptera: Culicidae) are vectors of several devastating human and animal pathogens, such as malaria, filaria, dengue and chikungunya viruses [1]. Several chemical products formulated to provide a high safety profile are commercially available, but their toxicity can lead to serious problems [2] including the incidence of resistances which increased rapidly in recent years [3].

Approximately 1500 naturally occurring microorganisms have been identified as potentially insecticidal agents. Metabolites from 942 microbial isolates were screened for insecticidal properties [4]. To date, it's *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *Bacillus sphaericus* formulations which are the predominant non-chemical tool used in mosquito-larvae control [5]. *B. thuringiensis* produces three classes of insecticidal proteins, *Cry* (Crystal proteins), *Cyt* (Cytolytic toxin) proteins during the sporulation phase as parasporal crystals, and *Vip* (vegetative insecticidal proteins) during the vegetative phase. It has been successfully used as a biopesticide for controlling pests that are harmful to crops, forests and humans [6].

Recently, resistance to its toxins are reported in field and laboratory studies [7]. It's the same thing for the *Bacillus sphaericus* in several countries [8][6]. The mechanisms of their resistance are widely studied in *Culex* and *Aedes* species [9][10][11][12][13]

New approaches and vector control tools are needed [14] and the most urgent need is to develop new insecticides to fight mosquito-borne diseases due to their crucial efficiency and their economic primordiality [15]. We explored in this study several clinical strains from the IHU-Méditerranée Infection (Marseille, France) bacterial strain collection (CSUR) as potential sources of entomopathogenic molecules. We studied the interactions between bacteria secondary metabolites and mosquitoes to identify potential novel compounds followed by a preliminary characterization of the highest active one for its probable future use in mosquito diseases control.

1. Materials and Methods

a) Bacterial strains used in this study

The selection of the species to be tested was based on the known toxic or pathogenic bacteria found in the literature or already used in living state against the insects (**Table S1**). Strains were selected from the CSUR collection of the laboratory of University Hospital Institute (Mediterranée Infection) in Marseille – France. They were isolated from clinical samples coming from patients by the culturomics technique [16], including an identification by MALDI-TOF MS [17] and the sequencing of whole 16S before being deposited in the collection.

We used *Bacillus thuringiensis* subspecies *israelensis* AM65-52 isolated from a commercial granular formulation (VectoBac GR, Valent Bioscience, USA) as a control to check and validate our protocol and to assess our strain's insecticidal activity.

Table S1: Details on the strains used in this study, isolated from clinical samples.

b) Preparation of samples

i. Bacteria culture

The strains were stored at -80 °C. The culture was carried out on solid medium Columbia agar + 5% sheep blood (Biomerieux SA. France) under aerobic or anaerobic condition at 37°C.Confirmation of species identification was performed by MALDI-TOF MS. Thereafter, for aerobic bacteria, colonies were transferred under sterile condition in 1 litter of liquid medium Tryptic soy broth T8907-1KG (Sigma-

Aldrich. France), while for anaerobic ones such as for *Clostridium spp*. colonies were transferred into blood culture bottles BacT/ALERT® FN plus (Biomerieux, France). After that, they were incubated for 3 days at 37° and 110 rpm in a shaker incubator in aerobic or anaerobic condition depending on strains. A negative control of culture consisting of free-bacteria medium was prepared and regularly checked for contamination.

The supernatant-pellet separation was carried out by centrifugation at 8000 g for 20 minutes at 4 ° C using a centrifuge (A98813 Bottle Assembly, J-Lite PP with JLA-8.1000 rotor, Beckman Coulter, France). The supernatant was immediately filtrated at 0.8µm then 0.45µm. Then, 50µL of the filtered supernatant were deposited on solid medium Columbia agar + 5% sheep blood (Biomerieux SA. France) to check its sterility. Finally, the supernatant was disposed in 75 mL flasks and frozen horizontally at -80°C before it was lyophilized the next day. The lyophilizate was stored at -20°C until the day of the assays.

ii. Bacteria inactivation and cell components extraction

The dry pellet was reconstituted in PBS 1X and then subjected to 3 freeze-thaw cycles for 5 minutes using liquid nitrogen and a hybridization incubator heated to 50°C. The tubes were then subjected to 3 sonication cycles at 50 Hz amplitude for 30 seconds. Subsequently, ultracentrifugation was carried out at 20,000 G for 20 minutes at 4°C. Thereafter, the supernatant was recovered and directly filtered at 0.45µm and then stored at -20°C. To check the sterility, 50µL of the filtered fraction was deposited on solid medium Columbia agar + 5% sheep blood (Biomerieux SA. France).

Once the larvae were ready, the sterile fractions (pellet and supernatant) were thawed at room temperature, then a Bradford protein assay was carried out. We tested the supernatant and pellet fractions of each stain separately at 2 mg/L and 6 mg/L of protein content. We also tested the mixture of the two fractions (pellet and supernatant) at 6 mg/L each to look if there was synergetic effect of secreted and cell constituent compounds.

c) Screening for insecticidal activity

Aedes albopictus colony was maintained at 27 ± 0.5°C and 80 ± 5% relative humidity. Adult mosquitoes were maintained on a constant exposure to 10% sucrose presented through cotton balls changed daily. For egg production, adult female mosquitoes were offered defibrinated human blood (French blood agency, France) via Hemotek membrane feeding system. Larvae were fed Tetra-Min fish food in clear water until pupa stage.

75 ml flasks were used for the insecticide screening assays. 25 of 3rd and early 4th larvae instars were placed in 100mL of clear distilled water. All tests of fraction were performed 25 larvae (N on replicates =4) so a total of 100 larvae as recommended by WHO [18]. Immediately after separating larvae in flasks containing 99 ml of clean distilled water, we added the fractions. Larvae are not fed until the 24thhour. Dead larvae are counted at 24, 48 and 72 hours. In each assay, 100 (4x25) larvae are used as negative control which did not receive any fraction, to assess eventually natural mortality. We consider that a fraction had an effect if it gave more than 20% of mortality.

d) Insecticidal activity prediction (investigation of Genes Encoding Toxins)

Total DNA extraction (Genomic and plasmid) was performed from colonies of the strains *L. sphaericus* CSUR P827 and *B. thuringiensis* CSUR P820 using EZ1 DNA kits (Qiagen, Courtaboeuf, France), according to the manufacturer's protocol. The DNA extracts were then stored at -20°C until PCR screening to identify the types of genes they might be carrying, especially *Cry* and *Cyt* proteins that are insecticidal to dipterans using Dipteran specific genes including *cry4A*/*4B*, *cry11*, *cry10*, *cyt1A*, *cyt1C*, *cyt2A*, *p19*, and *p20* (**Table S2**). The PCR amplifications were carried out as per [19]. The extracted DNA of the *Bti*AM65-52 was used as positive control.

Table S2: List of primers used in this study to explore the presence of genes encoding endotoxin, accessory proteins and *Cyt*.

e) Physicochemical properties of the active compounds of *Serratia marcescens*

Serratia marcescens P400 was chosen for further studies as the most effective strain in order to characterize the active compound. Bacterial supernatant was prepared from a liquid culture of *Serratia marcescens* P400 under the previously described conditions (see bacteria culture section) and was subsequently lyophilized. Then, it was reconstituted in sterile PBS 1X (300mg/mL). The stock solution was then aliquoted in small volumes of 1mL. One aliquot (1mL) of the stock solution was administrated as a positive control of larvicidal activity in each assay. The other aliquots were subjected to different treatments as shown below before the administration to the larvae. Negative controls corresponding to each different treatment were included. The pH was checked and adjusted to 7 after all treatments.

We performed the following experiments in order to characterise the chemical activity:

- Heating at 100C° for 60 minutes.
- Susceptibility to proteinase K (100µg/ml, 1 hour at 56C° pH 7.5).
- Protein precipitation with: acetonitrile or methanol (70% volume), Trichloroacetic acid (TCA 1.43g/ml at 20% volume).
- Solution adjusted to pH 2 and 10
- Extraction with various polarity solvents (50:50 v/v): ethyl acetate, di-ethyl ether, hexane and chloroform. Organic and aqueous layers were collected. Organic layers were dried and reconstituted in water.
- Molecular size filtration using 3 kDa spin columns (Amicon® Ultra-0.5 Centrifugal Filters, Merck KGaA, Darmstadt, Germany). The filtered fraction was recovered, and the non-filtered dry matter was resuspended in PBS 1X.

f) HPLC separation of the active compound

The chromatographic separation was performed depending on the results of the physicochemical properties identification of the active compounds. The 1mL aliquots were subjected to proteins precipitation using TCA (addition of 250µL of TCA 1.43g/ml). The mixture was incubated for 10 minutes at +4C°, then centrifugated at 13000 rpm for 5 minutes. The supernatant was then recovered (pH ≃ 2), and subjected to separation by high-performance liquid chromatography (Alliance 2690, Waters, Saint-Quentin-en-Yvelines, France). Eluting peaks were collected in order to test them for insecticidal activity.

First 1000µL were loaded into a reverse phase column intended to retain compounds with hydrophobic interactions (µBondapak C18 10 µm 3.9 × 300 mm, Waters) and eluted at 1 mL/min using a solvent gradient composition (97% water and 3% acetonitrile for 5 minutes [0-5 min]) followed by 5

minutes of cleaning-up with 5% water and 95% acetonitrile [5-10 min] then a return to initial conditions for 5 minutes [10-15 min].

Active fractions from this first reverse phase HPLC separation, were then subjected to further separation with Hydrophilic Interaction Chromatography (HILIC, Waters) permitting the retention of compounds according to hydrophilic interactions. 1500µL of direct collected active peaks from the C18 column were loaded into a XBRIDGE HILIC Column (5µm 4.6x250mm. Waters) and eluted using a solvent gradient composition as follow (Solvent A: 95% of acetonitrile and 5% of water 10 mM ammonium acetate pH 8 ; Solvent B: 50% -50% of same constituents): Isocratic elution at 1 mL/min with 95% solvent A for 5 minutes, wash step at 2 mL/min with 5 % solvent A for 5 minutes, return to initial condition for 5 minutes.

The different peaks were monitored at Max-Plot 210-400nm (PDA 996, Waters) and eluting peaks were collected in vials in order to be tested on mosquito larva. The number of fractions collected were accumulated (N=4) in order to enhance the biological effect (insecticidal activity). Blank injections were also collected the same way as negative controls. The fractions were dried at 40 °C under a stream of nitrogen and then reconstituted in 1mL of PBS 1X before tests. The pH was checked and adjusted when needed to pH-7 using NaOH (20%).

g) UHPLC-MS analysis of the active peaks

Active HILIC-collected peaks were analysed by UHPLC-MS using the following instruments: Acquity i-Class UPLC system connected to a Vion High Definition Q-TOF mass spectrometer. Samples were injected into a C18 column (ACQUITY UPLC BEH C18 1.7 µm, 2.1x50 mm, Waters) using water (A) and acetonitrile (B) supplemented with 0.1 % formic acid as mobile phases. The flow rate was set at 0.5 mL/min and a similar composition gradient was set as follow: 95 % of A during 0.5 minutes, then 5 to 20 % of B for 1.5 minutes, 80 % B for 1 minutes and return to initial conditions during 1 minute. A Data Independent Analysis was performed using a MS(e) method in order to survey the separated compounds (50-1000 m/z; 0.1 s per scan; 20-30 eV for CID). Positive and negative ionisation using a Z-spray electrospray source was performed (2 kV/40 V capillary and cone voltages; 120/450 ^{0}C source and desolvation temperatures). Raw data deconvolution was processed using UNIFI (Waters, version 1.9) and enabled the retrieve of each detected ion component information: retention time, mass-to-charge ratios (with or without parent fragmentation). The mass spectrometer was beforehand calibrated with a Major Mix solution (Waters) in order to calculated precise m/z ratios (below 2 ppm error). The Elucidate tool was used to identify known structures against isolated ion components within active retention times ([M+H] +, at least 1 fragment, 5 mDa mass error). Then a screening of the corresponding potential structures was performed with the following parameters in order to confirm the suspected chemicals in active fractions: 0.5 min retention time window, 2 ppm mass error on parents, at least 1 fragment.

h) Insecticidal activity assays of pure chemicals

- Given the results revealed by mass spectrometry, 3 fatty amines with the chemical formula $C_{22}H_{47}N$ were ordered (primary, secondary and tertiary amines), in order to be tested for potential insecticidal activity. Primary amine: Docosan-1-amine SY028561-1G, Sigma Aldrich, St. Louis, USA. Secondary amine: Diundecylamine S516511-250MG, Sigma Aldrich, St. Louis, USA. Tertiary amine: Dibutyltetradecylamine S810657-1G, Sigma Aldrich, St. Louis, USA.

The primary (Docosan-1-amine) and secondary amines (Diundecylamine) were obviously not soluble in water even at small concentrations. Subsequently, we only tested the tertiary amine Dibutyltetradecylamine against the mosquito larva (N=10 of *Aedes albopictus* L3-L4 larva stage, in a total final volume of 10 mL in a sterile flask). The assays were performed in triplicate using numerous dilutions ranging from 1µg/mL to 500µg/mL. The insecticidal activity of Dibutyltetradecylamine was compared to that given by Ivermectin.

A vial of IVOMEC 1%® injectable, 50 mL of 10mg/mL (Merial, Lyon, France) was used as an ivermectin solution. The assays were performed in triplicate using numerous dilutions ranging from 1ng/mL to 10µg/mL.

i) Toxicity tests for Dibutyltetradecylamine

The toxicity of Dibutyltetradecylamine is not reported elsewhere, not even in its Sigma Aldrich MSDS. Cell viability was evaluated using the MTT assay according to the manufacturer's recommendations (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (M5655-1G, Sigma Aldrich, St. Louis, USA). The cells that were used for the tests were: Human lung fibroblast cells (MRC-5); *Drosophila melanogaster* cell lines (S2) ; Murine fibroblast cell line (L929) ; Amphibian cell line, *Xenopus laevis* (African clawed frog) (XTC-2) ; *Aedes albopictus* cell line (C6/36).

Cell viability was measured at 4h, 8h, 12h and 24h post-administration. Briefly, $5*10^3$ cells/well were transferred to cell culture microplate 96 well (Greiner bio-one GmbH, Frickenhausen, Germany) and the plates were kept in the incubator for 24 h, at 28C° or 37 C° with 5% CO_2 depending on the different cells. Then, they were exposed to two concentrations, 100µg/mL and 1mg/mL of Dibutyltetradecylamine. The medium was replaced by a new medium and 10µL of MTT was added to each well reagent (MTT was solubilized in PBS 1X at a concentration of 5 mg/mL). The plate was then incubated for 4 hours at the adequate atmosphere depending on the different cells. After that, the contents of the well were discarded and replaced by 50µL of Dimethyl sulfoxide (D2650-5X5ML, Sigma Aldrich, St. Louis, USA). The plate was then incubated for 30 minutes in its adequate atmosphere. Data were acquired by spectrophotometry, using absorbance/Elisa microplate reader (BioTek, Winooski, USA). The assay was read at 570 nm and the cell viability percentage was calculated by Excel. We used paraformaldehyde 3% as positive control which may exhibit highest cells mortality, while we used PBS 1X as negative control which is not toxic to the cells. The tests were carried out 3 times and the conclusions were made on the mean of the 3 tests.

j) Statistical analysis

For strains insecticidal activity: The Epi Info version 7 program (http://www.cdc.gov/epiinfo/index.html), Addinsoft (2019) and XLSTAT statistical and data analysis solution Paris, France (https://www.xlstat.com) were used to compare mortality rates recorded at 72 h after administration of 6mg/L of the supernatant fractions.The Kruskal-Wallis test, comparison of k proportions and pairwise comparison was realized. A difference was statistically significant when p-values were ≤ 0.05. Dunn procedure (Bilateral test) performed to separate groups of strains according to their efficiencies.

Therefore, the compounds insecticidal activity (Characterized molecule versus Ivermectin), number of dead larvae observed trough the experiment at 10h and 24h were recorded. Then the number of dead larvae of all triplicates was transformed to the natural logarithm of (count + 1) for calculation of geometric means (GM) for each concentration. Multiple linear regression was modeled to test the effect

241 of each product and the exposure time on mosquito larvae. Significant effects were considered at
242 alpha=0.05. Statistical analysis was performed on Addinsoft software. A log-probit regression model
243 for the active compound and Ivermectin was performed. The larvicidal mortality was corrected by
244 Abbott's formula and an LC_{50} regression equation, and the 95% confidence limit was calculated by using
245 probit analysis. Probit analysis was performed by using the software by Dr. Alpha Raj. M "Free
246 LD50/LC50 Calculator (2020 web version)" based on the Probit Analysis method of Finney [20].

247

1. Results

a) Insecticidal activity of the clinical strains

After 72 hours post-administration of the samples at 6 mg/L of protein content, 9/13(69.23%) strains exhibited potential insecticidal effect on *A. albopictus* larvae (**Table 01**). The mortality raised with concentration using the supernatant fraction. No insecticidal effect was found using the pellet fractions for all of them including the *Bti* AM65-52.

Bti AM65-52, used a s a positive control, gave 33% of larvae mortality. The most effective strains belong to the entomopathogenic bacteria *Serratia marcescens* (non-pigmented). *S. marcescens* CSUR P400 giving 78% of mortality, CsurP587 giving 75% and CsurP833 giving 70%. *Microbacterium neimengenese* CsurP2323 gives 48% of larvae mortality. Similarly, the *Bacillus* family strains gave, 47% for *Lysinibacillus sphaericus* CsurP827 and 41% for *Bacillus thuringiensis* CsurP820. The anaerobic bacteria *Clostridium perfringens* CsurP813 gave 34%, *Yersinia enterolitica* CsurP840 gave 33% while the strain of *Enterobacter aerogenes* CsurP210 gave 22% of mortality. The other strains giving less than 20% of mortality were considered as non-effective. It is the case for the 3 strains of *Bacillus cereus*, CsurP754, CsurP2351 and CsurP329 giving 11%, 3% and 2% respectively and the strain *Aeromonas caviae* CsurP636 which gives 12%.

Mixing the pellet and the supernatant at 6 mg/L, did change considerably the rate of larvae mortality for all the strains **(Table 01).**

Table 01: Insecticidal activity exhibition of the secondary metabolites on 3rd and early 4th instar *Aedes albopictus* larvae at 72 hours post-administration

Strain	Reference	Pellet (2-6mg/L)	Supernatant 2mg/L	Supernatant 6mg/L	Supernatant + pellet (6mg/L each)	Control	Note
Serratia marcescens	CsurP400	0%	66%	78%	86.66%	0%	Potential Insecticidal activity
Serratia marcescens	CsurP587	0%	44%	75%	73.33%	0%	Potential Insecticidal activity
Serratia marcescens	CsurP833	0%	70%	72%	100%	0%	Potential Insecticidal activity
Microbacterium neimengenese	CsurP2323	0%	33%	48%	48%	0%	Potential Insecticidal activity
Lysinibacillus sphaericus	CsurP827	0%	25%	47%	48%	0%	Potential Insecticidal activity
Bacillus thuringiensis	CsurP820	0%	28%	41%	44%	0%	Potential Insecticidal activity
Clostridium perfringens	CsurP813	0%	2%	34%	44%	0%	Potential Insecticidal activity
Yersinia enterolitica	CsurP840	0%	5%	33%	32%	0%	Potential Insecticidal activity
Enterobacter aerogenes	CsurP210	0%	20%	22%	36%	0%	Potential Insecticidal activity
Bacillus cereus	CsurP754	0%	4%	11%	12%	0%	non-effective
Bacillus cereus	CsurP2351	0%	0%	3%	4%	0%	non-effective
Bacillus cereus	CsurP329	0%	0%	2%	0%	0%	non-effective
Aeromonas caviae	CsurP636	0%	9%	12%	16%	0%	non-effective
Bti	AM65-52	0%	15%	33%	34%	0%	Insecticidal activity

We performed the comparison of the difference by pairs including the reference strain (**Table S7**) and found that the difference was significant (critical value of $khi^2 = 22{,}362$, p-value ≤ 0.0001) between the insecticidal activity exhibition rates of the supernatant fractions from the different strains at 72h with 6 mg/L. A difference was considered statistically significant when p-values is$\leq$0.05.

The efficacy of the strains was compared to that of the *Bti* positive control (**Table 2**). Using the Dunn test, the strains were classified into six groups (from A to F) according to their efficiencies (Paris, France, https://www.xlstat.com). The most effective group on mosquitoes larvae was group E and was composed of the 3 strains of *S. marcescens* "mean ranks = [936 to 992]" and the group A which was the least effective including the non-effective strains in addition to the strain CsurP210 which records an insecticidal activity lower than the *Bti* AM65-52 (**Table S7**)"mean ranks = [460 to 600]".

Several strains proved to be more active than the reference strain of *Bti*. The difference was positively significant with strains killing more than 47% (group D, E and F), while it was negatively significant with strains killing less than 12% (Group B and A). The difference was not significant with strains exhibiting insecticidal activity between 22% and 47% which form the group C with the *Bti* AM65-52.

Table 02: Comparison of insecticidal rates between the clinical strains and *Bti* AM65-52. Extracted from Dunn pairwise test's results (**Table S7).**

Strains	Code	Mortality rate	STDEV	Mean rank	P-value	Result
Bti	**AM65-52**	**33%**	**Ref**	**Ref**	**Ref**	**Ref**
Serratia marcescens	CsurP587	75%	0,435	-294	≤ 0.0001	Pos. S
Serratia marcescens	CsurP833	70%	0,461	-259	≤ 0.0001	Pos. S
Serratia marcescens	CsurP400	78%	0,416	-315	≤ 0.0001	Pos. S
Lysinibacillus sphaericus	CsurP827	47%	0,502	-98	0.040	Pos. S
Aeromonas caviae	CsurP636	12%	0,327	147	0.002	Neg. S
Bacillus cereus	CsurP754	11%	0,314	154	0.001	Neg. S
Bacillus cereus	CsurP329	2%	0,141	217	≤ 0.0001	Neg. S
Bacillus cereus	CsurP2351	3%	0,171	210	≤ 0.0001	Neg. S
Enterobacter aerogenes	CsurP210	22%	0,416	77	0.106	NS
Clostridium perfringens	CsurP813	34%	0,476	-7	0.883	NS
Yersinia enterolitica	CsurP840	33%	0,473	0	1	NS
Microbacterium neimengenese	CsurP2323	48%	0,502	-105	0.028	Pos. S
Bacillus thuringiensis	CsurP820	41%	0,494	-56	0.24	NS

Pos. S: Positively significant Neg. S: Negatively significant NS: Non-significant STDEV: Standard deviation

b) Investigation of Genes Encoding Toxins

All the known genes were carried by the reference strain *Bti* AM65-52. The genes *cry11*, *cry 4A-4B* and the accessory genes *p20* and *p19* were detected in the *B. thuringiensis* CSUR820, while in the strain *L. sphaericus* CsurP827, also 4 genes were detected (*cry 11*, *cyt1c* and the accessory genes *p20* and *p19*).

c) physicochemical properties of the active compounds of *Serratia marcescens*

Serratia marcescens P400 was chosen for further studies as the most effective strain in order to characterize the active compound. We found that, the active compound was still effective after heating at 100C°, extreme pH variations or proteinase K treatment which indicated that the active compound was certainly not a protein.

The active compound was stable to a wide range of pH values (2 to 10). We found also that its molecular weight was lower than 3 kDa. Moreover, the precipitation of proteins using TCA treatment or the digestion using proteinase K enhanced the activity. More details are shown is the table S3.

Table S3: Detailed results of the different treatments aiming to determine the nature and physicochemical properties of the active compounds.

Common solvent extractions failed to extract the active compounds in the organic layer. The activity remained in the aqueous phase after the treatment. Protein precipitation with methanol or acetonitrile showed retention of the activity in the protein pellet (**Table S4**).

Table S4: Detailed results of the different treatments performed in order to extract the active compounds using solvents.

For HPLC analysis, we treated the initial sample with TCA to eliminate proteins. During the reverse phase C18 chromatographic separation, the activity was localised along the less hydrophobic part of the elution (5 first minutes). 6 peak fractions were recovered and tested on larvae (**Figure S1**) (**Table S5**). The first fraction was the most active with 28% of mortality then the 6th fraction with 8% of larva mortality. No mortality was observed for the other peaks and the following eluting much apolar compounds.

Table S5: Detailed results of the different collected peaks from the HPLC C18 separation of the TCA treated fraction against *Aedes albopictus* L3-L4 larva stage.

Figure S1: Active peaks after HPLCC18 column separation. The different peaks were here monitored at 270nm.

The subsequent analysis of Peak 1 and Peak 6 using the HILIC column revealed 2 major peaks (Peak 2-3 min and 7-8 min) (**Figure S2**). The first HILIC peak exhibited the insecticidal activity against larvae. These results indicate here a compound with poor retention along a hydrophilic column.

Table S6: Detailed results of the different collected peaks from the HPLC HILIC separation of the peak 1 from the C18 column against *Aedes albopictus* L3-L4 larva stage.

Figure S2: Active peaks collected from the HPLC HILIC separation of the peak 1 from the C18 column**.** The different peaks were monitored at Max-Plot 210-400nm.

Mass spectrometry analyses of the HPLC HILIC separation active peaks

We decided to analyse the active peaks to explore their mass content. The first eluting HILIC peak from both Peaks 1 and 6 revealed a compound after C18 reverse phase UHPLC-MS analysis. The most abundant mass peak (326.27 m/z) during positive ionisation was processed using the Elucidate tool set in the UNIFI software. The calculated chemical composition ($C_{22}H_{47}N$, 5 mDa error, +H adduct) matched a fatty amine structure from the Chemspider database and detected one theoretical fragment. A screening of the corresponding aliphatic amine formulae revealed its presence in both C18 collected peaks.

The properties of the amine function match the previously described properties (low molecular mass, hydrophilic, low retention on C18 or HILIC column). Indeed, aliphatic amines are defined by high pKa values (above 9 in general) indicating their hydrophilic properties. Nevertheless, aliphatic chains are hydrophobic because of their carbon chains and this should result in chromatographic retention on reverse phase columns. Pure commercial C22 aliphatic amines were evaluated: primary and secondary amines were not soluble in water; a tertiary amine was dissolved in water (below 100µg/mL) and showed a similar retention time as peak 6 in the HPLC C18 column. The same pure compound was injected in the HILIC column and poor retention time similar to active peak at 2 minutes was observed.

Using UHPLC-MS, the tested amine structure did not show the same exact retention time as the corresponding extracted ion mass from the samples. Nevertheless, using LC analysis, we could confirm that fatty amines had similar retention characteristics as the active peaks.

Insecticidal activity assays of a tertiary amine compared to Ivermectin

Table S8: LC50 of the tertiary amine Dibutyltetradecylamine against the mosquito larva *Aedes albopictus* L3-L4 stages after 24 hours of exposition.

Figure S3: The mortality curve of the larvae according to the tested concentrations of Dibutyltetradecylamine against the mosquito larva *Aedes albopictus* L3-L4 stages after 24 hours of exposition.

Both the ivermectin and Dibutyltetradecylamine had a significant effect on mosquito larvae. We compared the insecticidal activity of the Dibutyltetradecylamine to that given by the ivermectin. We tested the tertiary amine Dibutyltetradecylamine against the mosquito larva (*Aedes albopictus* L3-early L4 larva stage. It exhibited high insecticidal activity (LC50 =55.895 mg/L) (**Table S8**) (**Figure S3**). The ivermectin exhibited higher insecticidal activity (LC50 =332.214 µg/L) (**Table S9**) (**Figure S4**). We found that Ivermectin is 170 times more active than the Dibutyltetradecylamine 24 hours post exposure (when comparing the LC50). The ivermectin insecticidal effect reaches its maximum (100% of larva mortality) with 0.6 mg/L and 2 mg/L after respectively 24h and 10h post-exposure (**Figure S5**), while the Dibutyltetradecylamine activity did not change with time with 100% of larvae mortality with only 130 mg/L after 10 or 24 hours (**Figure S6**). Statistical results are displayed in table **Table S10**.

Table S9: LC50 of the ivermectin against the mosquito larva *Aedes albopictus* L3-L4 stages after 24 hours of exposition.

Figure S4: The mortality curve of the larvae according to the tested concentrations of ivermectin against the mosquito larva *Aedes albopictus* L3-L4 stages after 24 hours of exposition.

Figure S5: This grouped histogram shows the evolution of the mosquito larva *Aedes albopictus* L3-L4 stages mortality due to ivermectin according to time and doses administered.

Figure S6: This grouped histogram shows the evolution of the mosquito larva *Aedes albopictus* L3-L4 stages mortality due to Dibutyltetradecylamine according to time and doses administered.

Table S10: Comparison between ivermectin and Dibutyltetradecylamine insecticidal activity against mosquito larva *Aedes albopictus* L3-L4 stages after 10 and 24 hours of exposition.

Toxicity tests

At 24h of exposition to high concentrations, we found that Dibutyltetradecylamine was toxic on *Aedes albopictus* cell line, but it was also toxic to other different organism cell lines including humans (XTC, MRC-5, S2 and L929).

Table S11: Detailed results on the assessment of the toxicity of Dibutyltetradecylamine on different eukaryotic cell lines.

2. Discussion

Our first hypothesis was that bacteria could exhibit insecticidal effects through their secretome or within their cell components. Numerous studies previously reported high resistances to *Bti* toxins after only a few generations of selection and also suggested that different resistance mechanisms exist [7]. Samely, for *L. sphaericus*, various levels of resistance in laboratory and field populations ensued from different countries [8]. Two selected clinical strains carried the accessory genes: *B. thuringiensis* CsurP820 carried 2 *cry* genes (*cry11* and *cry4A-4B*) while *L. sphaericus* CsurP827 carried *cry 11* and *cyt1c*. Their effect was higher than the control strain *Bti* AM65-52 which carried more Dipteran specific genes. Which suggests that some toxins may be more effective than others which was already reported [6], or other unknown toxins may be encoded by these strains which was also already reported [21]. For this, the sequencing and study of their plasmids is already planned.

Concerning this study, we tested the supernatant and pellet fractions separately or mixed together of 13 bacteria strains isolated from clinical samples.

No insecticidal effect was found using pellet fractions for the entire set of tested strains including the *Bti* control (strain AM65-52), while some activity was found in the supernatant fractions. Nine out of thirteen (69.23%) of the strains exhibited insecticidal activity on the *A. albopictus* larvae (**Table 01**). Insecticidal activity was found to increase with concentration.

Serratia marcescens P400 was chosen for further studies as the most effective strain in order to characterize the active compound. Moreover, *M. neimengenese* CsurP2323, *Yersinia enterolitica* CsurP840 and *C. perfringens* CsurP813 exhibited good insecticidal activity compared to *Bti* AM65-52. Our results confirm that they form good candidates for the research in the field of vectors biological control. More studies will be needed for the identification of the secreted active compounds and the assessment of their activity on mosquitoes and other vectors.

S. marcescens is a well-known entomopathogenic and broad host range bacterium. When alive, it infects opportunistically both invertebrates and vertebrates but mostly infects or kills insects [22][23][24][25]. Different pigmented strains of *S. marcescens* promote different biological effects, especially those due to the red pigment prodigiosin such as antialgal, antibacterial, antifungal, antimalarial, antiprotozoal, anticancer, immunosuppressive, antiproliferative, UV-protective activities and also insecticidal activity [26]. A metalloprotease purified from locust pathogen *S. marcescens* HR-3 exhibited insecticidal activity against locusts (Tao et al., 2006). Here, we wanted to test not alive bacteria but their secondary metabolites in order to see if they could be active compounds against mosquitoes. Interestingly, the tested strains exhibited insecticidal activity on the *A. albopictus* larvae. All tested strains of *S. marcescens* showed insecticidal activities: the strain CsurP400 gives 78% of mortality, CsurP587 gives 75% and CsurP833 gives 70%. Such high activity given by the three strains using only 6mg / L of protein content, revealed for the first time that unpigmented *S. marcescens* strains appeared to contain active compounds which may be isolated. These compounds could therefore be considered in the field of biological control of infectious diseases transmitted by mosquitoes. HPLC separation followed by mass-spectrometry analyses of the active fraction revealed a molecular formula of a fatty amine. Further experiments using a commercial standard confirmed the chemical properties of the collected active compound.

Dibutyltetradecylamine, an aliphatic tertiary amine, was effective against *Ae. albopictus*. A few studies were previously performed on the activity of aliphatic amines against aquatic stages of mosquito

[28,29]. These studies showed their highest effectiveness against several important species and reported the absence of cross-tolerance between them and previously used insecticides with known resistance. Dibutyltetradecylamine was also known to be used for microbiocidal control in the processing of poultry [30]. We compared the efficiency of Dibutyltetradecylamine compared to Ivermectin, a highly effective and safe insecticide [31,32]. Several experiments were previously performed to assess the ivermectin's effects on important mosquito species, especially on *Ae. aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus* [33,34] and its potential to be used for mosquito borne diseases control such as malaria and dengue was previously suggested [35,36]. A few studies were performed on the effect of ivermectin on *Ae. albopictus* larva [37] and our present study reports its LC50 which is very low. An optimized protocol should be used to control aquatic stages of mosquitoes and avoid using mass drug administration to humans [38]. The study of the penetrating pathways of this amine, if associated with its hydrophilic character will help to define probable associations with other chemical or biological molecules. This will optimize its efficiency while reducing the amounts used in control to avoid toxic quantities. Another major interest of this amine in control is its cost because, amines are produced easily, quickly and cheaper (S810657-1G, Sigma Aldrich, St. Louis, USA).

In conclusion, this is the first study exploring clinical strains for insecticidal activity against mosquito larvae. We here revealed interesting bacteria exhibiting insecticidal activity. 13 strains isolated from clinical samples were tested against *A. albopictus* larva. Surprisingly, 69% of them secreted in the culture medium active compounds that were lethal to the mosquito larvae. This study opens new horizons for the search for new biological insecticidal molecules for the great replacement of chemical products. In this quest for the identification of the active compounds, the characterization of the active compound from *S. marcescens* revealed fatty amines to promote interesting insecticidal activity. Our findings focused on a tertiary fatty amine with a 22 carbon-backbone to be responsible for the death of larvae. Further experiments on this chemical family should be performed in the future, especially on the various structures of such fatty amines. NMR characterisation will be the next technique to be used in order to describe the exact structure of such active compounds.

References:

1. Benelli, G. Research in mosquito control: current challenges for a brighter future. *Parasitol. Res.* **2015**, *114*, 2801–2805.
2. Shukla, D.; Wijayapala, S.; Vankar, P.S. Effective mosquito repellent from plant based formulation. *Int. J. Mosq. Res.* **2018**, *5*, 19–24.
3. Moyes, C.L.; Vontas, J.; Martins, A.J.; Ng, L.C.; Koou, S.Y.; Dusfour, I.; Raghavendra, K.; Pinto, J.; Corbel, V.; David, J.-P.; et al. Contemporary status of insecticide resistance in the major Aedes vectors of arboviruses infecting humans. *PLoS Negl. Trop. Dis.* **2017**, *11*, e0005625.
4. Dhanasekaran, D.; Thangaraj, R. Microbial secondary metabolites are an alternative approaches against insect vector to prevent zoonotic diseases. *Asian Pacific J. Trop. Dis.* **2014**, *4*, 253–261.
5. Lacey, L.A. Bacillus thuringiensis serovariety israelensis and Bacillus sphaericus for mosquito control. *J. Am. Mosq. Control Assoc.* **2007**, *23*, 93–109.
6. Zhang, Q.; Hua, G.; Adang, M.J. Effects and mechanisms of Bacillus thuringiensis crystal toxins for mosquito larvae. *Insect Sci.* **2017**, *24*, 714–729.
7. Paris, M.; Tetreau, G.; Laurent, F.; Lelu, M.; Despres, L.; David, J.-P. Persistence of Bacillus thuringiensis israelensis (Bti) in the environment induces resistance to multiple Bti toxins in mosquitoes. *Pest Manag. Sci.* **2011**, *67*, 122–128.
8. Su, T. *Resistance and Its Management to Microbial and Insect Growth Regulator Larvicides in Mosquitoes*; 2016;
9. Bravo, A.; Soberón, M. How to cope with insect resistance to Bt toxins? *Trends Biotechnol.* **2008**, *26*, 573–579.
10. Georghiou, G.P.; Wirth, M.C. Influence of Exposure to Single versus Multiple Toxins of Bacillus thuringiensis subsp. israelensis on Development of Resistance in the Mosquito Culex quinquefasciatus (Diptera: Culicidae). *Appl. Environ. Microbiol.* **1997**, *63*, 1095–101.
11. Cadavid-Restrepo, G.; Sahaza, J.; Orduz, S. Treatment of an Aedes aegypti colony with the Cry11Aa toxin for 54 generations results in the development of resistance. *Mem. Inst. Oswaldo Cruz* **2012**, *107*, 74–9.
12. Stalinski, R.; Tetreau, G.; Gaude, T.; Després, L. Pre-selecting resistance against individual Bti Cry toxins facilitates the development of resistance to the Bti toxins cocktail. *J. Invertebr. Pathol.* **2014**, *119*, 50–53.
13. Paris, M.; Melodelima, C.; Coissac, E.; Tetreau, G.; Reynaud, S.; David, J.-P.; Despres, L. Transcription profiling of resistance to Bti toxins in the mosquito Aedes aegypti using next-generation sequencing. *J. Invertebr. Pathol.* **2012**, *109*, 201–208.
14. Toledo Marrelli, M.; Barretto Bruno WILKE, A.; Toledo MARRELLI, M. Genetic Control of Mosquitoes: population suppression strategies. *Rev. do Inst. Med. Trop. São Paulo.* **2012**, *54*, 287–292.
15. Raoult, D.; Abat, C. Developing new insecticides to prevent chaos: the real future threat. *Lancet Infect. Dis.* **2017**, *17*, 804–805.
16. Lagier, J.-C.; Khelaifia, S.; Alou, M.T.; Ndongo, S.; Dione, N.; Hugon, P.; Caputo, A.; Cadoret, F.; Traore, S.I.; Seck, E.H.; et al. Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat. Microbiol.* **2016**, *1*, 16203.
17. La Scola, B.; Raoult, D. Direct identification of bacteria in positive blood culture bottles by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry. *PLoS One* **2009**, *4*, e8041.
18. WHOCDCPE & WHOPES *GUIDELINES FOR LABORATORY AND FIELD TESTING OF MOSQUITO LARVICIDES*; 2005;
19. Dahmana, H.; Granjon, L.; Diagne, C.; Davoust, B.; Fenollar, F.; Mediannikov, O. Rodents as Hosts of Pathogens and Related Zoonotic Disease Risk. *Pathogens* **2020**, *9*, 202.
20. D. J. Finney. Probit Analysis. 2nd ed. *J. Am. Pharm. Assoc. (Scientific ed.)* **1952**, *41*, 627.
21. Nair, K.; Al-Thani, R.; Al-Thani, D.; Al-Yafei, F.; Ahmed, T.; Jaoua, S. Diversity of Bacillus thuringiensis Strains From Qatar as Shown by Crystal Morphology, δ-Endotoxins and Cry Gene Content. *Front. Microbiol.* **2018**, *9*, 708.
22. Mohan, M.; Selvakumar, G.; Sushil, S.N.; Bhatt, J.C.; Gupta, H.S. Entomopathogenicity of endophytic Serratia marcescens strain SRM against larvae of Helicoverpa armigera (Noctuidae: Lepidoptera). *World J. Microbiol. Biotechnol.* **2011**, *27*, 2545–2551.
23. Raymann, K.; Coon, K.L.; Shaffer, Z.; Salisbury, S.; Moran, N.A. Pathogenicity of Serratia marcescens Strains in Honey Bees. *MBio* **2018**, *9*, e01649-18.
24. O'Callaghan, M.; Garnham, M.L.; Nelson, T.L.; Baird, D.; Jackson, T.A. The pathogenicity of Serratia strains to Lucilia sericata (Diptera: Calliphoridae). *J. Invertebr. Pathol.* **1996**, *68*, 22–27.
25. Pietri, J.E.; Liang, D. Virulence of entomopathogenic bacteria in the bed bug, Cimex lectularius. *J.*

Invertebr. Pathol. **2018**, *151*, 1–6.
26. Kurbanoglu, E.B.; Ozdal, M.; Ozdal, O.G.; Algur, O.F. Enhanced production of prodigiosin by Serratia marcescens MO-1 using ram horn peptone. *Braz. J. Microbiol.* **2015**, *46*, 631–7.
27. Tao, K.; Long, Z.; Liu, K.; Tao, Y.; Liu, S. Purification and Properties of a Novel Insecticidal Protein from the Locust Pathogen Serratia marcescens HR-3. *Curr. Microbiol.* **2006**, *52*, 45–49.
28. Georghiou, G.P.; Calman, J.R.; Mulla, M.S. Aliphatic Amines Against Insecticide-Susceptible and Resistant Strains of Culex pipiens quinquefasciatus and Anopheles albimanus. *J. Econ. Entomol.* **1969**, *62*, 171–173.
29. Mulla, M.S.; Darwazeh, H.A.; Gillies, P.A. Evaluation of Aliphatic Amines Against Larvae and Pupae of Mosquitoes. *J. Econ. Entomol.* **1970**, *63*, 1472–1475.
30. Eric W. Liimatta; Laura B. Gage Microbiocidal Control in the Processing of Poultry 2018.
31. Navarro, M.; Camprubí, D.; Requena-Méndez, A.; Buonfrate, D.; Giorli, G.; Kamgno, J.; Boussinesq, M.; Mu ~ Noz, J.; Krolewiecki, A. Safety of high-dose ivermectin: a systematic review and meta-analysis. *J Antimicrob Chemother* **2020**, *75*, 827–834.
32. Mekuriaw, W.; Balkew, M.; Messenger, L.A.; Yewhalaw, D.; Woyessa, A.; Massebo, F. The effect of ivermectin® on fertility, fecundity and mortality of Anopheles arabiensis fed on treated men in Ethiopia. *Malar. J.* **2019**, *18*, 357.
33. Deus, K.M.; Saavedra-Rodriguez, K.; Butters, M.P.; Black, W.C.; Foy, B.D. The Effect of Ivermectin in Seven Strains of Aedes aegypti (Diptera: Culicidae) Including a Genetically Diverse Laboratory Strain and Three Permethrin Resistant Strains. *J. Med. Entomol.* **2012**, *49*, 356–363.
34. Derua, Y.A.; Malongo, B.B.; Simonsen, P.E. Effect of ivermectin on the larvae of Anopheles gambiae and Culex quinquefasciatus. *Parasites and Vectors* **2016**, *9*, 1–6.
35. Chaccour, C.J.; Kobylinski, K.C.; Bassat, Q.; Bousema, T.; Drakeley, C.; Alonso, P.; Foy, B.D. Ivermectin to reduce malaria transmission: A research agenda for a promising new tool for elimination. *Malar. J.* **2013**, *12*, 1–8.
36. Xu, T.-L.; Han, Y.; Liu, W.; Pang, X.-Y.; Zheng, B.; Zhang, Y.; Zhou, X.-N. Antivirus effectiveness of ivermectin on dengue virus type 2 in Aedes albopictus. *PLoS Negl. Trop. Dis.* **2018**, *12*, e0006934.
37. Molinari, G.; Soloneski, S.; Reigosa, M.A.; Larramendy, M.L. Genotoxic and cytotoxic in vitro evaluation of ivermectin and its formulation ivomec® on Aedes albopictus larvae (CCL-126TM) cells. *Toxicol. Environ. Chem.* **2010**, *92*, 1577–1593.
38. Kobylinski, K.C.; Sylla, M.; Chapman, P.L.; Sarr, M.D.; Foy, B.D. Ivermectin mass drug administration to humans disrupts malaria parasite transmission in Senegalese villages. *Am. J. Trop. Med. Hyg.* **2011**, *85*, 3–5.

Supplementary material

Table S1: Strains used in this study isolated from clinical samples

Strain name	CSUR code	Strain code	Origin	Sample specimens
Serratia marcescens	CSURP587	12206589H	Human	Blood
Serratia marcescens	CSURP833	/	Human	Feces
Serratia marcescens	CSURP400	DK333	Human	Urine
Lysinibacillus sphaericus	CSURP827	/	Human	Feces
Aeromonas caviae	CSURP636	/	Human	Feces
Bacillus cereus	CSURP754	/	Human	Feces
Bacillus cereus	CSURP329	/	Human	Feces
Bacillus cereus	CSURP2351	/	Human	Skin (soap)
Enterobacter aerogenes	CSURP210	NCDC 819-56	Human	ND
Clostridium perfringens	CSURP813	mammouth8	Animal	Mammoth feces
Yersinia enterolitica	CSURP840	/	Human	Feces
Microbacterium neimengenese	CSURP2323	tim4	Environment	Contact lens
Bacillus thuringiensis	CSURP820	/	Human	Feces

Table S2: List of primers used in this study to explore the presence of genes encoding endotoxin, accessory proteins and *Cyt*.

Gene	Primers	Sequences	Amplicon size	References
cry4a/4b	dip2a dip1b	GGTGCTTCCTATTCTTTGGC ATGGCTTGTTTCGCTACATC	1290 bp	Carozzi et al., 1991
cry10	cry10-1 cry10-2	ATATGAAATATTCAATGCTC ATAAATTCAAGTGCCAAGTA	614 bp	Porcar et al., 1999
cry11	cry11-1 cry11-2	TTAGAAGATACGCCAGATCAAGC CATTTGTACTTGAAGTTGTAATCCC	304 bp	Bravo et al., 1998
cyt1a	cyt1A1 cyt1A2	GTTGTAAGCTTATGGAAAAT TTAGAAGCTTCCATTAATA	701 bp	Zghal et al., 2008
cyt1c	cyt1C1 cyt1C2	CAAAATCTACGGGAGCAAGG GGAAGGATCCCTTTGACTTTT	1320 bp	(Nair et al., 2018)
cyt2a	cyt2A1 cyt2A2	AATACATTTCAAGGAGCTA TTTCATTTTAACTTCATATC	471 bp	Guerchicoff et al., 1997
p19	p19-1 p19-2	GCAGGAGGAACATCACCATT GGATTTGCTGAGCAGGTCAT	291 bp	(Nair et al., 2018)
p20	p20-1 p20-2	TGACGAGGAAACAGAGTATACGA TGAAAGGTTAAACGTTCCGATT	704 bp	(Nair et al., 2018)

Table S3: Detailed results of the different treatments aiming to determine the nature and physicochemical properties of the active compounds.

Treatment	Mortality at 72 hours		Mean of percentage of mortality (%)
	1st replicate	2nd replicate	
Positive control (500µL of untreated fraction)	8/25	9/25	34
Heated at 100C° for 60 minutes.	5/25	7/25	24
Protein digestion using proteinase K	12/25	13/25	50
Negative control of Proteinase K	0/25	0/25	0
Protein precipitation with Trichloroacetic acid (TCA) the pH of the supernatant fraction is adjusted to 7 using NaOH 20%	12/25	14/25	52

Negative control of Trichloroacetic acid (TCA)	0/25	0/25	0
pH 10 for 2 hours	6/25	5/25	22
pH 2 for 2 hours	7/25	9/25	32
PBS 1X	0/25	0/25	0

Table S4: Detailed results of the different treatments performed in order to extract the active compounds using solvents.

Extraction treatment	Mortality at 72 hours		Mean of percentage of mortality (%)
	1st replicate	2nd replicate	
Acetonitrile 70% (supernatant after protein precipitation)	0/25	0/25	0
Acetonitrile 70% pellet	7/25	8/25	30
Methanol 70%	1/25	0/25	2
Methanol pellet	5/25	6/25	22
Ethyl acetate 70%	0/25	0/25	0
Ethyl acetate pellet	3/25	5/50	16
Di-ethyl ether 70%	0/25	0/25	0
Di-ethyl ether pellet	2/25	4/25	12
Hexane 70%	0/25	0/25	0
Hexane pellet	3/25	3/25	12
Chloroform 70%	0/25	0/25	0
Chloroform pellet	2/25	1/25	6
Cytospin filtration > 3 kDa	2/25	3/25	10
Cytospin filtration < 3 kDa	5/25	6/25	22
Positive control (500µL of untreated fraction)	9/25	8/25	34
PBS 1X	0/25	0/25	0

Table S5: Detailed results of the different collected peaks from the HPLC C18 separation of the TCA treated fraction against *Aedes albopictus* L3-L4 larva stage.

Fraction	Mortality at 72 hours		Mean of percentage of mortality (%)
	1st replicate	2nd replicate	
Control fraction	10/25	8/25	36
C18-Peak-1	8/25	6/25	28
C18-Peak-1 negative control	0/25	0/25	0
C18-Peak-2	0/25	0/25	0
C18-Peak-2 negative control	0/25	0/25	0
C18-Peak-3	0/25	0/25	0
C18-Peak-3 negative control	0/25	0/25	0
C18-Peak-4	0/25	0/25	0
C18-Peak-4 negative control	0/25	0/25	0
C18-Peak-5	0/25	0/25	0
C18-Peak-5 negative control	0/25	0/25	0
C18-Peak-6	1/25	3/25	8
C18-Peak-6 negative control	0/25	0/25	0
Negative control of the hydrophobic part of the chromatogram [5-10 min]	0/25	0/25	0

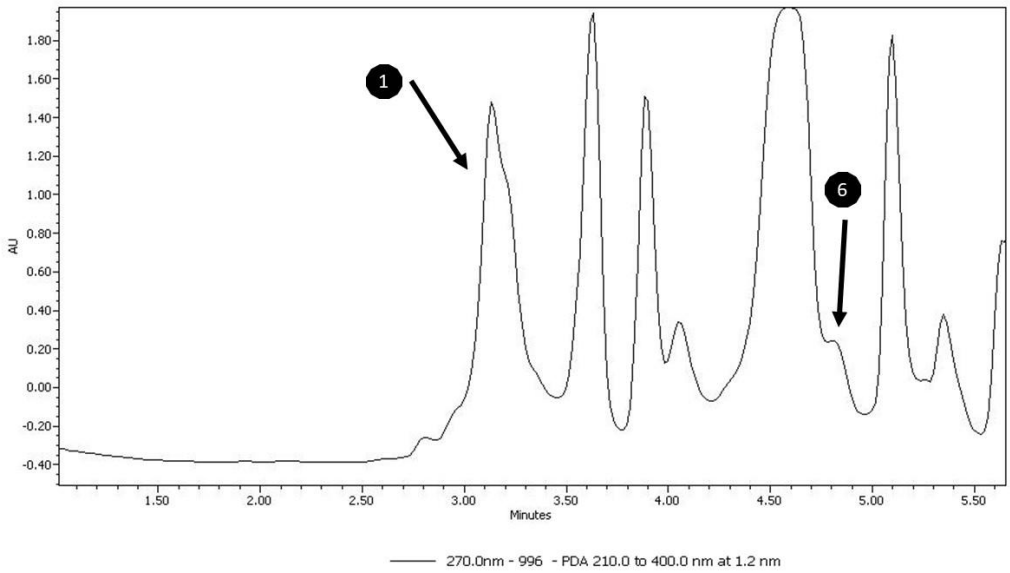


Figure S1: Active peaks after HPLC C18 column separation. The different peaks were monitored at 270nm.

Table S6: Detailed results of the different collected peaks from the HPLC HILIC separation of the peak 1 from the C18 column against *Aedes albopictus* L3-L4 larva stage.

Fraction	Mortality at 72 hours		Mean of percentage of mortality (%)
	1st replicate	2nd replicate	
Control fraction	8/25	11/25	38
HILIC-Peak-2_3 min	25/25	25/25	100
HILIC-Peak-2_3 min negative control	0/25	0/25	0
HILIC-Peak-7_8 min	25/25	25/25	100
Negative control of the hydrophobic period 0-2 min]	0/25	0/25	0
Negative control of the hydrophobic period 3-7 min]	0/25	0/25	0
Negative control of the hydrophobic period 8-11 min]	0/25	0/25	0
PBS 1X negative control	0/25	0/25	0

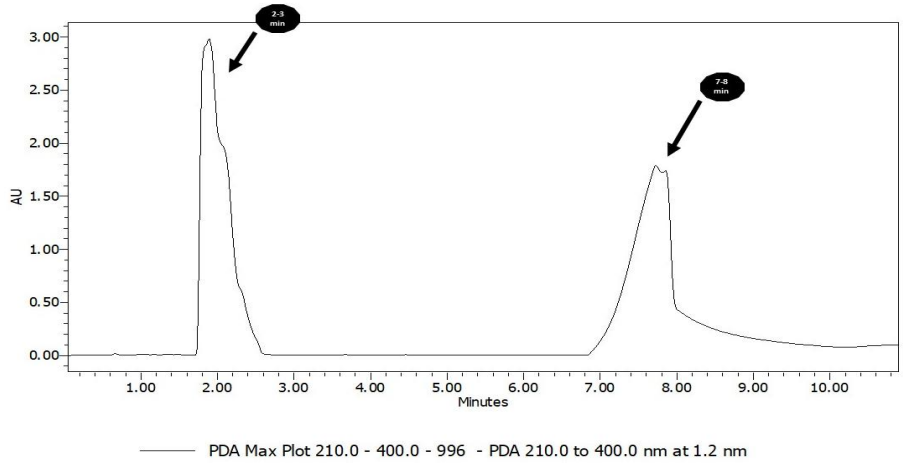


Figure S2: Active peaks collected from the HPLC HILIC separation of the peak 1 from the C18 column**.** The different peaks were monitored at Max-Plot 210-400nm.

561 **Table S8:** LC50 of the tertiary amine Dibutyltetradecylamine against the mosquito larva *Aedes albopictus* L3-L4
562 stages after 24 hours of exposition.

LD/LC	LD/LC	95%Fiducial CI	
(%)	**10^3 ng/mL**	**Lower**	**Upper**
LD46	53.246	48.692	58.226
LD47	53.898	49.288	58.939
LD48	54.556	49.890	59.659
LD49	55.222	50.499	60.387
LD50	**55.895**	**51.114**	**61.123**
LD51	56.576	51.738	61.868
LD52	57.267	52.369	62.623
LD53	57.966	53.008	63.388
LD54	58.676	53.657	64.163
LD55	59.396	54.316	64.951
LD56	60.127	54.984	65.750
LD57	60.870	55.663	66.563

563

564 **Table S9:** LC50 of the ivermectin against the mosquito larva *Aedes albopictus* L3-L4 stages after 24 hours of
565 exposition.

LD/LC	LD/LC	95%Fiducial CI	
(%)	**ng/mL**	**Lower**	**Upper**
LD46	294.766	219.042	396.670
LD47	303.733	225.705	408.736
LD48	312.954	232.557	421.145
LD49	322444	239.609	433.915
LD50	**332.214**	**246.869**	**447.064**
LD51	342.281	254.350	460.611
LD52	352.660	262.062	474.578
LD53	363.367	270.019	488.986
LD54	374.420	278.232	503.860
LD55	385.839	286.718	519.227
LD56	397.644	295.490	535.113
LD57	409.857	304.566	551.549

566

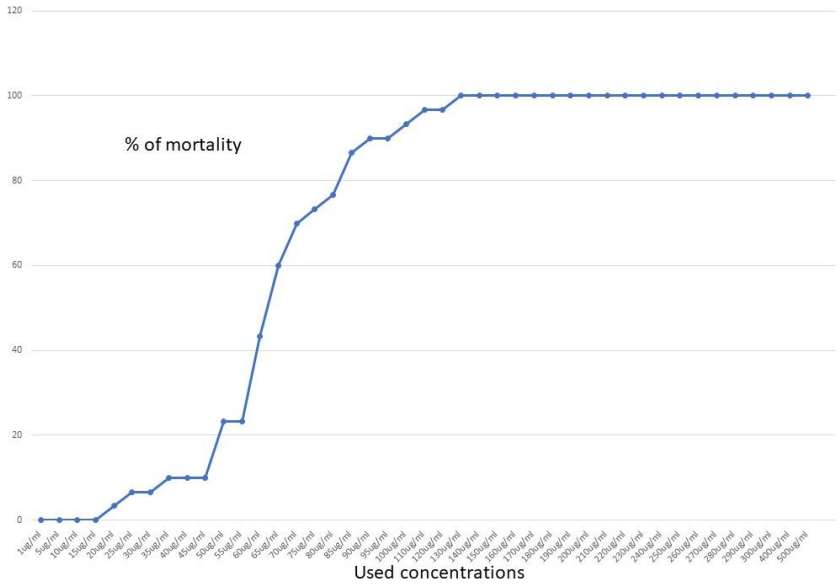


Figure S3: The mortality curve of the larvae according to the tested concentrations of Dibutyltetradecylamine against the mosquito larva *Aedes albopictus* L3-L4 stages after 24 hours of exposition.

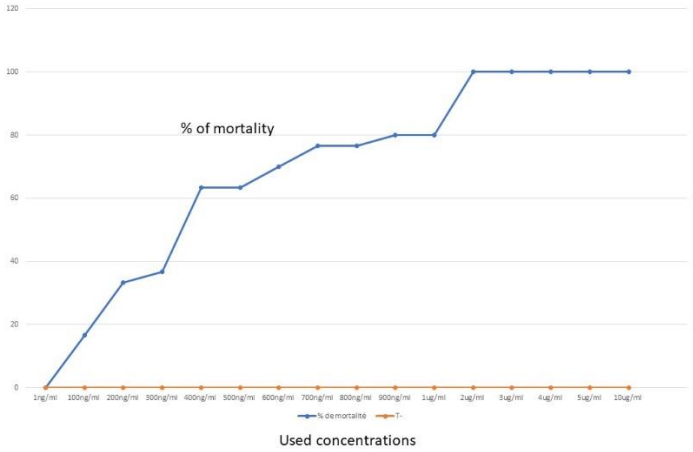


Figure S4: The mortality curve of the larvae according to the tested concentrations of ivermectin against the mosquito larva *Aedes albopictus* L3-L4 stages after 24 hours of exposition.

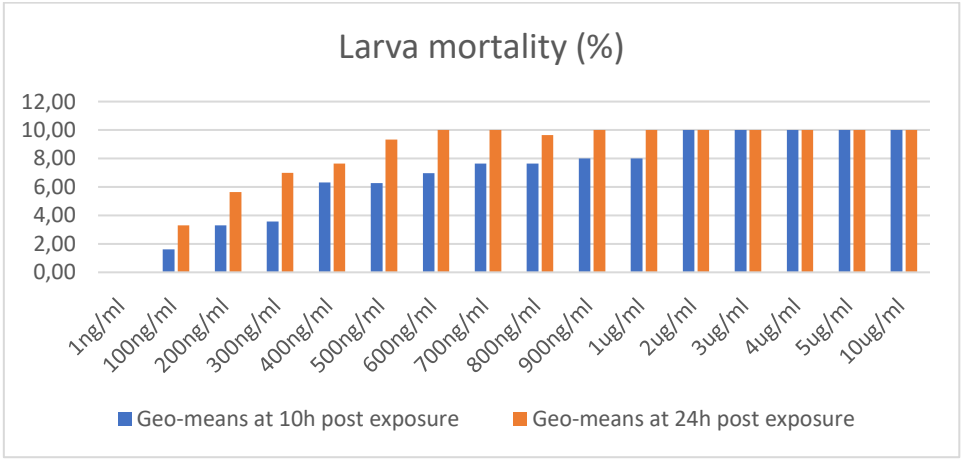


Figure S5: This grouped histogram shows the evolution of the mosquito larva *Aedes albopictus* L3-L4 stages mortality due to ivermectin according to time and doses administered.

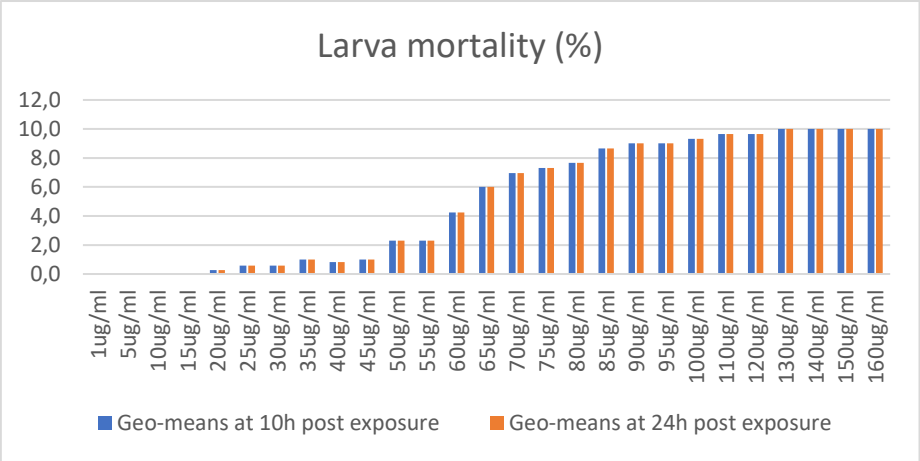


Figure S6: This grouped histogram shows the evolution of the mosquito larva *Aedes albopictus* L3-L4 stages mortality due to Dibutyltetradecylamine according to time and doses administered.

Table S10: Comparison between ivermectin and Dibutyltetradecylamine insecticidal activity against mosquito larva *Aedes albopictus* L3-L4 stages after 10 and 24 hours of exposition "linear regression".

Dibutyltetradecylamine			Ivermectin		
Concentration (10^3 ng/ml)	10h	24h	Concentration (ng/ml)	10h	24h
1	0.0	0.0	1	0.00	0.00
5	0.0	0.0	100	1.62	3.31
10	0.0	0.0	200	3.31	5.65
20	0.3	0.3	300	3.58	7.00
30	0.6	0.6	400	6.32	7.65
40	0.8	0.8	500	6.27	9.32
50	2.3	2.3	600	6.96	10
60	4.2	4.2	700	7.65	10
70	7.0	7.0	800	7.65	9.66
80	7.7	7.7	900	8	10
85	8.7	8.7	1000	8	10
90	9.0	9.0	2000	10	10
100	9.3	9.3	3000	10	10
110	9.7	9.7	4000	10	10
120	9.7	9.7	5000	10	10
≥130	10.0	10.0	10000	10	10
t	6.96	< 0.0001	t	7.65	-2.83
Pr > |t|	6.96	< 0.0001	Pr > |t|	< 0.0001	0.014
R^2	0.54		R^2	0.93	
F	48.44		F	84.06	
Pr > F	< 0.0001		Pr > F	< 0.0001	

585 **Table S11**: Detailed results on the assessment of the toxicity of Dibutyltetradecylamine on different eukaryotic

586 cell lines.

c	Exposition time	Cell viability (%)				Conclusion
		1mg/mL	100µg/mL	PFA	PBS 1X	
XTC	4h	23	45	28	96	Toxic
	8h	22	25	30	95	
	12h	18	22	32	97	
	24h	16	22	25	95	
MRC-5	4h	38	50	41	92	Toxic
	8h	25	36	34	96	
	12h	18	23	22	89	
	24h	17	20	25	95	
C6/36	4h	53	64	32	95	Toxic
	8h	29	54	26	95	
	12h	16	26	22	97	
	24h	11	12	27	96	
S2	4h	38	60	24	96	Toxic
	8h	21	40	30	92	
	12h	18	36	26	94	
	24h	16	20	24	96	
L929	4h	20	83	23	92	Toxic
	8h	11	41	30	91	
	12h	9	12	26	95	
	24h	10	10	28	90	

587

Publication N°11

Hymenopteran parasitoids of hard ticks in Western Africa and the Russian Far East

Mapenda Gaye[1,2], Nadia Amanzougaghene[1,2], El Hadji Amadou Niang[1,2,3], **Younes Laidoudi**[1,2], Zuzana Sekeyová[4], Maureen Laroche[2,5], Jean-Michel Bérenger[2,5], Didier Raoult[1,2], Mária Kazimírová[6], Florence Fenollar[2,5], Oleg Mediannikov[1,2*]

[1] Aix Marseille Univ, IRD, AP-HM, MEΦI, Marseille, France
[2] IHU-Méditerranée Infection, Marseille, France
[3] Laboratoire d'Ecologie Vectorielle et Parasitaire, Faculté des Sciences et Techniques, Université Cheikh Anta Diop (UCAD) de Dakar, Sénégal
[4] Biomedical Research Center, Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic
[5] Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France [6] Institute of Zoology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 06, Bratislava, Slovak Republic

ARTICLE DE RECHERCHE

Soumis à : Scientific Reports - Nature

Scientific Reports.

Research article

Hymenopteran parasitoids of hard ticks in Western Africa and the Russian Far East

Mapenda Gaye[1,2], Nadia Amanzougaghene[1,2], El Hadji Amadou Niang[1,2,3], Younes Laidoudi[1,2], Zuzana Sekeyová[4], Maureen Laroche[2,5], Jean-Michel Bérenger[2,5], Didier Raoult[1,2], Mária Kazimírová[6], Florence Fenollar[2,5], Oleg Mediannikov[1,2*]

[1] Aix Marseille Univ, IRD, AP-HM, МЕФИ, Marseille, France
[2] IHU-Méditerranée Infection, Marseille, France
[3] Laboratoire d'Ecologie Vectorielle et Parasitaire, Faculté des Sciences et Techniques, Université Cheikh Anta Diop (UCAD) de Dakar, Sénégal
[4] Biomedical Research Center, Institute of Virology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic
[5]Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France [6] Institute of Zoology, Slovak Academy of Sciences, Dúbravská cesta 9, 845 06, Bratislava, Slovak Republic

*Corresponding author:
Dr. Oleg MEDIANNIKOV
Address: MEPHI, IRD, APHM, IHU-Méditerranée Infection,19-21 Boulevard Jean Moulin, 13385 Marseille Cedex 05. Tel: +33 (0)4 13 73 24 01, Fax: +33 (0)4 13 73 24 02, E-mail: olegusss1@gmail.com

Word abstract count: 199

Word text count: 2575

ABSTRACT

Some parasitoids of the genus *Ixodiphagus* (Hymenoptera, Chalcidoidea: Encyrtidae) are well-known natural enemies of ticks and have the ability to control them. In this study, we aimed to investigate the occurrence of parasitoid wasps in adult hard ticks from Western Africa (Côte d'Ivoire and Senegal) and Far Eastern Europe (Russia) using molecular methods. The morphological identification allowed the classification of 785 collected specimens of six species of ticks: *Rhipicephalus* (*Boophilus*) *microplus* (41%), *Ixodes persulcatus* (33%), *Dermacentor silvarum* (11%), *Haemaphysalis concinna* (7%), *Amblyomma variegatum* (5%) and *Haemaphysalis japonica* (3%). The MALDI-TOF mass spectrometry protocol allowed even the identification of ticks stored dried or in 70% ethanol for a long period. Molecular screening of ticks by a new standard PCR system developed in this study revealed the presence of parasitoid wasp DNA in 3% (28/785) of analyzed ticks. Among the positive tick samples, 86% (24/28) were identified as *Ixodiphagus hookeri.* The remaining 14% (4/28) of *I. persulcatus* ticks probably contained DNA from as yet undescribed parasitoid wasp species of the family Braconidae. This is the first report of species belonging to the family Braconidae in hard ticks. Parasitoids could be used as a promising approach for biological control of ticks.

Keywords: Parasitoid wasps, Ticks, Western Africa, Russia

INTRODUCTION

Ticks (Ixodida) are obligate blood-sucking ectoparasites of vertebrates almost all over the world [1]. About 10% of the 900 currently identified tick species are known to transmit different types of pathogens, such as viruses (e.g. tick-borne encephalitis virus, Crimean–Congo hemorrhagic fever virus), prokaryotes (*Rickettsia* spp., *Borrelia* spp., *Anaplasma* spp., *Ehrlichia* spp.) and eukaryotes (*Babesia* spp., *Theileria* spp.) that infect humans, domestic and wild animals [2].

In general, ticks involved in the spread of human pathogens often feed on small mammals and birds during their immature stages, while adult ticks tend to feed on bigger herbivores and carnivores [3]. As a result, because of the diseases they transmit and the enormous economic losses they cause, the epidemiological importance of ticks is increasing dramatically worldwide [4]. Recently, the recognized number of human diseases caused by microorganisms transmitted by ticks has increased [5]. Ticks are currently considered the second-largest group of vectors of human infectious diseases worldwide, after mosquitoes [6].

Three families of ticks are known: Argasidae (soft ticks), Nuttalliellidae and Ixodidae (hard ticks). Soft and hard ticks are well known as vectors of human diseases [7,8]. This stresses the need to develop targeted methods and diversify management programs for the control of these harmful vectors.

For the prevention of diseases transmitted by arthropods, vector control is necessary. Several tick-specific pesticides (acaricides), such as organophosphates, carbamates and pyrethroids are used to control them [9]. However, these products became less effective due to the development of resistance in targeted tick populations [9]. In addition, some of them are highly toxic to humans [9] and [9]many of them were banned. For this reason, biological control methods are increasingly been considered as a viable alternative to the use of pesticides, and as having a very low impact on the environment [10]. Potential biological control agents include entomopathogenic fungi, nematodes and parasitoid wasps, which represent the most promising candidates [10].

Hymenopteran parasitoids, also called parasitoid wasps, belong to the order Hymenoptera containing two suborders (Symphyta and Apocrita), classified in 27 superfamilies (9 in Symphyta and 18 in Apocrita), 132 families and about 8,423 existing genera [11]. The order Hymenoptera is one of the most diverse groups of insects with 153,088 currently existing known species, in addition to 2,429 extinct species [11]. Most of the known parasitoids belong to the group of terebrants (Apocrita), comprising the superfamilies of Chalcidoidea (23 families), Cynipoidea (8 families) and Ichneumonoidea (3 families) [11].

Because of their relatively high host specificity, hymenopteran parasitoids have long been recognized as important biological control agents of pests in agriculture [12]. However, the biology and

ecology of these parasitoids remain largely unknown [12]. In particular, females of parasitoid wasps use their ovipositor, a spawning organ at the end of their abdomen, to lay their eggs on or into their hosts, where hatched larvae will feed-on [13]. The host eventually dies due to parasitism, although there appear to be examples where the host can survive and continue to reproduce [14–16].

Ticks have few natural enemies and are generally parasitized by chalcid wasps belonging to the Encyrtidae family, which have been described in many tick species worldwide [14]. Since the beginning of the 20thcentury, wasps of the genus *Ixodiphagus* (Chalcidoidea: Encyrtidae) are known as the only tick parasitoid. Currently, eight species of the genus *Ixodiphagus* are identified: *Ixodiphagus texanus* (Howard, 1907), *Ixodiphagus hookeri* (Howard, 1908), *Ixodiphagus mysorensis* (Mani, 1941), *Ixodiphagus hirtus* (Nikolskava, 1950), *Ixodiphagus theilerae* (Fielder, 1953), *Ixodiphagus biroi* (Erdos, 1956), *Ixodiphagus sagarensis* (Geevarghese, 1977) and *Ixodiphagus taiaroaensis* (Heath and Cane, 2010) [17,18].

These wasps, in particular *I. hookeri*, have been found in several species of ticks belonging to the genera *Ornithodoros*, *Amblyomma*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, *Ixodes* and *Rhipicephalus* [16,19–22]. In contrast, the efficacy of *Ixodiphagus* wasps in tick control is still debated by researchers, as their biology has not been fully clarified [10]. The largest study to date has demonstrated the ability of these parasitoids to reduce the number of *Amblyomma variegatum* ticks in a Kenyan cattle population [20]. Recently, similar studies have demonstrated the potential role of *I. hookeri* in the circulation of certain bacteria, such as *Arsenophonus nasoniae*, rickettsiae (*Rickettsia helvetica* and *Rickettsia monacensis*) and *Wolbachia* spp. in ticks including *Ixodes ricinus* [23–25]. Unfortunately, there is a critical lack of data on the existence and/or prevalence of parasitoid wasps in ticks in Africa and Eurasia.

The main objectives of the present study are, i) to develop a powerful molecular tool to specifically detect hymenopteran parasitoids in ticks, ii) to screen hard ticks from Western Africa and the Russian Far East, and iii) to characterize these parasitoids genetically.

RESULTS

Tick collection and morphological identification

A total of 785 adult hard ticks were collected in Western Africa (Senegal and Côte d'Ivoire; n=368) and the Russian Far East (n=417). Morphological identification revealed the presence of six tick species, belonging to five genera (Fig. 2). In Western Africa, the species identified were: *A. variegatum* (43 including 5 males and 38 females) from Senegal and *Rhipicephalus (Boophilus) microplus* (325 including 65 males and 260 females) collected from Côte d'Ivoire. Ticks from the Russian Far East belonged to the following species: *I. persulcatus* (256 of which 132 were males and 124 females), *D. silvarum* (83: 48 males

and 35 females), *H. concinna* (54: 29 males and 25 females) and *H. japonica* (24: 8 males and 16 females) (Table 1).

Analysis of MALDI-TOF MS spectra

A total of 84 tick specimens (7 males and 7 females per species) representing the six different species were analyzed by MALDI-TOF (MS). Visual comparison of spectrum profiles using the gel view tool and superposition of spectrum profiles in each state using ClinProTools software (Bruker) revealed a clear difference in spectral profiles of the different tick species. Analysis of the spectral profiles using this new protocol (rehydration) exhibited good reproducibility quality of the spectra of the six tick species. In the same way, this protocol also showed a difference between the two species of the same genus, *H. concinna* and *H. japonica* (Fig. 3).

All spectra of the 84 specimens, including 14 *D. silvarum*, 14 *I. persulcatus*, 14 *A. variegatum*, 14 *R. (B.) microplus*, 14 *H. concinna* and 14 *H. japonica*, were screened against the arthropod database (DB) presenting tick reference spectra. Among the six species, only two spectra (*A. variegatum* and *R. (B.) microplus*) existed in the DB. The blind test against DB revealed an identification of only two species *A. variegatum* and *R. (B.) microplus*. Then, the spectra of the other morphologically identified species, namely *D. silvarum*, *I. persulcatus*, *H. concinna* and *H. japonica*, were added to this database.

Molecular detection of parasitoid wasps and phylogenetic analysis

Designed primers were found to be highly specific both *in silico* and *in vitro*: they did not amplify DNA of any of negative control DNA (Table S1).

In total, 785ticks were screened for the presence of parasitoid wasps' DNA and 3% (28/785) were found to be positive. Specifically, parasitoid wasps' DNA was detected in *I. persulcatus* (17), *R. (B.) microplus* (9), 3.5% *D. silvarum* (1) and 3.5% *H. concinna* (1). No positive samples were found in *A. variegatum* and *H. japonica*. All positive samples as well as positive controls (*I. hookeri*) were subsequently sequenced. After assembly and correction of electrophoreograms by ChromasPro software, 28 sequences were obtained.

Comparison with the GenBank database sequences showed that 24 of the 28 analyzed sequences were identified as *I. hookeri,* sharing 99.6-100% similarities with the sequence of *I. hookeri* that we obtained from positive control (GenBank accession number MH077537). More specifically, these 24 sequences harbored nine different genotypes (Table 2). The distribution of these *I. hookeri* genotypes according to tick species and collection site are presented in Table 2. The phylogenetic position of these nine genotypes within the order Hymenoptera is given in Fig. 4.

The remaining four sequences (4/28), amplified from *I. persulcatus* from Russia, were all identical to each other (*Aphidiinae* 100% identity) (Table 2)**.** A BLAST search showed that these sequences probably represent the DNA of yet undescribed species within the subfamily, as only 97.7% (503/515-bps), 97.6% (528/541-bps) and 97.2% (526/541-bps) similarities were observed, respectively, with the 28SrRNA gene of *Aphidius funebris* (KP983290), *Pseudopraon* spp.(FJ396357) and Aphidiinae spp.(FJ396381). In the phylogenetic tree (Fig. 4), the sequence of this potential new species, provisionally referred here as"Aphidiinae sp.GP4", is clustered together with the parasitoid wasp species belonging to the Aphidiinae subfamily within the Braconidae family.

The partial nucleotide sequence of the 28S rRNA gene obtained from this study was deposited in the GenBank under accession numbers from MN956813 to MN956823.

DISCUSSION

A new standard PCR system targeting the partial sequence of the 28S rRNA gene was developed for the detection of parasitoid Hymenoptera and could be used for routine screening of wasps in ticks as well as in other hosts. This system seems to be sensitive and specific. The amplified 28S rRNA gene portion makes it possible to distinguish between the different species of hymenopterans.

Screening for the presence of parasitoid wasp's DNA revealed a positivity of 3.6% (28/785). Such a low prevalence of parasitoid wasp infestation can be explained by the fact that only adult tick specimens were analysed. Indeed, infected ticks die because of parasitism, but, eventually, some can survive and transform into adult [14–16,18], so the infection level is usually much more important at immature stages compared to adults [36]. A recent study on *I. ricinus* showed that the *I. hookeri* wasp infestation was higher in nymphs (infestation rate 7.2% to 14.6%) than in adults (0.6%; 3/ 481) [36]. In the site in Slovakia, from where the *I. hookeri* positive controls originated, the infestation rate of *I. ricinus* nymphs during 2015-2017 ranged between 4.1% and 23.5% (M. Kazimírová, unpublished). Here, we show that parasitoid wasps' infestation can occur in adult ticks, and with a higher prevalence than previously reported. If wasps are detected in adult ticks, the development of parasitoid wasp larvae (e.g. *I. hookeri*) is unlikely to occur successfully [36].

In this study, we report for the first time the presence of *I. hookeri* in ticks from Western Africa (Côte d'Ivoire) in *R. (B.) microplus*) and in Russian Far East (in *I. persulcatus*, *D. silvarum* and *H. concinna*). It has been shown that wasps of the genus *Ixodiphagus* have a wide host range [18]. Recent studies have reported the ability of *I. hookeri* to infest several tick species belonging to the genera *Ornithodoros*, *Amblyomma*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, *Ixodes* and *Rhipicephalus* [18,19,21]. These results confirm

the global distribution of *I. hookeri* reported by other studies, particularly in Africa [20], America [19] and Europe [23,25].

Parasitoid sequences obtained from four (4/28) *I. persulcatus* ticks were 100% identical to each other. These sequences probably represent a not yet described species within the Aphidiinae subfamily, Braconidae family, as low identity with only 97.2-97.7% and its position on phylogenetic tree suggest it. All closest matches for these sequences belong to parasitoid wasps of the Braconidae family (Hymenoptera: Braconidae). Parasitoids from this family were not previously known to parasitize hard ticks. To date, only parasitoid wasps of the genus *Ixodiphagus* (Hymenoptera: Encyrtidae) have been reported as tick parasites [18].

The genetic variant identified in this study probably belongs to a new wasp species of the family Braconidae. Amplification of other genes (such as mitochondrial genes) followed by phylogenetic analysis, may be necessary to better describe this new genetic variant. Species of the *Aphidius* genus are known to parasitize aphids, but no species of this genus have been reported so far in ticks [37]. Therefore, the Braconidae parasitoid wasp found in *I. persulcatus* should be necessarily identified by prospective entomological studies. It is interesting to note that the Russian Far East is the only region from which *I. hirtus*, another encyrtid wasp parasitizing *I. persulcatus* is known [22]. However, even taking in consideration morphological differences between *I. hirtus* and other species of *Ixodiphagus* genus, the sequence obtained during the present study cannot be attributed to *I. hirtus*, because28S-based genetic identities between Encyrtidae (for example *Ixodiphagus sp.*) and Braconidae are never higher than 92%. Therefore, hymenopteran sequences identified in *I. persulcatus* ticks certainly do not belong to Encyrtidae.

On the other hand, species of the family Braconidae are well known as potential biological control agents against harmful insects [38,39]. Indeed, a recent study showed that species of the genus *Aphidius* can reduce the cotton aphid population, thus constituting a very effective biological control tool [37]. The identification of the potentially new Braconidae species parasitizing ticks in this study is of particular interest. Indeed, the fact that a braconid wasp could parasitize ticks may be used in the development of a new biological control tools against ticks. However, further studies on the biology of hymenopteran parasitoids of the Braconidae family and on the potential environmental impact of using these wasps in biological control of ticks are needed.

The identification of ticks collected in Western Africa and the Russian Far East by MALDI-TOF MS using a new rehydration protocol, has allowed the incrementation of our arthropods database with the new spectra of four hard tick species, namely *I. persulcatus*, *D. silvarum*, *H. concinna* and *H. japonica*. Moreover, the new protocol, proposed in this study based on the rehydration of alcohol-stored

specimens, generated reproducible spectra even for old tick samples stored dry or in 70% ethanol. Indeed, even if kept dry for at least fifteen years, this protocol could give profiles of mass spectrometry spectra (MS) specific to different tick species. Therefore, new protocol used during this study could be standardized for the routine identification of arthropod specimens kept for a long time making them difficult to identify by classical morphological identification keys. These results confirm findings of recent studies indicating that MALDI-TOF (MS) could be used as a rapid, reliable, inexpensive and effective tool for the identification of tick species [31,33] as well as species of other arthropod vectors [40]. However, the conservation status of arthropod specimens may influence the quality and reproducibility of spectra, and an earlier study showed that the use of fresh or frozen specimens could improve the reproducibility and quality of spectra [29]. The MALDI-TOF (MS) has revolutionized clinical microbiology by its effectiveness in the routine identification of microorganisms [41]. Now, it appears to be effective for the rapid identification of arthropods of medical interest requiring no expertise in arthropod identification [29,33,42]. Currently, this innovative tool is increasingly being used, as it presents simple and fast data analysis compared to morphological and molecular methods [43].

CONCLUSION

In this study, we detected for the first time the presence of *I. hookeri* in adult hard ticks from Western Africa (*R. (B.) microplus*) and the Russian Far East (*I. persulcatus*, *D. silvarum* and *H. concinna*). This confirms, on the one hand, that *I. hookeri* has an extensive geographical distribution and, on the other, that this wasp can infest several species of hard ticks even at the adult stage. In addition, we reported for the first time the existence of another parasitoid wasp from the family Braconidae infesting *I. persulcatus*. Once discovered, it may be a promising biological control tool against ticks. Future studies should be conducted to better understand the biology and ecology of these parasitoid wasps and their implications for the epidemiology of tick-borne diseases.

It would be interesting to continue these studies in other parts of the world in order to better understand the role of tick parasitoids in the control of tick populations and to consider them as possible options for biological control.

METHODS

Study area and tick collection

A total of 785 hard ticks were collected from two regions of two continents (Western Africa and Russian Far East) (Fig. 1). In Western Africa, ticks were collected from two countries, Côte d'Ivoire and Senegal. In Côte d'Ivoire, ticks were collected manually from cattle in the Savannahand Bandama Valley, over a period ranging from October 30 to November 8, 2014. In Senegal, ticks were collected

manually from cattle in the village Bandafassi in the region of Kedougou. The collection was performed in August 17, 2004. Ticks were stored in 70% ethanol. Far Eastern questing ticks were collected in Russia near the city of Khabarovsk. The collection was performed in the Khekhtsir forest using the flag technique in May 2002 and 2003. The ticks collected were kept dried. In addition, DNA of 16 adult *Ixodiphagus hookeri* (parasitoids wasps) obtained from *Ixodes ricinus* nymphs collected in Slovakia [23] were used as positive controls in this study.

Morphological identification

All tick species were identified morphologically using taxonomic keys [26–28], then transported and stored at the laboratory of the IHU-Mediterranean infection, Marseille (France) until further investigations.

Dissection and preparation of samples

The dissection was performed using a sterile surgical blade. Four legs on one side of each tick specimen were cut, then used for analysis by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF). Afterward, a longitudinal section was performed to obtain two equal parts of the ticks. One part was used for molecular analyzes and the second one was kept in a sterile tube and stored at -20° C for further analysis.

Identification of ticks by MALDI-TOF MS analysis

Optimization of the preparation of tick samples before MALDI-TOF (MS). Prior to MALDI-TOF analysis, a new protocol based on rehydration of dry-stored specimens by adding 40 µl of HPLC water to each of the tubes containing the tick samples for 24 hours before homogenization was applied. Following the homogenization of the tick legs, a pinch of glass powder (Sigma, Lyon, France) was added to each sample, plus 40 µl of a 70% (v / v) mixture of formic acid and 50% (v / v) acetonitrile (Fluka, Buchs, Switzerland). Finally, using a Tissue Lyser (QIAGEN, Germany), the legs were ground with the configuration parameters as previously described [29].

Setting up samples on the target plate. The crushed legs were centrifuged at 2000 g for 30 seconds and 1 µl of the supernatant of each sample was carefully placed on a MALDI-TOF target plate, with four replicates for each sample as previously described [29]. After subsequent drying, 1 µl of CHCA matrix solution composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma, Lyon France), 50% of acetonitrile (v / v), 2.5% of acid trifluoroacetic acid (v/v) (Aldrich, Dorset, UK) and HPLC grade water was deposited on each spot of the target plate. The target plate was dried at room temperature and was then introduced directly into the MALDI-TOF Microflex LT mass spectrometry device (Bruker Daltonics, Bremen, Germany) for analysis, as previously described [30].

MALDI-TOF parameters (MS). In this study, the protein mass profiles for each tick sample were obtained using a MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics) according to the parameters as previously described [31]. The analysis of the spectrum profiles obtained was made by Flex analysis software v.3.3. Spectrum profiles were subsequently exported to another ClinProTools v.2.2 and MALDI-Biotyper v.3.0 software (Bruker Daltonics) [32].

Database implementation and blind test. Four species of ticks (*Dermacentor silvarum, Haemaphysalis concinna, Haemaphysalis japonica* and *Ixodes persulcatus*), whose spectral profiles were not in MALDI-TOF (MS) database of arthropods of IHU laboratory have been added to the database. In order to identify the ticks using the MALDI-TOF, a blind test was performed as previously described [33].

Molecular analysis

DNA extraction. The genomic DNA was extracted from a half-part of each tick as well as that of *I. hookeri* after being subjected to enzymatic lysis with proteinase K followed by incubation at 56 °C overnight. The extraction was performed using the QIAamp Tissue Extraction Kit (Qiagen, Courtaboeuf, France), in the QiagenEZ1 automated system, following the manufacturer's instructions. DNA was eluted in 100 µl and stored at -20°C until further use.

Standard PCR and sequencing. To detect the DNA of hymenopteran parasitoids, we designed a new specific set of primers for the standard PCR targeting a 560-bps fragment of the 28S ribosomal RNA (28S rRNA) gene. Primers were designed using free web Primer3 software, version 4.0 (http://frodo.wi.mit.edu/primer3/) following the general rules described elsewhere [34]. The specificity and sensibility of the PCR assay was tested *in silico* using primer-BLAST (NCBI, USA) and validated using a panel of arthropods and bacterial DNAs (Table S1). The oligonucleotide sequences of the forward and reverse primers were as follows: 28S-hym-F (5'-AGACCGATAGCGAACAAGTA-3') and 28S-hym -R (5'-GGTCCTGAAAGTACCCAAA-3').

The DNA extracted from all the ticks was then analyzed using the newly designed system for potential hymenopteran parasitoid DNA.

All the standard PCR reactions were performed in a Thermal Cycler Peltier PTC200 cycler thermal model (MJ Research Inc., Watertown, MA, USA). Each reaction was conducted in a final volume of 50 µl, containing 5 µl of DNA of each sample, 25 HotstarTaq - AmpliTaq Gold (Life Technologies, Carlsbad, California, USA), 1.5 µl of primers (Forward and Reverse) and 17 µl water DNAse/RNAse free, using the following amplification profile: an initial denaturation step at 95 °C for 15 minutes, followed by 40 denaturation cycles at 95°C for 30 seconds, step hybridization at a temperature of 59°C for 30 seconds and an elongation at 72°C for 30 seconds. The DNA of *I. hookeri* was used as a positive

297 control and master mixture as a negative control for each experiment. The success of the amplification
298 was confirmed by electrophoresis on 1.5% agarose gel.

299 Purification of the PCR products of the positive samples was performed using NucleoFast 96
300 PCR plates (Macherey-Nagel EURL, Hoerdt, France) according to the manufacturer's instructions. The
301 purified amplicons were then sequenced using the Big Dye Terminator Cycle sequencing kit (Perkin
302 Elmer Applied Biosystems, Foster City, CA) with an automated ABI sequencer (Applied Biosystems).

303 **Phylogenetic analysis**

304 Obtained sequences were then assembled and edited using ChromasPro software (ChromasPro 1.7,
305 Technelysium Pty Ltd., Tewantin, Australia), and then compared using NCBI BLAST with sequences
306 deposited in the GenBank database (www.ncbi.nlm.nih.gov/blast/Blast.cgi). The phylogenetic tree was
307 created by MEGA 7 software using the maximum-likelihood method [35].

REFERENCES

1. de la Fuente, J. *et al.* Tick-Pathogen Interactions and Vector Competence: Identification of Molecular Drivers for Tick-Borne Diseases. *Front Cell Infect Microbiol* **7**, (2017).
2. Karasartova, D. *et al.* Bacterial and protozoal pathogens found in ticks collected from humans in Corum province of Turkey. *PLOS Neglected Tropical Diseases* **12**, e0006395 (2018).
3. Gargili, A. *et al.* The role of ticks in the maintenance and transmission of Crimean-Congo hemorrhagic fever virus: A review of published field and laboratory studies. *Antiviral Res* **144**, 93–119 (2017).
4. Jongejan, F. & Uilenberg, G. The global importance of ticks. *Parasitology* **129**, S3 (2005).
5. Singer, M. & Bulled, N. Ectoparasitic Syndemics: Polymicrobial Tick-borne Disease Interactions in a Changing Anthropogenic Landscape: Tick-borne Disease Syndemics. *Medical Anthropology Quarterly* **30**, 442–461 (2016).
6. Parola, P. & Raoult, D. Ticks and Tickborne Bacterial Diseases in Humans: An Emerging Infectious Threat. *Clinical Infectious Diseases* **32**, 897–928 (2001).
7. Mans, B. J., de Klerk, D., Pienaar, R. & Latif, A. A. Nuttalliella namaqua: A Living Fossil and Closest Relative to the Ancestral Tick Lineage: Implications for the Evolution of Blood-Feeding in Ticks. *PLoS One* **6**, (2011).
8. Lafri, I. *et al.* Detection of relapsing fever *Borrelia* spp., *Bartonella* spp. and Anaplasmataceae bacteria in argasid ticks in Algeria. *PLoS Negl Trop Dis* **11**, (2017).
9. Kirby, C. Stafford III. Tick Management Handbook https://www.lymediseaseassociation.org/resources/book-list/1646-tick-management-handbook-prepared-by-kirby-c-stafford-iii.
10. Collatz, J. *et al.* A hidden beneficial: biology of the tick-wasp *Ixodiphagus hookeri* in Germany: Biology of Ixodiphagus hookeri in Germany. *Journal of Applied Entomology* **135**, 351–358 (2011).
11. Aguiar, A. P. *et al.* Order hymenoptera. *Zootaxa* **3703**, 51–62 (2013).
12. Franck, P., Maalouly-Matar, M. & Olivares, J. Molecular Tools for the Detection and the Identification of Hymenoptera Parasitoids in Tortricid Fruit Pests. *Int J Mol Sci* **18**, (2017).
13. Parasitic Wasps. https://pubs.ext.vt.edu/content/pubs_ext_vt_edu/en/ENTO/ENTO-74/ENTO-74.html.
14. Davis, A. J. & Davis, A. J. Bibliography of the Ixodiphagini (Hymenoptera, Chalcidoidea, Encyrtidae), parasites of ticks (Acari, Ixodidae), with notes on their biology. *Tijdschrift voor entomologie.* **129**, 181–190 (1986).
15. Dheilly, N. M. *et al.* Who is the puppet master? Replication of a parasitic wasp-associated virus correlates with host behaviour manipulation. *Proc Biol Sci* **282**, (2015).
16. Hu, R. & Hyland, K. E. Effects of the feeding process of *Ixodes scapularis* (Acari: Ixodidae) on embryonic development of its parasitoid, *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae). *J. Med. Entomol.* **35**, 1050–1053 (1998).
17. Heath, A. C. & Cane, R. P. A new species of *Ixodiphagus* (Hymenoptera: Chalcidoidea: Encyrtidae) parasitizing seabird ticks in New Zealand. *New Zealand Journal of Zoology* **37**, 147–155 (2010).
18. Hu, R., Hyland, K. E. & Oliver, J. H., Jr. A review on the use of *Ixodiphagus* wasps (Hymenoptera: Encyrtidae) as natural enemies for the control of ticks (Acari: Ixodidae). *Systematic and Applied Acarology* **3**, 19 (1998).
19. Bezerra Santos, M. A. *et al.* Larvae of *Ixodiphagus* wasps (Hymenoptera: Encyrtidae) in *Rhipicephalus sanguineus* sensu lato ticks (Acari: Ixodidae) from Brazil. *Ticks Tick Borne Dis* **8**, 564–566 (2017).
20. Mwangi, E. N., Hassan, S. M., Kaaya, G. P. & Essuman, S. The impact of *Ixodiphagus hookeri*, a tick parasitoid, on Amblyomma variegatum (Acari: Ixodidae) in a field trial in Kenya. *Exp. Appl. Acarol.* **21**, 117–126 (1997).
21. Stafford, K. C., Denicola, A. J. & Magnarelli, L. A. Presence of *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae) in two Connecticut populations of *Ixodes scapularis* (Acari: Ixodidae). *J. Med. Entomol.* **33**, 183–188 (1996).
22. Triapitsyn, V. A. [Redescription of Ixodiphagus hirtus (Hymenoptera, Encyrtidae), a parasite of the taiga tick *Ixodes persulcatus* in the Far East of the USSR]. *Parazitologia* **16**, 489–493 (1982).
23. Bohacsova, M., Mediannikov, O., Kazimirova, M., Raoult, D. & Sekeyova, Z. *Arsenophonus nasoniae* and Rickettsiae Infection of *Ixodes ricinus* Due to Parasitic Wasp *Ixodiphagus hookeri*. *PLoS One* **11**, (2016).
24. Plantard, O. *et al.* Detection of Wolbachia in the Tick Ixodes ricinus is Due to the Presence of the Hymenoptera Endoparasitoid *Ixodiphagus hookeri*. *PLoS One* **7**, (2012).
25. Tijsse-Klasen, E., Braks, M., Scholte, E.-J. & Sprong, H. Parasites of vectors - *Ixodiphagus hookeri* and its Wolbachia symbionts in ticks in the Netherlands. *Parasit Vectors* **4**, 228 (2011).
26. Filippova, N. A. Ixodoid ticks of the subfamily Ixodinae. *Fauna of the USSR. Arachnida* (1977).
27. Filippova, N. A. Fauna of Russia and neighbouring countries. Ixodid ticks of subfamily Amblyomminae. *Fauna of Russia and neighbouring countries. Ixodid ticks of subfamily Amblyomminae.* (1997).
28. Walker, A. R., Matthews, J. & Preston, P. M. The development of electronic keys for the identification of ticks. *International Journal of Tropical Insect Science* **25**, (2005).

29. Nebbak, A. *et al.* Comparative analysis of storage conditions and homogenization methods for tick and flea species for identification by MALDI-TOF MS. *Med. Vet. Entomol.* **31**, 438–448 (2017).
30. Yssouf, A. *et al.* Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry: An Emerging Tool for the Rapid Identification of Mosquito Vectors. *PLoS ONE* **8**, e72380 (2013).
31. Kumsa, B. *et al.* Morphological, molecular and MALDI-TOF mass spectrometry identification of ixodid tick species collected in Oromia, Ethiopia. *Parasitol Res* **115**, 4199–4210 (2016).
32. Fotso Fotso, A. *et al.* MALDI-TOF mass spectrometry detection of pathogens in vectors: the Borrelia crocidurae/Ornithodoros sonrai paradigm. *PLoS Negl Trop Dis* **8**, e2984 (2014).
33. Diarra, A. Z. *et al.* Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali. *PLoS Negl Trop Dis* **11**, (2017).
34. Thornton, B. & Basu, C. Real-time PCR (qPCR) primer design using free online software. *Biochem Mol Biol Educ* **39**, 145–154 (2011).
35. Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol* **30**, 2725–2729 (2013).
36. Ramos, R. A. N. *et al.* Occurrence of *Ixodiphagus hookeri* (Hymenoptera: Encyrtidae) in Ixodes ricinus (Acari: Ixodidae) in Southern Italy. *Ticks and Tick-borne Diseases* **6**, 234–236 (2015).
37. Ali, A., Desneux, N., Lu, Y., Liu, B. & Wu, K. Characterization of the natural enemy community attacking cotton aphid in the Bt cotton ecosystem in Northern China. *Sci Rep* **6**, 24273 (2016).
38. Peixoto, L. *et al.* When taxonomy and biological control researchers unite: Species delimitation of Eadya parasitoids (Braconidae) and consequences for classical biological control of invasive paropsine pests of Eucalyptus. *PLoS ONE* **13**, e0201276 (2018).
39. Solà, M., Castañé, C., Lucas, E. & Riudavets, J. Optimization of a Banker Box System to Rear and Release the Parasitoid Habrobracon hebetor (Hymenoptera: Braconidae) for the Control of Stored-Product Moths. *J. Econ. Entomol.* (2018) doi:10.1093/jee/toy219.
40. Yssouf, A. *et al.* Identification of flea species using MALDI-TOF/MS. *Comp. Immunol. Microbiol. Infect. Dis.* **37**, 153–157 (2014).
41. Bizzini, A. & Greub, G. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. *Clin. Microbiol. Infect.* **16**, 1614–1619 (2010).
42. Yssouf, A. *et al.* Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Rapid Identification of Tick Vectors. *J Clin Microbiol* **51**, 522–528 (2013).
43. Laroche, M. *et al.* MALDI-TOF MS as an innovative tool for detection of Plasmodium parasites in Anopheles mosquitoes. *Malar. J.* **16**, 5 (2017).

Acknowledgments
The authors thank L.Ivanov, N. Zdanovskaya and R. Vorobyova, C. Sokhna, M. Sambou, B. Davoust, K.P. Yao KP, and JD N'Guessan for their help in collecting ticks.

Author Contributions
DR, MK, FF and OM conceived and designed research. GM, NA and ML conducted experiments. GM, NA, EAN, YL, ML, JMB and ZS contributed analytical tools. GM, NA and YL analyzed data. GM, NA, EAN and YL, and wrote the manuscript. All authors read and approved the manuscript.

Competing of interest
The authors declare that they have no conflict of interest.

Ethics declarations
This article does not contain any studies with human participants or vertebrates performed by any of the authors.

Supplementary information

Table S1 Analytical specificities: a panel of arthropods and bacterial species tested with Hymenoptera-specific 28S rRNA based standard PCR assays

Figure legends

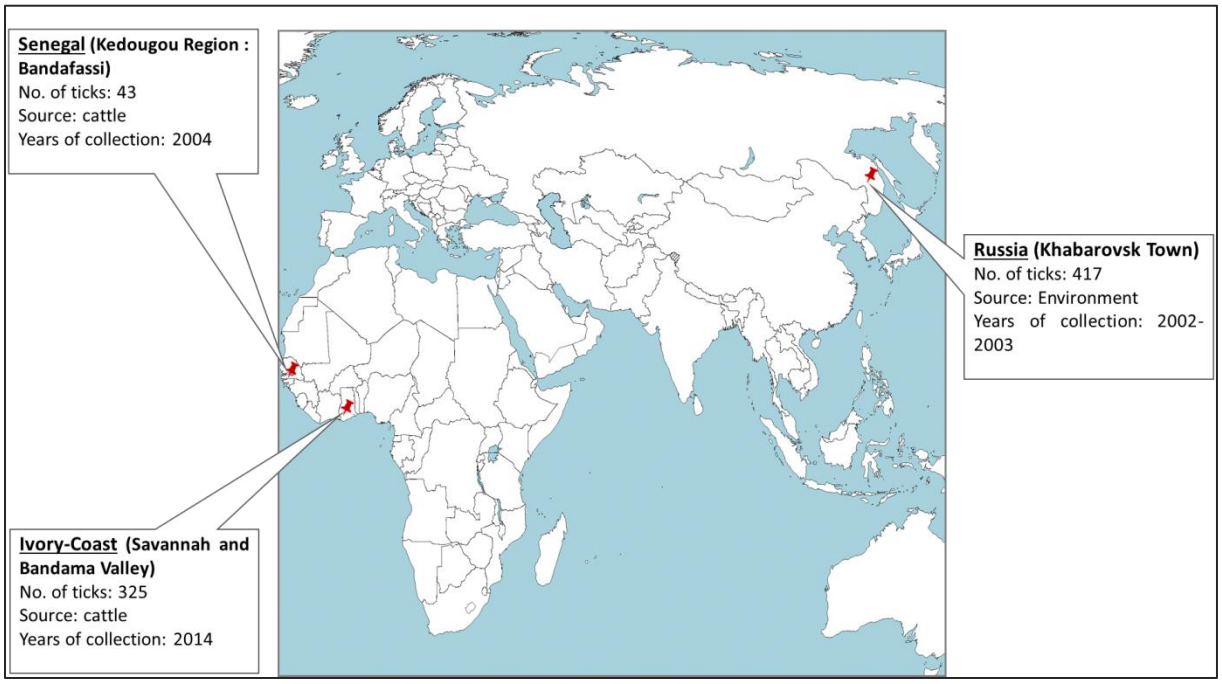


Fig. 1 Map showing the origin, period and number of hard ticks collected in Africa and the Far East

Fig. 2 Pictures of hard tick species collected and studied from Western Africa and the Far East. A) *Rhipicephalus (Boophilus) microplus*, B) *Amblyomma variegatum* C) *Haemaphysalis japonica*, D) *Haemaphysalis concinna*, E) *Ixodes persulcatus* and F) *Dermacentor silvarum*,

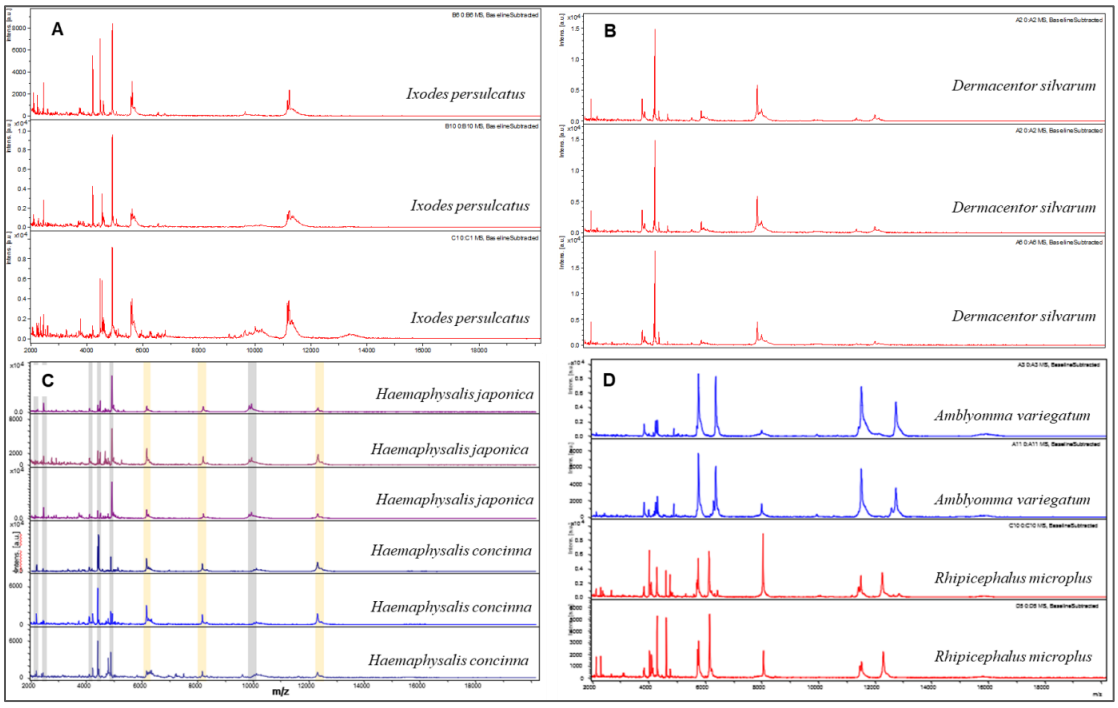


Fig. 3 MS spectra profiles of tick species using the new "rehydration" protocol. A) *Ixodes persulcatus*, B) *Dermacentor silvarum*, C) *Haemaphysalis concinna* and *Haemaphysalis japonica* and C) *Rhipicephalus (Boophilus)microplus* and *Amblyomma variegatum.*

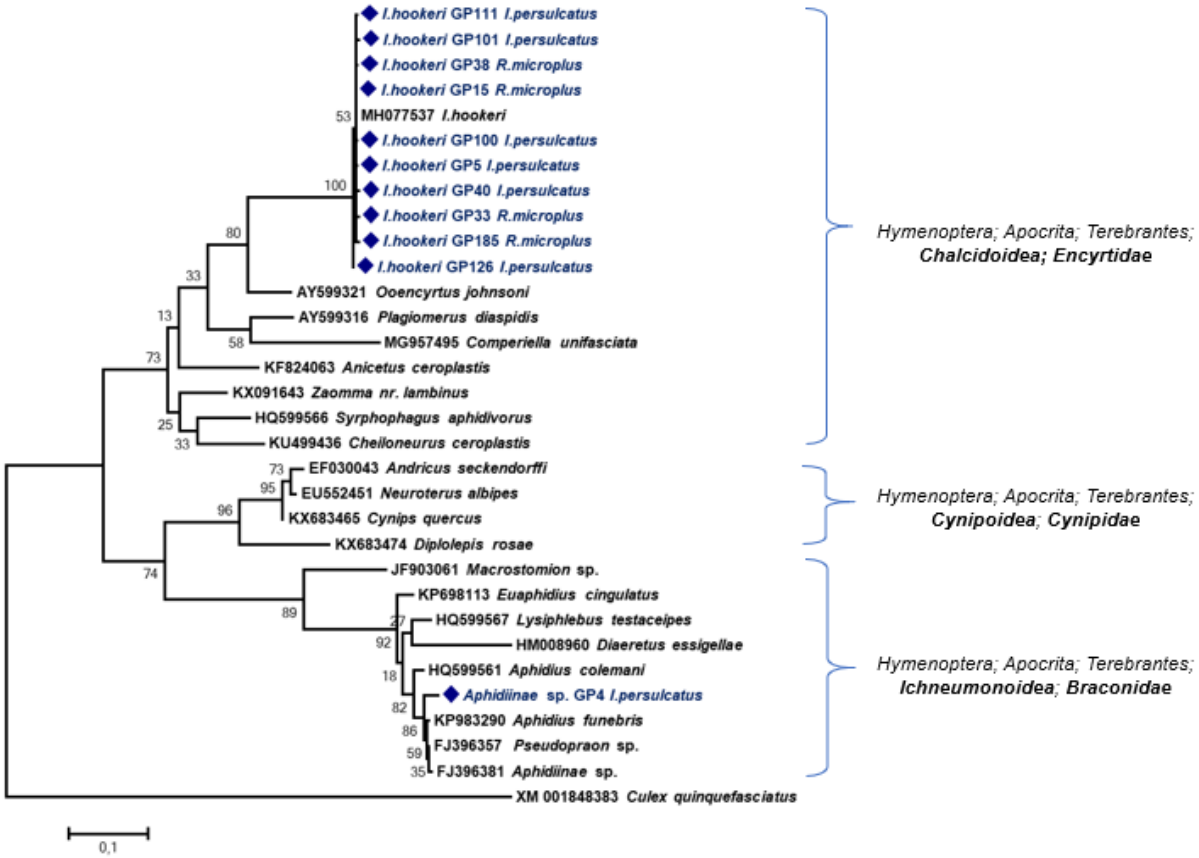


Fig. 4 Maximum-likelihood (ML) phylogenetic tree based on the 28S rRNA gene showing the position of parasitoid wasps amplified in this study within the order of Hymenoptera. Phylogenetic inference was conducted in MEGA 7 using the Maximum Likelihood method based on the Tamura 3-parameter model with 500 bootstrap replicates. The analysis involved 32 nucleotide sequences. The scale bar represents a 10% nucleotide sequence divergence.

429 **Tables**

430 **Table 1** Ticks collected from animals in Western Africa and from the vegetation in the Far East Russia as well as
431 the results of the search for Hymenoptera parasitoids.

Country / Region	Geographic coordinates	Tick species	Number (male/female)	% of ticks positive for parasitoid wasps
Russia / Khabarovsk	48°28′57″ N 135°05′01″ E	*Ixodes persulcatus*	256 (132/124)	17 (7%)
		Dermacentor silvarum	83 (48/35)	1 (1%)
		Haemaphysalis concinna	54 (29/25)	1 (2%)
		Haemaphysalis japonica	24 (8/16)	0
Côte d'Ivoire/Bandama Valley and Savannah	8°8′ N 5°6′ W	*Rhipicephalus (Boophilus) microplus*	325 (65/260)	9 (3%)
Senegal / Bandafassi	12° 32′ N, 12° 19′ W	*Amblyomma variegatum*	43 (5/38)	0
	Total		785 (287/498)	28 (3%)

432

433 **Table 2** Sequences of parasitoid wasps amplified in this study and deposited in GenBank

Sequences type	Tick species (no.)	Collection site	Total	Ascension number
*I.hookeri*_GP15	*R. (B.) microplus* (6), *I. persulcatus* (7), *D. silvarum* (1), *H. concinna* (1)	Côte d'Ivoire, Russia	15	MN956813
*I.hookeri*_GP5	*I. persulcatus* (1)	Russia	1	MN956814
*I.hookeri*_GP100	*I. persulcatus* (1)	Russia	1	MN956815
*I.hookeri*_GP111	*I. persulcatus* (1)	Russia	1	MN956816
*I.hookeri*_GP38	*R. microplus* (1)	Russia	1	MN956817
*I.hookeri*_GP40	*I. persulcatus* (1)	Russia	1	MN956818
*I.hookeri*_GP101	*I. persulcatus* (1)	Russia	1	MN956819
*I.hookeri*_GP126	*I. persulcatus* (1)	Russia	1	MN956820
*I.hookeri*_GP185	*R. (B.) microplus* (1)	Côte d'Ivoire	1	MN956821
*I.hookeri*_GP33	*R. (B.) microplus* (1)	Côte d'Ivoire	1	MN956822
*Aphidiinae*_sp._GP4	*I. persulcatus* (4)	Russia	4	MN956823
Total	4		28	

434

Chapitre 3 :

Surveillance et contrôle de maladies vectorielles chez le réservoir animal

3.2 : Surveillance épidémiologique de maladies vectorielles chez des animaux réservoirs

Préambule

Les maladies vectorielles sont des maladies de l'homme et de l'animal, causées par des parasites, des virus et des bactéries transmis par des vecteurs. Selon l'Organisation mondiale de la santé, il y a plus de 700 000 décès annuels dus à ces maladies, telles que le paludisme, la dengue, la schistosomiase, la trypanosomiase humaine africaine, la leishmaniose, la maladie de Chagas, la fièvre jaune, l'encéphalite japonaise et l'onchocercose (WHO 2014). Elles sont, particulièrement, rencontrées en zone tropicale et subtropicale, là où elles affectent de manière grave les populations les plus pauvres. Depuis 2014, des flambées majeures de dengue, de paludisme, de chikungunya, de fièvre jaune et de Zika ont affligé des populations entières, causé une morbidité et une mortalité importantes et submergé les systèmes de santé de plusieurs pays. D'autres maladies telles que la leishmaniose et la filariose lymphatique provoquent des souffrances chroniques, une morbidité à vie, des incapacités et une stigmatisation occasionnelle. La diversité écologique, associée à d'importantes disparités économiques et sociales, ont fait du contrôle et de l'éradication de ces maladies un défi permanent pour les responsables de la santé publique (da Costa et al. 2005; Horta et al. 2004, Calic et al. 2004).

Les animaux sont utilisés comme des sentinelles pour la détection de dangers environnementaux et de ceux en rapport avec la transmission de maladies infectieuses, et même comme des indicateurs d'événements liés au bioterrorisme (Backer et al. 2001, Duncan et al. 2004, Rabinowitz et al. 2006). Les estimations selon lesquelles 75% des maladies infectieuses émergentes, récemment découvertes, sont de nature zoonotique sont bien connues (Taylor et al. 2001). Des études impliquant des animaux de compagnie et d'autres animaux domestiques ou sauvages peuvent compléter les études épidémiologiques relatives directement à la santé des collectivités humaines. Étant donné que les animaux de compagnie sont en proximité de l'homme, les données sur les maladies à transmission vectorielle concernant ces animaux

peuvent fournir des informations uniques sur l'incidence, les facteurs de risque et les sources d'exposition avant même l'apparition de foyers de maladies chez l'homme. Les chiens sont sensibles à plusieurs infections zoonotiques à transmission vectorielle, notamment, émergentes ou ré-émergentes. En tant qu'animaux sentinelles, les chiens sont, fréquemment, exposés aux tiques infectées, aux puces, aux triatomes, aux moustiques et aux phlébotomes. De plus, les chiens développent une forte réponse immunitaire spécifique contre de nombreux agents pathogènes à transmission vectorielle. Ils développent des signes cliniques aigus et parfois chroniques de maladies très similaires aux manifestations de la maladie rapportée chez l'homme (Lindenmayer 1991). A cela s'ajoute le fait que les chiens sont, généralement, accessibles pour une manipulation et la réalisation d'un prélèvement biologique en toute sécurité (Cleaveland 2006), ce qui renforce leur utilité en surveillance épidémiologique.

J'ai mené des études exploratoires sur le rôle sentinelle joué par des animaux, particulièrement, le chien pour des maladies vectorielles. Mon étude sur les filarioses chez le chien en Guyane (**Publication N°12**), montre l'existence d'au moins quatre génotypes de filaires dont trois sont zoonotiques ou, potentiellement, zoonotiques à savoir : *Dirofilaria immitis* (17,34% - 17/98), *Acanthocheilonema reconditum* (3,06% - 3/98), *Cercopithifilaria bainae* (2,9% - 2/98) et *Brugia* sp. (4,08% - 4/98). J'ai également démontré la similarité du génotype de *Brugia* sp. circulant chez les chiens et les singes-hurleurs de Guyane (**Publication N°6**), suggérant ainsi la proximité de ce parasite potentiellement zoonotique avec l'homme. En France métropolitaine, j'ai décrit un foyer de dirofilarioses à *Dirofilaria immitis* et *D. repens* avec une prévalence de 35,2% (n=17) chez des chiens militaires dans le département de l'Indre. En plus du risque zoonotique pour les personnes, les résultats de cette étude confirment la propagation de ces parasitoses vers le Nord de la France du fait de l'extension de la présence de vecteurs (**Publication N°13**).

Plusieurs études ont souligné le rôle des renards en tant que réservoir pour des maladies d'intérêt médical, en particulier, la rage, l'échinococcose, la maladie de Lyme, la leptospirose, et toutes les autres maladies vectorielles des canidés (Atawodi et al. 2013, Pluemer et al. 2019). Nous avons dépisté par biologie moléculaire un total de 93 renards roux (*Vulpes vulpes*) chassés entre 2008 et 2018, dans les départements des Bouches-du-Rhône et du Var, au Sud-Est de la France. Cette étude nous a permis d'avoir une image claire du rôle de ces canidés en tant que sentinelles pour des maladies vectorielles. Nous avons détecté la présence de l'ADN de nématodes chez 3% (3/93) des renards : *Spirocerca vulpis* a été identifié chez un renard et *Dirofilaria immitis* chez deux autres. Nous avons également mis en évidence l'ADN d'hémoparasites : *Hepatozoon canis* (92%), *Leishmania infantum* (15%) et *Babesia vogeli* (3%). Les agents bactériens à transmission vectorielle détectés étaient *Anaplasma platys* (2%) et le génotype 3 de *Coxiella burnetii* (3%). Il est important de noter que le même génotype de *Coxiella burnetii* a déjà circulé chez l'homme dans la même région (**Publication N°14**).

Dans le nord de l'Algérie, nous avons montré qu'en plus de la diversité des filarioses canines (*Dirofilaria immitis, Dirofilaria repens, Acanthocheilonema reconditum, Cercopithifilaria bainae* et de *Cercopithifilaria* sp. II), décrite au cours de notre précédente étude (**Publication N°3**), les chiens de cette région sont également porteurs de nombreux hémoparasites d'intérêt médical et vétérinaire. L'étude portant sur un ensemble de 227 chiens, a aussi révélé une forte prévalence de *Leishmania infantum* (37,5%), de *Trypanosoma* spp. (6,6%), de *Trypanosoma evansi* (3,1%), d'une potentielle sous-espèce de *Trypanosoma congolense*, de *Babesia vogeli* (13,2%) et d'*Hepatozoon canis* (41%) (**Publication N°15**). En Afrique de l'Ouest, on a eu également accès à 123 échantillons de sang de chiens prélevés en 2018, en Côte d'Ivoire. Les analyses PCR ont révélé la présence de *Leishmania infantum* (15,4%), de *Trypanosoma congolense* (4,1%), de bactéries appartenant à la famille des Anaplasmataceae (17,1 %) dont *Ehrlichia canis* (12,2%), de *Babesia vogeli* (1,6 %) et de 10,6%

d'infections par des filaires incluant *Dirofilaria immitis, D. repens* et *Acanthocheilonema reconditum* (**Publication N°16**). Etant donné le caractère massif des infections mises en évidence, ainsi que le risque zoonotique qui est associé à la leishmaniose, les autorités sanitaires médicales et vétérinaires ivoiriennes informées vont accroître la surveillance épidémiologique.

En plus des animaux domestiques, la faune sauvage joue aussi un rôle de sentinelle pour les maladies infectieuses en général et les maladies vectorielles en particulier. Des études ont rapporté une corrélation entre les individus ayant un contact avec la faune sauvage en zone forestière tels que les éco-gardes, les éco-touristes, les forestiers et les chasseurs et le risque de rickettsioses, de coxiellose et d'anaplasmose. En Italie, il a été rapporté que 37% et 23,8% de ces personnes sont positives aux recherches d'anticorps anti-*Rickettsia* et anti-*Coxiella* respectivement (Livio et al. 2007, Mancini et al. 2015, Fenga et al. 2015). Pour autant, on constate que les réservoirs sauvages de ces maladies vectorielles sont mal étudiés. Ainsi, nous démontrons dans une étude (**Publication N°17**) le rôle des reptiles en tant que sentinelles pour la présence et la transmission des agents pathogènes du groupe des fièvres boutonneuses (rickettsies), de la coxiellose, et de l'anaplasmose dans les zones boisées et périurbaines du Sud de l'Italie. Un total de 172 reptiles (4 serpents et 168 lézards) a été prélevé dont 94,18% étaient infestés par des tiques. Parallèlement, un ensemble de 50 sérums humains a été prélevé sur des personnes vivant à proximité de ces zones forestières et ayant des antécédents de piqures de tiques. Les résultats montrent une prévalence de *Rickettsia monacensis*, *R. helvetica* et Candidatus *Cryptoplasma* sp. qui était, respectivement, de 83,17%, 16,82% et 0,8% chez les ectoparasites des reptiles. Seuls 2,4% des reptiles étaient positifs pour Candidatus *Cryptoplasma* sp., et un seul cas d'*Anaplasma* sp. a été détecté. Les analyses sérologiques des sérums humains ont montré une prévalence de 8% pour *Rickettsia* spp., 16% pour *Coxiella burnetti* et 22% pour *Anaplasma phagocytophilum*. Cette étude suggère que les reptiles

(lézards) peuvent avoir aussi un rôle de réservoir pour les rickettsies du groupe boutonneux dans le Sud de l'Europe.

Publication N°12

Detection of Canine Vector-Borne Filariasis and their *Wolbachia* Endosymbionts in French Guiana

Younes Laidoudi[1,2], Jean-Lou Marié[3], Djamel Tahir[1,2], Stéphanie Watier-Grillot[3,4], Oleg Mediannikov [1,2] and Bernard Davoust [1,2,3,*]

[1] Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France
[2] IHU Méditerranée Infection, Marseille, France
[3] Animal Epidemiology Working Group of the Military Health Service, Marseille, France
[4] Epidemiology and Public Health Center of the French Armed Forces, Marseille, France

ARTICLE DE RECHERCHE

Publié dans : Microorganisms 2020, 8(5), 770

(doi.10.3390/microorganisms8050770)

microorganisms

MDPI

Article

Detection of Canine Vector-Borne Filariasis and Their *Wolbachia* Endosymbionts in French Guiana

Younes Laidoudi [1,2], Jean-Lou Marié [3], Djamel Tahir [1,2], Stéphanie Watier-Grillot [3,4], Oleg Mediannikov [1,2] and Bernard Davoust [1,2,3,*]

[1] Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, 13385 Marseille, France; younes.laidoudi@yahoo.com (Y.L.); djamel.tahir@yahoo.fr (D.J.); olegusss1@gmail.com (O.M.)

[2] IHU Méditerranée Infection, 13385 Marseille, France

[3] Animal Epidemiology Working Group of the Military Health Service, 13014 Marseille, France; jean-lou.marie@wanadoo.fr (J.-L.M.); s.watier.grillot@gmail.com (S.W.-G.)

[4] Epidemiology and Public Health Center of the French Armed Forces, 13014 Marseille, France

* Correspondence: bernard.davoust@gmail.com; Tel.: 33-4-13-73-24-01

Received: 10 April 2020; Accepted: 18 May 2020; Published: 21 May 2020

Abstract: In French Guiana, canine heartworm disease is well known, but the diversity of filarial parasites of dogs remains largely unknown. A total of 98 canine blood samples from Cayenne and Kourou were assessed by a blood wet mount preparation, heartworm antigen test and molecular exploration of filarioid and *Wolbachia* DNAs, followed by a multiplex species-specific qPCR's identification and a subsequent sequencing analysis. Thereafter, a phylogeny based on maximum likelihood was carried out to facilitate specific identification. Five dogs were microfilaremic. Heartworm antigens were detected in 15 (15.3%) dogs. Of these, six (6.1%) were considered as occult infections as neither microfilariae nor *Dirofilaria immitis* DNA were detected. The 11 (11.2%) *D. immitis* isolates corresponded to a low virulent strain. Six of the *D. immitis* isolates were positive for *Wolbachia* endosymbionts of *D. immitis* belonging to the clade C DNA. *Acanthocheilonema reconditum* DNA was detected in 3 (3.1%) samples. Of these latter, one was found co-infected with the *Brugia* sp. genotype and the DNA of the clade D of the *Wolbachia* endosymbiont of *Brugia* species. This latter was also detected in two filarioid DNA-free samples. Finally, two samples were positive for *Cercopithifilaria bainae* genotype, which is distinct from those identified in Europe. The present study highlights the urgent need to implement chemoprophylaxis associated with anti-*Wolbachia* drugs to control these potential zoonoses.

Keywords: canine vector-borne helminth; filariasis; *Wolbachia*; species diversity; zoonosis; French Guiana

1. Introduction

Canine vector-borne diseases (CVBDs) constitute a worldwide group of illnesses affecting dogs. They are caused by a wide range of bacteria, viruses, protozoa and helminths, all transmitted to dogs by parasitic arthropods bites [1]. These diseases constitute an important public health concern in tropical and subtropical regions from the Old and New World, where they are endemic [2]. In French Guiana, dogs seem to be affected by several major CVBDs, such as leishmaniasis [3], anaplasmosis [4], trypanosomiasis [5], ehrlichiosis [6] and arbovirosis [7].

Canine filariasis are a group of canine vector-borne helminth (CVBH) caused by several nematodes belonging to the Onchocercidae family. Canids constitute suitable hosts for many filarial parasites of veterinary and human importance, such as the zoonotic *Dirofilaria immitis* (Leidy 1856), the agent of cardiopulmonary dirofilariasis (also known as heartworm) in dogs and pulmonary

dirofilariasis in human [8], *D. repens* which causes in humans, like in dogs the subcutaneous filariasis [9]. These two species, together with brugian parasites that cause lymphatic filariasis (e.g., *Brugia timori, B. malayi* and *B. pahangi*) [10,11], constitute the most thread-like filarial worms causing millions of canine and human cases throughout the world [12–14]. These parasites have bloodstream microfilariae and are transmitted by mosquito bites. Dogs may also be affected with another less or completely avirulent group of CVBH transmitted by parasitic arthropods other than mosquitoes, such as *Acanthocheilonema reconditum* and *Cercopithifilaria* spp. parasite of the sub-cutaneous connective tissues which are actively transmitted through the bites of fleas/lice and ticks, respectively [15–18]. Furthermore, *A. dracunculoides* infests the peritoneal cavity of dogs [19] and a little known filarioid *Onchocerca lupi* inducing ocular nodules on the eyelids, conjunctiva and sclera in dogs as in human [20].

Some filarioids of the subfamilies Onchocercinae and Dirofilariinae are associated with an endosymbiotic intracellular bacterium of the genus *Wolbachia* [21], which can be found in all filarioid developmental stages and is essential for the long-term survival of the adult filarioids [22]. Moreover, *Wolbachia* are host-specific, and each filarial species that harbor *Wolbachia* is associated with a specific bacterial genotype [23]. These features constitute make its a suitable target for the diagnosis of filarial infection, especially when occurring in dead-end hosts as is the case of *D. immitis* in humans and cats [24,25]. Recent studies showed that the combined detection of *Wolbachia* and filarioid DNA improves the diagnosis of these infections [26,27].

CVBH are strongly related to the distribution of their vectors and are therefore geographically varied. Few studies data are available on canine filariasis from Latin America. The current species reported from canids in Brazil are potentially zoonotic and are often caused by *A. reconditum*, *C. bainae*, *D. grassii*, *D. immitis* and *D. repens* [28]. In addition, two cases of human filariasis have been reported across the continent, caused by lymphatic filariasis in Brazil, Dominican Republic, Guyana and Haiti and onchocerciasis in Brazil, Colombia, Ecuador, Guatemala, Mexico and Venezuela [29]. However, the information on CVBH in French Guiana is very scarce.

In the present study, we aimed to determine the presence of CVBH in dog blood samples from French Guiana as well as the strains of their endosymbiotic *Wolbachia* using molecular assays. also aimed to investigating *D. immitis* infection rate using heartworm antigen test. The study provides preliminary information that could use in the future for the control of the potentially zoonotic filariasis in dogs from French Guiana.

2. Materials and Methods

2.1. Sampling and Study Area

In January 2016, in two sites of French Guiana separated by 60 km, 67 adult dogs, including 18 stray dogs and 49 shelter dogs were sampled in Cayenne (4° 56′ 4.6″ N, 52° 19′ 49.19″ W), the main city of French Guiana with 57,000 residents and 31 adult shelter dogs from Kourou (5° 9′ 34.92″ N, 52° 39′ 1.08″ W). All dogs were subjected to a blood sampling via a cephalic venipuncture using a BD Vacutainer™ K3EDTA tubes (Fisher Scientific, Illkirch, France), then conditioned at 4 °C and transported to our laboratory. Ethical aspects related to dog sampling were treated in accordance with the French law. Owner consent was obtained for all shelter dogs of both cities. Likewise, the consent of the director of the Cayenne dog pound was obtained for all stray dogs. All dogs (n = 98) were apparently healthy, including 52 females and 46 males.

2.2. Ethics Approval

Dogs were examined by veterinarians with the assistance and acceptance of their owners. Ethical aspects related to dog sampling were treated in accordance with the French law. The owner consents were obtained for all shelter dogs of both cities. Likewise, the consent of the director of the Cayenne dog pound was obtained for all stray dogs.

2.3. Microfilaria and Heartworm Antigen Tests

Immediately after sampling, the dog's blood samples were processed for the detection of heartworm antigens using the WITNESS® *Dirofilaria* (Zoetis, Lyon, France). According to the manufacturer's recommendations, this immunochromatographic test allowed the identification of heartworm antigens from bloodstream. The blood wet mount preparation was also performed on each blood sample for microfilariae detection. One drop of homogenized EDTA blood was examined under a microscope at low magnification. Microfilaria-positive samples were confirmed by the visualization of live microfilariae moving like snakes between blood cells. In the present study, samples with *D. immitis* antigen-positive, but negative in PCR were considered as heartworm occult infection.

2.4. DNA Extraction

Genomic DNA was extracted individually from all blood samples. The detail of the extraction protocol is described elsewhere [9]. Briefly, DNAs were obtained after two lysis steps using a powder glass and proteinase K for the mechanical and enzymatic digestion, respectively. The extraction was carried out in Biorobot EZ1 System with the EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions. Each extraction was eluted in a total volume of 100 µL and stored at −20 °C until analysis.

2.5. Molecular Screening for Filarioid and Wolbachia DNAs

First, all samples were tested using the combined multiplex approach, recently developed for the detection of filarioid and *Wolbachia* DNAs [27]. The screening was processed as follows: (i) the exploration of filarial and *Wolbachia* DNAs using, respectively, the pan-filarial [Pan-Fil 28S] and the pan-*Wolbachia* [All-Wol-16S] qPCRs (Table 1), (ii) followed by species specific identification from samples tested positive for filarioid-*Wolbachia* DNAs using, respectively, the triplex [Triplex TaqMan COI] qPCR targeting *D. immitis*, *D. repens* and *A. reconditum* and the duplex [Wol-Diro *ftsZ*] qPCR targeting the prokaryotic homolog of the eukaryotic protein tubulin gene (*ftsZ*) of *Wolbachia* of *D. immitis* and that of *D. repens* (Table 1).

Table 1. Primers and probes used in this study.

Application	System Name's	Target Gene	Primer & Probes Name's	Sequences 5′–3′	Amplicon Size (pb)	Tm /Elongation Time	Specificity	References
qPCR	Pan-Fil 28S	28S LSU rRNA	qFil-28S-F	TTGTTTGAGATTGCAGCCCA	151	60 °C/30″	Filarial species	[27]
			qFil-28S-R	GTTTCCATCTCAGCGGTTTC				
			qFil-28S-P	6FAM-5′-CAAGTACCGTGAGGGAAAGT-3′-TAMRA				
	All-Wol-16S	16S rRNA gene	all.Wol.16S.301-F	TGGAACTGAGATACGGTCCAG	177	60 °C/30″	*Wolbachia* sp.	
			all.Wol.16S.478-R	GCACGGAGTTAGCCAGGACT				
			all.Wol.16S.347-P	6FAM-5′-AATATTGGACAATGGGCGAA-3′-TAMRA				
	Triplex TaqMan COI	*cox1*	Fil.COI.749-F	CATCCTGAGGTTTATGTTATTATTTT	166	60 °C/30″	FAM: *D. immitis* VIC: *D. repens* Cy5: *A. reconditum*	
			Fil.COI.914-R	CWGTATACATATGATGRCCYCA				
			D.imm.COI.777-P	6FAM-CGGTGTTTGGGATTGTTAGTG-TAMRA				
			D.rep.COI.871-P	6VIC-TGCTGTTTTAGGTACTTCTGTTTGAG-TAMRA				
			A.rec.COI.866-P	Cy5-TGAATTGCTGTACTGGGAACT-BHQ-3				
	Wol-Diro ftsZ	Prokaryotic homolog of the eukaryotic protein tubulin	WDiro.ftsZ.490-F	AAGCCATTTRGCTTYGAAGGTG	111	60 °C/30″	FAM: *Wolbachia* of *D. immitis* VIC: *Wolbachia* of *D. repens*	
			WDiro.ftsZ.600-R	AAACAAGTTTTGRTTTGGAATAACAAT				
			WDimm.ftsZ.523-P	6FAM-CGTATTGCAGAGCTCGGATTA-TAMRA				
			WDrep.ftsZ.525-P	6VIC-CATTGCAGAACTGGGACTGG-TAMRA				
PCR	16S W-Spec	16S rRNA	W-Specf	CATACC TATTCGAAGGGATAG	438	60 °C/1'	*Wolbachia* sp.	[30]
			W-Specr	AGCTTCGAGTGAA ACCAATTC				
	Pan-Nem 18S	18S SSU rRNA	Fwd.18S.631	TCGTCATTGCTGCGGTTAAA	1127–1155	54 °C/1′30″	Nematodes	[9]
			Rwd.18S.1825r	GGTTCAAGCCACTGCGATTAA				
	Pan-Fil *cox*1	*cox1*	Fwd.957	ATRGTTTATCAGTCTTTTTTTATTGG	509	52 °C/45″	Filarial species	[27]
			Rwd.1465	GCAATYCAAATAGAAGCAAAAGT				
	Pan-Fil 12S	12S rRNA	Fwd.12S.110	TCCAGAATAATCGGCTATACATTTT	497 to 570	56 °C/45″	Filarial species	The present study
			Rwd.12S.681	CCATTGACGGATGGTTTGTA				

2.6. Molecular and Phylogenetic Characterization of Filarioid and Wolbachia

First, all samples that tested positive for filarial DNA were subjected to PCR amplification and sequencing analysis using the following systems: The standard (Pan-Nematoda) PCR [9] was used to amplify 1194 pb from the small subunit rRNA gene and the filarial specific (Pan-fil COI) PCR [27] targeting 509 pb from the Cytochrome c oxidase subunit I (*cox*1) gene. The third PCR was developed to amplify 497–570 pb from the 12S rDNA gene of filarial nematodes (Table 1), while all *Wolbachia*-positive samples were tested using the standard (W16S-Spec) PCR [30] targeting 438 pb fragment from the 16S rDNA.

When the filarial co-infections were found, we performed a serial 2-fold dilution of blood using Hank's balanced salt solution (GIBCO®) followed by a DNA extraction as described above. This was performed in duplicate. The objective was to concentrate only one species of microfilariae before the extraction. Once, the DNA were obtained from each dilution they were processed for amplification using the 18S, *cox*1 and 12S PCR primers, then the last two positive dilution by each PCR were subjected to the sequencing analysis.

All PCR reactions were carried out in a total volume of 50 µL, consisting of 25 µL of AmpliTaq Gold master mix (Thermo Fisher Scientific, Waltham, MA, USA), 18 µL of ultra-purified water DNAse-RNAse free, 1 µL of each primer and 5 µL of genomic DNA. PCR reactions with all systems were run using the following protocol: incubation step at 95 °C for 15 min, 40 cycles of one minute at 95 °C, 30 s for the annealing at a different annealing temperature for each PCR assay and elongation from 45 s to 1 min and 30 s (Table 1) at 72 °C with a final extension for five minutes at 72 °C. PCR reactions were performed in a Peltier PTC-200 model thermal cycler (MJ Research, Inc., Watertown, MA, USA).

DNA amplicons generated through the PCRs were purified using filter-plate Millipore NucleoFast 96 PCR kit following the manufacturer's recommendations (Macherey Nagel, Düren, Germany). Purified DNAs were subjected to the second reaction using the BigDye® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), then the products were purified on the Sephadex G-50 Superfine gel filtration resin prior sequencing on the ABI Prism 3130XL.

Nucleotide sequences were assembled and edited by ChromasPro 2.0.0. The absence of co-amplification of nuclear mitochondrial genes (numts) was verified as recommended [31]. In addition, the visual verification of sequence chromatograms ambiguities, indels and stop codons of the translated sequences were performed by Chromas Pro 2.0.0 software. Sequences amplified from the filarial 18S, *cox*1 and 12S rDNA as well as the 16S rDNA of *Wolbachia* were subjected separately to a preliminary analysis using Basic Local Alignment Search Tool (BLAST) [32].

Filarial (18S, *cox*1 and 12S) and *Wolbachia* (16S) sequences obtained in this study were aligned with the closely related sequences retrieved from GenBank or Worm databases [33]. The alignment was performed using the ClustalW application within Bioedit v. 7.2.5. software [34]. DNA sequences of the non-filarial nematodes *Dracunculus medinensis (*AY852268), *Heliconema longissimum* (GQ332423) and (NC 016127) were used as outgroups for the 18S, *cox*1 and 12S trees, respectively and *Rickettsia* sp. (AB795333) for the *Wolbachia* 16S phylogram. Finally, the best nucleotide substitution model was chosen according to the Akaike Information Criterion (AIC) option in MEGA6 [35]. Phylograms were generated using the maximum likelihood (ML) method based on Kimura 2-parameter (+G) model [36] for both the 18S and the 16S and General Time Reversible (+G, +I) [37], Hasegawa-Kishino-Yano 5+G) models [38] for the *cox*1 and 12S phylograms, respectively. All phylograms were generated with 1000 bootstrap replicates using all sites of the sequences.

3. Results

The detailed results of the dogs tested positive by at least one assay (parasitological, serologic or molecular) are shown in Table 2. The blood wet mount preparation revealed the presence of microfilariae in 6 (6.1%) samples. One of these dogs was a dangerous stray dog that had to be euthanized according to regulations. We performed the autopsy and reported 12 adult filariae (*D. immitis*) in the right ventricle (6 males and 6 females) (Figure 1). The heartworm antigen test detected

15 (15.3%) positive samples. The molecular screening revealed the presence of at least one molecular marker of filarioid and *Wolbachia* in 19 (19.4%) samples. Of these, 7 (7.1%) samples tested positive for both *Wolbachia* and filarial DNAs, 9 (9.2%) samples for filarial DNA only and 3 (3.1%) for *Wolbachia* DNA only.

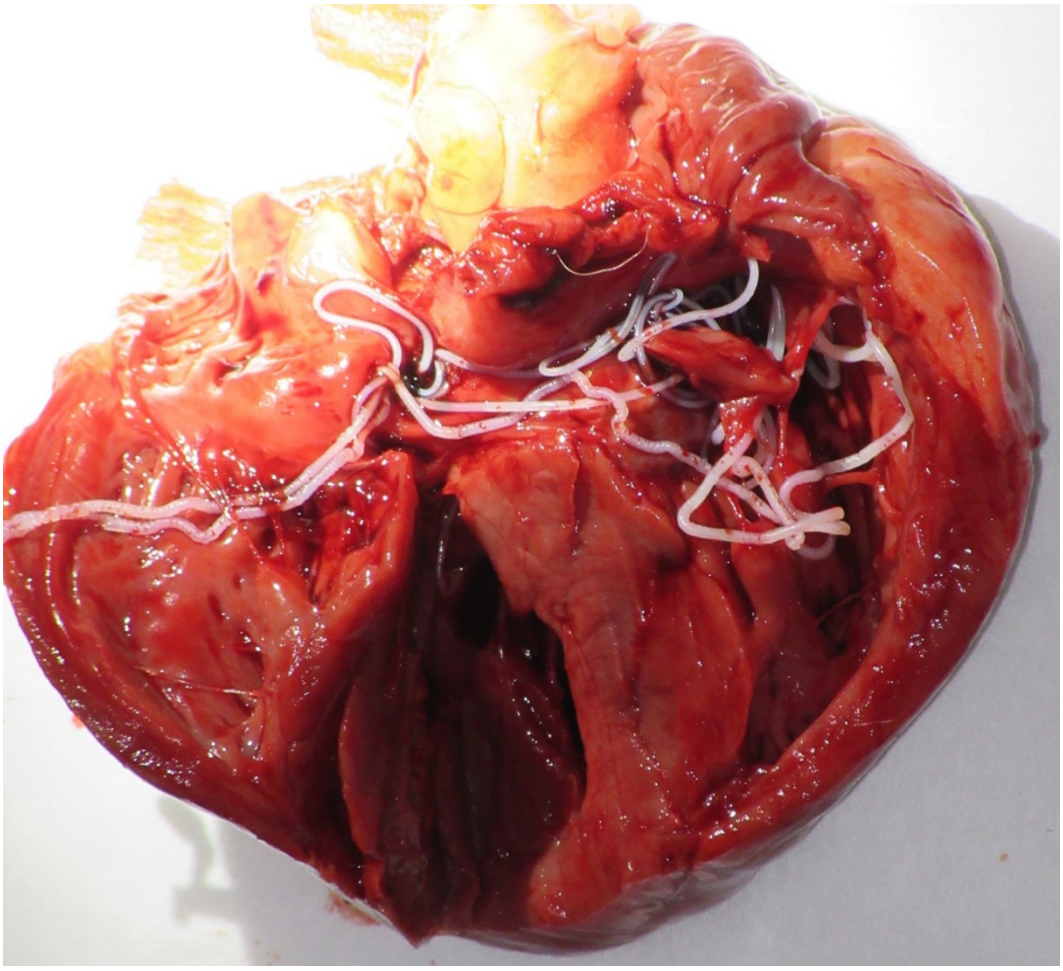


Figure 1. Heartworm (*Dirofilaria immitis*) in the canine heart of dog.

Table 2. Results of dogs positive for at least one assays (parasitologic, serologic and molecular assays).

Sample Description		Parasitology	Serology	Filarioid							*Wolbachia*				Decision
				qPCR-Based Detection			Multi-Locus Genotyping				qPCR-Based Detection		16S Genotyping		
							Accession Number (AN)								
Dog Code	Location	Thin Smear	Witness *Dirofilaria*	Filarial DNA	*D. imm*	*A. rec*	18S	*Cox*1	12S	Species	*Wolbachia*	*WDim*	16S (AN)	*Wolbachia* Clade/Strains	
CMT 01	Cayenne	Neg.	Neg.	Pos.	N/A	Pos.	MN795082 MN795087	MT193075 MT193074	MT252011	*A. reconditum Brugia* sp.	Pos.	N/A	MT231951	*D/WBr.*	*A. reconditum Brugia* sp.
CMT 12	Cayenne	Neg.	Neg.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	Pos.	N/A	MT231952	*D/WBr.*	*Brugia* sp.
CMT 13	Cayenne	Neg.	Neg.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	Pos.	N/A	MT231953	*D/WBr.*	*Brugia* sp.
CMT 14	Cayenne	Neg.	Pos.	Pos.	N/A	Pos.	MN795083	MT193076	MT252012	*A. reconditum*	N/A	N/A	..-..	..-..	*A. reconditum*/ O-Heartworm
CMT 18	Cayenne	Pos.	Neg.	Pos.	N/A	Pos.	MN795084	MT193077	MT252013	*A. reconditum*	N/A	N/A	..-..	..-..	*A. reconditum*
CMT 19	Cayenne	Pos.	Pos.	Pos.	Pos.	N/A	MN795071	MT193078	MT252014	*D. immitis*	Pos.	Pos.	MT231954	*C/WDim.*	*D. immitis*
CMT 32	Kourou	Neg.	Pos.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	N/A	N/A	..-..	..-..	O-Heartworm
CMT 34	Kourou	Neg.	Neg.	Pos.	Pos.	N/A	MN795072	MT193079	MT252015	*D. immitis*	N/A	N/A	..-..	..-..	*D. immitis*
CMT 36	Kourou	Neg.	Pos.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	N/A	N/A	..-..	..-..	O-Heartworm
CMT 38	Kourou	Neg.	Pos.	Pos.	Pos.	N/A	MN795073	MT193080	MT252016	*D. immitis*	N/A	N/A	..-..	..-..	*D. immitis*
CMT 40	Kourou	Pos.	Pos.	Pos.	Pos.	N/A	MN795074	MT193081	MT252017	*D. immitis*	N/A	N/A	..-..	..-..	*D. immitis*
CMT 41	Kourou	Neg.	Pos.	Pos.	Pos.	N/A	MN795075	MT193082	MT252018	*D. immitis*	Pos.	Pos.	MT231955	*C/WDim.*	*D. immitis*
CMT 43	Kourou	Neg.	Neg.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	Pos.	N/A	MT231956	*D/WBr.*	*Brugia* sp.
CMT 52	Kourou	Neg.	Pos.	Pos.	Pos.	N/A	MN795076	MT193083	MT252019	*D. immitis*	N/A	N/A	..-..	..-..	*D. immitis*
CMT 53	Kourou	Neg.	Pos.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	N/A	N/A	..-..	..-..	O-Heartworm

CMT 54	Kourou	Neg.	Pos.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	N/A	N/A	..-..	..-..	O-Heartworm
CMT 61	Cayenne	Neg.	Pos.	N/A	N/A	N/A	..-..	..-..	..-..	..-..	N/A	N/A	..-..	..-..	O-Heartworm
CMT 71	Cayenne	Pos.	Pos.	Pos.	Pos.	N/A	MN795077	MT193084	MT252020	*D. immitis*	Pos.	Pos.	MT231957	*C/WDim.*	*D. immitis*
CMT 75	Cayenne	Pos.	Pos.	Pos.	Pos.	N/A	MN795078	MT193085	MT252021	*D. immitis*	Pos.	Pos.	MT231958	*C/WDim.*	*D. immitis*
CMT 76	Cayenne	Neg.	Neg.	Pos.	Pos.	N/A	MN795079	MT193086	MT252022	*D. immitis*	N/A	N/A	..-..	..-..	*D. immitis*
CMT 89	Cayenne	Neg.	Neg.	Pos.	N/A	N/A	MN795085	..-..	MN795631	*C. bainae*	N/A	N/A	..-..	..-..	*C. bainae*
CMT 90	Cayenne	Neg.	Neg.	Pos.	N/A	N/A	MN795086	..-..	MN795632	*C. bainae*	N/A	N/A	..-..	..-..	*C. bainae*
CMT 91	Cayenne	Pos.	Pos.	Pos.	Pos.	N/A	MN795080	MT193087	MT252023	*D. immitis*	Pos.	Pos.	MT231959	*C/WDim.*	*D. immitis*
CMT 97	Cayenne	Neg.	Pos.	Pos.	Pos.	N/A	MN795081	MT193088	MT252024	*D. immitis*	Pos.	Pos.	MT231960	*C/WDim.*	*D. immitis*

Neg.: negative, Pos.: positive, N/A: no amplification, AN: Accession number, D. imm: *D. immitis*, A. rec: *A. reconditum*, WBr: *Wolbachia* endosymbiont of *Brugia* sp., WDim: *Wolbachia* endosymbiont of *D. immitis*, O-heartworm: occult heartworm.

Of those 16 dogs tested positive for filarial DNA, 11 (11.2%) and 3 (3%) samples were, respectively positive for *D. immitis* and *A. reconditum* by the triplex qPCR. Two samples remained unidentified by this assay. However, the duplex qPCR identified the specific DNA of *Wolbachia* endosymbiont of *D. immitis* in 6 (6.1%) samples. These latter were also positive for *D. immitis* DNA. However, the duplex qPCR did not amplify *Wolbachia* DNA from 4 samples. Compared to the molecular assays, the heartworm antigens were detected in 9 (9.1%) samples among those positive for *D. immitis* DNA and in 5 (6.1%) samples negative for *D. immitis* by qPCR. Of these latter, one sample was positive for *A. reconditum* DNA.

A nearly full-length DNA sequence of the 18S rDNA gene (1194 pb) was obtained from all samples tested positive for filarial DNA (n = 16). Filarioid single species DNA was obtained from 15 of them. The last one was found co-infected and yielded two amplicon sequences (Table S1).

Four genotypes were identified: 11 sequences of *D. immitis* (MN795071 to MN795081) were identical each other and showed 100% identity with *D. immitis* isolated from dogs in Japan (AB973231), 2 similar sequences of *C. bainae* (MN795085, MN795086) were identical to that isolated from dog in the USA (MH390715), 3 sequences of *A. reconditum* (MN795082, MN795083, MN795084) were 100% identical to that isolated from dogs in Côte d'Ivoire (MK495733). Of these, one sequence of *Brugia* sp. (MN795087) closely related to lymphatic filariasis with 99.9% identity with *Brugia malayi* (AF036588) and *Wuchereria bancrofti* (AY843436).

The *cox*1 and 12S sequences were obtained from all *D. immitis* and *A. reconditum* previously amplified by the 18S. *D. immitis cox*1 (MT193078 to MT193088) and 12S (MT252014 to MT252024) sequences were identical to each other for each gene and exhibited 99.78% and 100% of identity, respectively with *cox*1 (MT027229) and 12S (KF707482) sequences of *D. immitis* isolated from dogs, but were different from the virulent strain of *D. immitis* that occurred in Latin America [39]. This was observed for both *cox*1 and 12S, where identity was 91.71% and 95.12% with *cox*1 (HQ540424) and 12S (HQ540423), respectively. However, *A. reconditum cox*1 (MT193075 to MT193077) and 12S (MT252011 to MT252013) sequences showed an identity of 99.55% and 100% of identity with *A. reconditum cox*1 (JF461456) and 12S (AJ544853) sequences, respectively. The *cox*1 sequence of *Brugia* sp. was also amplified (MT193074) and displayed 93.39% identity with *B. timori* (AP017686), 92.73% with *B. malayi* (MK250713) and 91.19% with *B. pahangi* (MK250710). However, the *C. bainae* 12S sequences (MN795631, MN795631) showed, respectively 97.12% and 97% of identity and query cover with *C. bainae* (KF381408) isolated from dogs in Italy. Despite several attempts, the standard PCRs targeting the *cox*1 and the 12S rRNA gene failed to amplify the *C. bainae* and *Brugia* sp., respectively.

Phylogenetic analyses using ML method of the 18S, *cox*1 and 12S genes showed that the isolate of both *D. immitis* and *A. reconditum* from Guiana dogs clustered together with those usually isolated around the world (Figures 2–4). Moreover, both *cox*1 and 12S trees (Figures 3 and 4) indicate that *D. immitis* isolate is distinguished by a clearly separated branch from the virulent isolate from Brazil [39]. The isolate of *Brugia* sp. was clustered with brugian species and was a clearly separated branch although it was placed in the same genus. This was observed for both 18S (Figure 2) and *cox*1 inferences (Figure 3). While *C. bainae* isolates were clustered with the same species isolated from dogs in the USA (Figure 2). However, the 12S tree showed its separation from the isolated one in Europe (Figure 4).

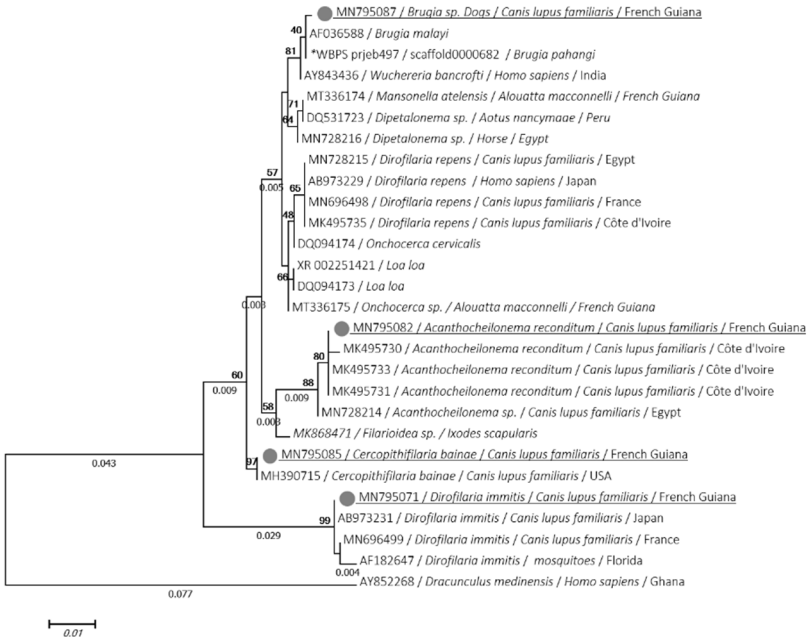


Figure 2. Phylogram of the 18S rRNA gene generated by maximum likelihood method from 28 partial (969) sequences. The tree with the highest log likelihood (−2078,7662) is shown. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 01254)). Numbers above and below branches are the display of bootstrap replicate values and branches length, respectively. Host, geographical location (when available) and GenBank accession number are indicated in each node. The sequences of the present study are underlined. *: indicates DNA sequences retrieved from Worm database.

Figure 3. Phylogram of the *cox*1 gene generated by maximum likelihood method from 29 partial (449) sequences. The tree with the highest log likelihood (−2690.9892) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4403)). Numbers above and below branches are the display of bootstrap replicate values and branches length, respectively. Host, geographical location (when available) and GenBank accession number are indicated in each node. The sequences of the present study are underlined. *: indicates the most virulent strain of *D. immitis* from Latin America [39].

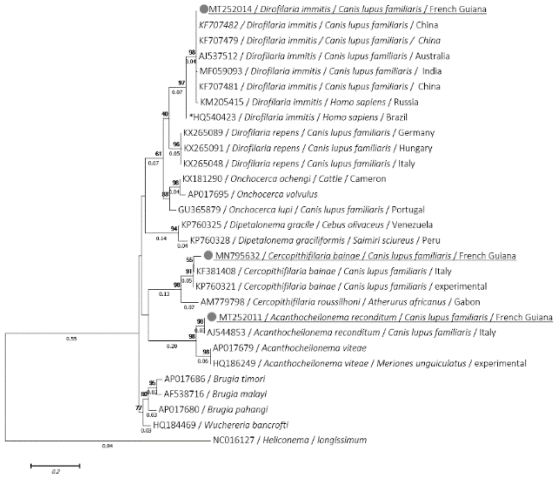


Figure 4. Phylogram of the 12S rRNA gene generated by maximum likelihood method from 29 partial (421) sequences. The tree with the highest log likelihood 5-2356.1682) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6437)). Numbers above and below branches are the display of bootstrap replicate values and branches length, respectively. Host, geographical location (when available) and GenBank accession number are indicated in each node. The sequences of the present study are underlined. *: indicates the most virulent strain of *D. immitis* from Latin America [39].

Wolbachia 16S partial sequences were successfully amplified and sequenced from all samples detected by qPCR. A total of 10 samples were amplified and were split into two distinct genotypes according to the BLAST results. Six identical sequences (MT231954, MT231955, MT231957, MT231958, MT231959, MT231960) showed an identity ranging from 99.3% to 99.6% with *Wolbachia* endosymbiont of *D. immitis* (MH062176, AF088187). These latter were isolated from samples positive for *D. immitis* single-specie-DNA. While three sequences were isolated from samples tested negative for filarial DNA and another one was obtained from a co-infected sample by *A. reconditum* and *Brugia* sp. All were identical to each other (MT231951, MT231952, MT231953, MT231956) and displayed 100% identity with the *Wolbachia* endosymbiont of *B. malayi* (CP034333), *B. pahangi* (AJ012646) and *B. timori* (AJ012646). The phylogenetic inference classified these genotypes into the clade C and D supergroups of *Wolbachia* and were clustered with *Wolbachia* endosymbiont of *D. immitis* and *Brugia* spp., respectively (Figure 5).

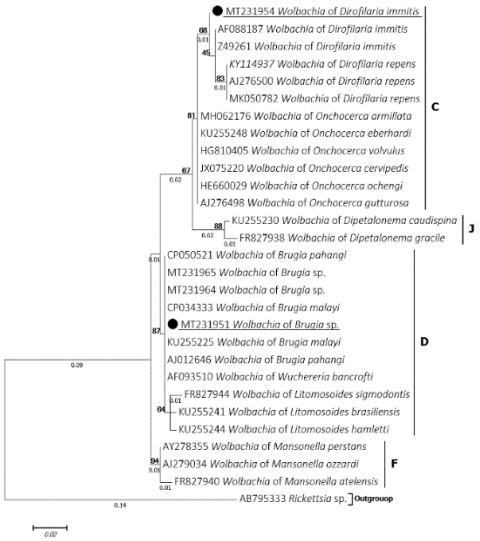


Figure 5. Phylogram of the *Wolbachia* 16S gene generated by maximum likelihood method from 29 partial (295) sequences. The tree with the highest log likelihood (−735.8600) is shown. A discrete

Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2679)). Numbers above and below branches are the display of bootstrap replicate values and branches length, respectively. Host, geographical location (when available) and GenBank accession number are indicated in each node. The sequences of the present study are underlined (black circle).

4. Discussion

This is the first molecular report of filaria and *Wolbachia* infections from dogs in French Guiana. Dogs are the most implicated reservoir for filariasis [9–11,15,17,19]. The control of these vector-borne helminths is based on epidemiological information and the use of adequate diagnostic methods [40]. The present study highlighted the presence of four filarial species in Guiana's dogs: *D. immitis*, *A. reconditum* and for the first time *C. bainae* and *Brugia* sp. Compared to the parasitological assays, the molecular diagnosis remains the most adequate tool to study the diversity of these parasites [28]. This tool had gained an important increase in research area [41,42]. In addition, several studies have recently associated the molecular detection of *Wolbachia* in the diagnosis of canine filariasis [9,10,27].

Despite the several diagnostic assays we performed, our results remained not exhaustive and are limited by the sampling method. In the absence of skin samples, we are not able to achieve the exploration of filarial infections from Guiana's dogs, especially those with cutaneous microfilariae, such as *Onchocerca lupi* and *Cercopithifilaria* spp. [17].

Microfilariae were not found in the blood samples of twelve dogs that tested positive for filarial DNA. The blood wet mount preparation is not a microfilariae concentration test, and only detects an integral microfilariae with a threshold of 30 microfilaria (mf)/mL [43,44]. Due to the lack of an adequate equipment in the field we were not able to perform a microfilariae concentration tests, such as Knott or filtration tests [45]. This represents a limitation of this study, which decrease the accuracy of our results in terms of sensitivity and specificity. Likewise, the molecular assays are able to detect the DNA from a micro-fragment of microfilaria with an analytical sensitivity threshold of 1.5×10^{-4} mf/mL [27].

It is worth highlighting that *D. immitis* infection is the most diagnosed canine filariasis in the world. In French Guiana, this parasite is known to be enzootic, as veterinarians often prescribe chemoprophylaxis. However, epidemiological data are lacking. The *D. immitis* infection rate of 16.3% ($n = 98$) reported here is complementary to a study carried out in Guyana using the Knott's test (14.1%, $n = 2135$) [46] and elsewhere in Venezuela (15.2%, $n = 138$) and in Dominican Republic (18.2%, $n = 104$) [46,47].

In the field as in veterinary clinics, heartworm antigens tests are the most widely used assays. These tests detect the adult antigens of *D. immitis* from both occult and non-occult infections with different levels of sensitivity and specificity [48]. Here, the WITNESS® Dirofilaria test revealed the presence of *D. immitis* antigens in 15 dogs, five of which were considered as an occult infection. Of these, one sample was also found positive for *A. reconditum* DNA. Despite the fact that this filarioid may cross-react on heartworm antigen tests [49], the occult infection cannot be excluded. In Latin America, *A. reconditum* and *D. immitis* are often present as co-infections in dogs [28]. Furthermore, the presence of *Spirocerca lupi* in Guiana dogs [50] could interfere with heartworm antigen and produce such results [41]. On the other hand, no antigens were detected from two *D. immitis* DNA-positive samples (CMT-34, CMT76) (Table 2). This could be related the presence of immune-complex that block the antigen detection process or to the low secretion of antigens by adults worms [8].

To the best of our knowledge, no human infection by *D. immitis* has been reported in French Guiana to date. Since humans can be infected, doctors could be confronted with this parasitosis, which is mainly characterized by the presence of pulmonary nodules. In South America, fifty sporadic cases have been reported: pulmonary (Venezuela, Colombia, Brazil, Argentina) and subcutaneous/ocular nodules (Brazil, Chile) [8,51,52]. *D. immitis* implicated in human ocular dirofilariasis was classified as a more virulent strain [51]. Fortunately, the phylogenetic analysis we

conducted indicated that it is not the virulent *D. immitis* (Figures 3 and 4) which could explain the lower prevalence of human cases [39].

It is not surprising that no dog was found parasitized by *D. repens* responsible for subcutaneous dirofilariasis [34]. This benign, but more zoonotic dirofilariasis is only described in the Old World. In America, a few doubtful cases of *D. repens* were reported [53–55] wherein a lack of information was provided on the origin of the dogs examined. The possibility that this is an imported case cannot be ruled out.

A. reconditum is the causative agent of canine subcutaneous filariasis [15]. This affection, characterized by subcutaneous nodules, is largely neglected as it usually remains unnoticed with no clinical consequences The infection has a worldwide distribution, including the United States, South America, Oceania and many African and European countries [15]. Human infections by *A. reconditum* are quite exceptional. One case (subconjunctival infection) has been described in Australia [36] and two cases in Turkey [56].

Surprisingly, we found the DNA of *C. bainae* in the blood of two dogs. This filarioid was first described in Brazil and has since been reported in Europe, Africa, Australia and Americas [57]. The adult form of these tick-borne filaroids usually dwell beneath the cutaneous tissues of infected dogs, while their microfilaria are distributed unevenly in superficial dermal tissues [58]. Our study constitutes the first report of its DNA in blood. The study conducted by Rojas et. al. (2015), reported the presence of a filarioid DNA from the blood, wherein a low-quality inclusive DNA sequence of a dermal filarioid was detected using the HRM real time qPCR. The same dog was skin-positive for *C. bainae* DNA [57]. These results are not exhaustive and further investigation of this filarioid from skin samples are needed to describe the real prevalence of this filariasis. In addition, our results are complementary to the phylogenetic analysis of *C. bainae* carried out on isolates from Brazil, which revealed the evolutionary separation of isolates from Europe and Latin America [59].

Another surprising finding was the detection of a filarioid species from the genus *Brugia*. Canine brugian infections are often caused by *Brugia* species associated with lymphatic filariasis in Asia (*B. malayi, B. pahangi* and *B. timori*) [12]. While the other brugian infections are encountered in wild animals, such as the Asian primates, raccoons and rabbits in the USA [60]. Recently, in Brazil, blood microfilaria from the genus *Brugia* were reported from the ring-tailed coatis (*Nasua nasua*) [61]. In Guiana, the unique species of *Brugia* was described morphologically from the lymphatic system of the coatimundi (*Nasua nasua vittata*) and was named *Brugia guyanensis* (Orihel 1964) [62]. The possibility that the *Brugia* sp. herein we detected is the same *B. guyanensis* cannot be ruled out in the absence of morphologic identification. Our findings highlighted the circulation of a potential zoonotic *Brugia* in French Guiana dogs. In addition, human health could be at risk if the nematode reported here in domestic dogs had zoonotic potential.

Finally, our results reported the presence of two *Wolbachia* strains from the clade C and D of filarial nematodes, wherein the first one was clustered with those associated with *D. immitis* and the second one with those of lymphatic filarial parasites. In terms of host-relationship, *Wolbachia* strain is filaria species-specific [23], which allows them to serve as a diagnostic target [10,24,45]. Moreover, in heartworm infected dogs and cats, these bacteria are implemented in pulmonary disease [24,25]. Clarifying these bacteria by antibiotic treatments induces infertility and death of filarioid worms and has reduced the incidence of inflammatory pulmonary lesions and thrombi associated with heartworm disease, providing an effective treatment strategy for the control and eradication of the filarial infection in human and dogs [22,26].

5. Conclusions

The present study molecularly detected *D. immitis*, *A. reconditum*, *C. bainae* and *Brugia* sp. and the associated *Wolbachia* endosymbionts from canine blood in French Guiana. *Brugia* sp. and *C. bainae* were detected for the first time in French Guiana dogs. It would be interesting to know whether *Brugia* sp. DNA we detected corresponds to a new species or if it is *Brugia guyanensis*. To this end, a morphologic based-taxonomy should be investigated simultaneously with molecular studies in the future. In addition, further studies based on blood and skin samples are required to

expand the epidemiological knowledge of these nematodes in French Guiana. Finally, there is an urgent need for the implementation of preventive chemoprophylaxis against these vector-borne helminths. The use of the anti-*Wolbachia* drugs should also be explored in the future.

Supplementary Materials: The following are available online at www.mdpi.com/2076-2607/8/5/770/s1, Table S1: PCR/Sequencing results of the 18S.

Author Contributions: Y.L., D.T., J.L.M. and B.D.: conceptualization, methodology; Y.L. and D.T.: formal analysis; J.L.M., S.W.G. and B.D.: field investigations; Y.L., D.T., B.D., O.M.: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the French Armed Forces Health Service, the "Association pour la Recherche en Infectiologie", Ceva Santé Animale company and the Institut Hospitalo-Universitaire Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: We thank Christophe Calleja (Zoetis) for supply of serologic kits.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Dantas-Torres, F. Canine vector-borne diseases in Brazil. *Parasit. Vectors* **2008**, *1*, 25, doi:10.1186/1756-3305-1-25.
2. Otranto, D.; Dantas-Torres, F.; Breitschwerdt, E.B. Managing canine vector-borne diseases of zoonotic concern: Part one. *Trends Parasitol.* **2009**, *25*, 157–163, doi:10.1016/j.pt.2009.01.003.
3. Medkour, H.; Davoust, B.; Dulieu, F.; Maurizi, L.; Lamour, T.; Marié, J.-L.; Mediannikov, O. Potential animal reservoirs (dogs and bats) of human visceral leishmaniasis due to *Leishmania infantum* in French Guiana. *PLoS Negl. Trop. Dis.* **2019**, *13*, e0007456, doi:10.1371/journal.pntd.0007456.
4. Dahmani, M.; Marié, J.-L.; Mediannikov, O.; Raoult, D.; Davoust, B. First Identification of *Anaplasma* platys in the blood of dogs from French Guiana. *Vector Borne Zoonotic Dis.* **2015**, *15*, 170–172, doi:10.1089/vbz.2014.1720.
5. Tahir, D.; Davoust, B.; Heu, K.; Lamour, T.; Demar, M.; Marié, J.-L.; Blanchet, D. Molecular and serological investigation of *Trypanosoma cruzi* infection in dogs in French Guiana. *Vet. Parasitol. Reg. Stud. Rep.* **2017**, *12*, 106–109, doi:10.1016/j.vprsr.2017.06.004.
6. Davoust, B.; Keundjian, A.; Rous, V.; Maurizi, L.; Parzy, D. Validation of chemoprevention of canine monocytic ehrlichiosis with doxycycline. *Vet. Microbiol.* **2005**, *107*, 279–283, doi:10.1016/j.vetmic.2005.02.002.
7. Llagonne-Barets, M.; Icard, V.; Leparc-Goffart, I.; Prat, C.; Perpoint, T.; Andre, P.; Ramière, C. A case of Mayaro virus infection imported from French Guiana. *J. Clin. Virol.* **2016**, *77*, 66–68, doi:10.1016/j.jcv.2016.02.013.
8. Simón, F.; Siles-Lucas, M.; Morchón, R.; González-Miguel, J.; Mellado, I.; Carretón, E.; Montoya-Alonso, J.A. Human and animal dirofilariasis: The emergence of a zoonotic mosaic. *Clin. Microbiol. Rev.* **2012**, *25*, 507–544, doi:10.1128/cmr.00012-12.
9. Laidoudi, Y.; Ringot, D.; Watier-Grillot, S.; Davoust, B.; Mediannikov, O. A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France. *Parasite* **2019**, *26*, 72, doi:10.1051/parasite/2019073.
10. Satjawongvanit, H.; Phumee, A.; Tiawsirisup, S.; Sungpradit, S.; Brownell, N.; Siriyasatien, P.; Preativatanyou, K. Molecular analysis of canine filaria and its *Wolbachia* endosymbionts in domestic dogs collected from two animal university hospitals in Bangkok Metropolitan Region, Thailand. *Pathogens* **2019**, *8*, 114, doi:10.3390/pathogens8030114.
11. Ambily, V.; Pillai, U.N.; Arun, R.; Pramod, S.; Jayakumar, K. Detection of human filarial parasite *Brugia malayi* in dogs by histochemical staining and molecular techniques. *Vet. Parasitol.* **2011**, *181*, 210–214, doi:10.1016/j.vetpar.2011.04.041.
12. Ravindran, R.; Varghese, S.; Nair, S.N.; Balan, V.M.; Lakshmanan, B.; Ashruf, R.M.; Kumar, S.S.; Gopalan, A.K.K.; Nair, A.S.; Malayil, A.; et al. Canine filarial infections in a human *Brugia malayi* endemic area of India. *BioMed Res. Int.* **2014**, *2014*, 1–9, doi:10.1155/2014/630160.

13. Drake, J.; Wiseman, S. Increasing incidence of *Dirofilaria immitis* in dogs in USA with focus on the southeast region 2013–2016. *Parasit. Vectors* **2018**, *11*, 39, doi:10.1186/s13071-018-2631-0.
14. World Health Organization. Lymphatic Filariasis. Available online: https://www.who.int/news-room/fact-sheets/detail/lymphatic-filariasis (accessed on 12 May 2020).
15. Brianti, E.; Gaglio, G.; Napoli, E.; Giannetto, S.; Dantas-Torres, F.; Bain, O.; Otranto, D. New insights into the ecology and biology of *Acanthocheilonema reconditum* (Grassi, 1889) causing canine subcutaneous filariosis. *Parasitology* **2012**, *139*, 530–536, doi:10.1017/s0031182011002198.
16. Otranto, D.; Brianti, E.; Latrofa, M.S.; Annoscia, G.; Weigl, S.; Lia, R.P.; Gaglio, G.; Napoli, E.; Giannetto, S.; Papadopoulos, E.; et al. On a *Cercopithifilaria* sp. transmitted by *Rhipicephalus sanguineus*: A neglected, but widespread filarioid of dogs. *Parasit.Vectors* **2012**, *5*, 1, doi:10.1186/1756-3305-5-1.
17. Cortes, H.; Cardoso, L.; Giannelli, A.; Latrofa, M.S.; Dantas-Torres, F.; Otranto, D. Diversity of *Cercopithifilaria* species in dogs from Portugal. *Parasit. Vectors* **2014**, *7*, 261, doi:10.1186/1756-3305-7-261.
18. Nelson, G.S. *Dipetalonema reconditum* (Grassi, 1889) from the dog with a note on its development in the flea, *Ctenocephalides felis* and the louse, *Heterodoxus spiniger*. *J. Helminthol.* **1962**, *36*, 297, doi:10.1017/s0022149x00023968.
19. Muñoz, C.; Gonzálvez, M.; Rojas, A.; Martínez-Carrasco, C.; Baneth, G.; Berriatua, E.; Ortiz, J. Massive microfilaremia in a dog subclinically infected with *Acanthocheilonema dracunculoides*. *Parasitol. Int.* **2020**, *76*, 102070.
20. Franchini, D.; Giannelli, A.; Di Paola, G.; Cortes, H.; Cardoso, L.; Lia, R.P.; Campbell, B.; Dantas-Torres, F.; Lenoci, D.; Abu Assad, E.; et al. Image diagnosis of zoonotic onchocercosis by *Onchocerca lupi*. *Vet. Parasitol.* **2014**, *203*, 91–95, doi:10.1016/j.vetpar.2014.03.007.
21. Martin, C.; Gavotte, L. The bacteria *Wolbachia* in filariae, a biological Russian dolls' system: New trends in antifilarial treatments. *Parasite* **2010**, *17*, 79–89, doi:10.1051/parasite/2010172079.
22. Sabūnas, V.; Radzijevskaja, J.; Sakalauskas, P.; Petkevicius, S.; Karvelienė, B.; Žiliukienė, J.; Lipatova, I.; Paulauskas, A. *Dirofilaria repens* in dogs and humans in Lithuania. *Parasit. Vectors* **2019**, *12*, 177, doi:10.1186/s13071-019-3406-y.
23. Bandi, C.; Anderson, T.J.C.; Genchi, C.; Blaxter, M. Phylogeny of *Wolbachia* in filarial nematodes. *Proc. R. Soc. B Boil. Sci.* **1998**, *265*, 2407–2413, doi:10.1098/rspb.1998.0591.
24. Turba, M.E.; Zambon, E.; Zannoni, A.; Russo, S.; Gentilini, F. Detection of *Wolbachia* DNA in blood for diagnosing filaria-associated syndromes in cats. *J. Clin. Microbiol.* **2012**, *50*, 2624–2630, doi:10.1128/jcm.00528-12.
25. Dingman, P.; Levy, J.K.; Kramer, L.H.; Johnson, C.M.; Lappin, M.R.; Greiner, E.C.; Courtney, C.H.; Tucker, S.J.; Morchón, R. Association of *Wolbachia* with heartworm disease in cats and dogs. *Vet. Parasitol.* **2010**, *170*, 50–60, doi:10.1016/j.vetpar.2010.01.037.
26. Maia, C.; Altet, L.; Serrano, L.; Cristóvão, J.; Tabar, M.-D.; Francino, O.; Cardoso, L.; Campino, L.; Roura, X. Molecular detection of *Leishmania infantum*, filariae and *Wolbachia* spp. in dogs from southern Portugal. *Parasit. Vectors* **2016**, *9*, 170, doi:10.1186/s13071-016-1452-2.
27. Laidoudi, Y.; Davoust, B.; Varloud, M.; El Hadj, A.N.; Fenollar, F.; Mediannikov, O. Development of a multiplexed qPCRs-based approach for the diagnosis of *Dirofilaria immitis*, *D. repens*, *Acanthocheilonema reconditum* and the others filariosis. *BioRxiv* **2019**, *24*, 842575, doi:10.1101/842575.
28. De Argôlo, E.G.G.; Reis, T.; Fontes, D.A.T.; Gonçalves, E.C.; Giese, E.G.; Melo, F.T.V.; Dos Santos, J.N.; Furtado, A.P. Canine filariasis in the Amazon: Species diversity and epidemiology of these emergent and neglected zoonoses. *PLoS ONE* **2018**, *13*, e0200419, doi:10.1371/journal.pone.0200419.
29. Schneider, M.C.; Aguilera, X.; Junior, J.B.D.S.; Ault, S.K.; Najera, P.; Martínez, J.; Requejo, R.; Nicholls, R.S.; Yadon, Z.E.; Silva, J.C.; et al. Elimination of neglected diseases in Latin America and the Caribbean: A mapping of selected diseases. *PLoS Negl. Trop. Dis.* **2011**, *5*, e964, doi:10.1371/journal.pntd.0000964.
30. Werren, J.H.; Windsor, D.M. *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? *Proc. R. Soc. B Boil. Sci.* **2000**, *267*, 1277–1285, doi:10.1098/rspb.2000.1139.
31. Song, H.; Buhay, J.E.; Whiting, M.F.; Crandall, K.A. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 13486–13491, doi:10.1073/pnas.0803076105.
32. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410.

33. Howe, K.L.; Bolt, B.J.; Shafie, M.; Kersey, P.; Berriman, M. WormBase ParaSite—A comprehensive resource for helminth genomics. *Mol. Biochem. Parasitol.* **2017**, *215*, 2–10.
34. Hall, T.; Biosciences, I.; Carlsbad, C. BioEdit: An important software for molecular biology. *GERF Bull. Biosci.* **2011**, *2*, 60–61.
35. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Boil. Evol.* **2013**, *30*, 2725–2729, doi:10.1093/molbev/mst197/.
36. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120, doi:10.1007/bf01731581.
37. Nei, M.; Kumar, S. *Molecular Evolution and Phylogenetics*; Oxford University Press: New York, NY, USA, **2000**, p.333.
38. Hasegawa, M.; Kishino, H.; Yano, T. aki Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol*. **1985**, *22*,160–174.
39. Dantas-Torres, F.; Otranto, D. Dirofilariosis in the Americas: A more virulent *Dirofilaria immitis*? *Parasit. Vectors* **2013**, *6*, 288, doi:10.1186/1756-3305-6-288.
40. Otranto, D.; Dantas-Torres, F.; Breitschwerdt, E. Managing canine vector-borne diseases of zoonotic concern: Part two. *Trends Parasitol.* **2009**, *25*, 228–235, doi:10.1016/j.pt.2009.02.005.
41. Aroch, I.; Rojas, A.; Slon, P.; Lavy, E.; Segev, G.; Baneth, G. Serological cross-reactivity of three commercial in-house immunoassays for detection of *Dirofilaria immitis* antigens with *Spirocerca lupi* in dogs with benign esophageal spirocercosis. *Vet. Parasitol.* **2015**, *211*, 303–305, doi:10.1016/j.vetpar.2015.06.010.
42. Mallawarachchi, C.H.; Chandrasena, N.T.G.A.; Wickramasinghe, S.; Premaratna, R.; Gunawardane, N.Y.I.S.; Mallawarachchi, N.S.M.S.M.; De Silva, N.R. A preliminary survey of filarial parasites in dogs and cats in Sri Lanka. *PLoS ONE* **2018**, *13*, e0206633, doi:10.1371/journal.pone.0206633.
43. Bowman, D.D.; Mannella, C. Macrocyclic lactones and *Dirofilaria immitis* microfilariae. *Top. Companion Anim. Med.* **2011**, *26*, 160–172, doi:10.1053/j.tcam.2011.07.001.
44. Rossi, M.I.D.; Paiva, J.; Bendas, A.; Mendes-De-Almeida, F.; Knackfuss, F.; Miranda, M.; Guerrero, J.; Fernandes, O.; Labarthe, N.V. Effects of doxycycline on the endosymbiont *Wolbachia* in *Dirofilaria immitis* (Leidy, 1856)—Naturally infected dogs. *Vet. Parasitol.* **2010**, *174*, 119–123, doi:10.1016/j.vetpar.2010.07.019.
45. Evans, C.C.; Bradner, J.L.; Savadelis, M.D.; Nelson, C.T.; Moorhead, A.R. Acetic acid as an alternative reagent in the modified Knott test. *Vet. Parasitol.* **2019,** *276*,108975, doi: 10.1016/j.vetpar.2019.108975.
46. Orihal, T.C. Canine filariasis in British Guiana. *J. Parasitol*. **1964**, *50*, 33.
47. Guilarte, D.V.; Martínez, E.G.; El Hen, F.; Guzmán, R.; Blondell, D.; Díaz, M.T.; Santiago, J. Diagnóstico de *Dirofilaria immitis* en el municipio Sucre, estado Sucre, Venezuela. *Bol. Mal. Salud Amb*. **2011**, *51*, 51–58.
48. Duran-Struuck, R.; Jost, C.; Hernandez, A.H. *Dirofilaria immitis* prevalence in a canine population in the Samana Peninsula (Dominican Republic) – June 2001. *Vet. Parasitol.* **2005**, *133*, 323–327, doi:10.1016/j.vetpar.2005.06.008.
49. Gillis, J.M.; Smith, R.D.; Todd, K.S. Diagnostic criteria for an enzyme-linked immunosorbent assay for occult heartworm disease: Standardization of the test system in naturally exposed dogs. *Am. J. Veter Res.* **1984**, *45*, 2289–2292.
50. Bartolo, A. La spirocercose canine à *Spirocerca lupi*. *Point Vét.* **2006**, *269*, 20-26.
51. Bain, O.; Otranto, D.; Diniz, D.; Dos Santos, J.N.; De Oliveira, N.P.; Almeida, I.; De Almeida, R.N.F.; De Almeida, L.N.F.; Dantas-Torres, F.; Sobrinho, E.F.D.A. Human intraocular filariasis caused by *Pelecitus* sp. Nematode, Brazil. *Emerg. Infect. Dis.* **2011**, *17*, 867–869. doi:10.3201/eid1705.101309.
52. Mar, P.-H.; Yang, I.-C.; Chang, G.-N.; Fei, C.-Y. Specific polymerase chain reaction for differential diagnosis of *Dirofilaria immitis* and *Dipetalonema reconditum* using primers derived from internal transcribed spacer region 2 (ITS2). *Vet. Parasitol.* **2002**, *106*, 243–252, doi:10.1016/s0304-4017(02)00032-8.
53. López, J.; Valiente-Echeverría, F.; Carrasco, M.; Mercado, R.; Abarca Villaseca, K. Morphological and molecular identification of canine filariae in a semi-rural district of the Metropolitan Region in Chile. *Rev. Chil. Infectol*. **2012**, *29*, 284–289.
54. Ramos-Lopez, S.; León-Galván, M.F.; Salas-Alatorre, M.; Lechuga-Arana, A.A.; Valencia-Posadas, M.; Gutiérrez-Chávez, A.J. First molecular identification of *Dirofilaria repens* in a dog blood sample from Guanajuato, Mexico. *Vector Borne Zoonotic Dis.* **2016**, *16*, 734–736, doi:10.1089/vbz.2016.1948.
55. Lent, H.; De Freitas, J.F.T. Dirofilariose sub-cutanea dos cães no Brasil. *Memórias Inst. Oswaldo Cruz* **1937**, *32*, 443–448, doi:10.1590/S0074-02761937000300007.

56. Huynh, T.; Thean, J.; Maini, R. *Dipetalonema reconditum* in the human eye. *Br. J. Ophthalmol.* **2001**, *85*, 1391–1392.
57. Rojas, A.; Rojas, D.; Montenegro, V.M.; Baneth, G. Detection of *Dirofilaria immitis* and other arthropod-borne filarioids by an HRM real-time qPCR, blood-concentrating techniques and a serological assay in dogs from Costa Rica. *Parasit. Vectors* **2015**, *8*, 170, doi:10.1186/s13071-015-0783-8.
58. Otranto, D.; Brianti, E.; Abramo, F.; Gaglio, G.; Napoli, E.; Latrofa, M.S.; Ramos, R.A.; Dantas-Torres, F.; Bain, O. Cutaneous distribution and localization of *Cercopithifilaria* sp. microfilariae in dogs. *Vet. Parasitol.* **2012**, *190*, 143–150, doi:10.1016/j.vetpar.2012.05.016.
59. Ramos, R.A.N.; Rêgo, A.G.D.O.D.; Firmino, E.D.D.F.; Ramos, C.A.D.N.; De Carvalho, G.A.; Dantas-Torres, F.; Otranto, D.; Alves, L.C. Filarioids infecting dogs in northeastern Brazil. *Vet. Parasitol.* **2016**, *226*, 26–29, doi:10.1016/j.vetpar.2016.06.025.
60. Paniz-Mondolfi, A.E.; Gárate, T.; Stavropoulos, C.; Fan, W.; González, L.M.; Eberhard, M.; Kimmelstiel, F.; Sordillo, E.M. Zoonotic filariasis caused by novel *Brugia* sp. nematode, United States, 2011. *Emerg. Infect. Dis*. **2014**, *20*, 1248–1250.
61. Moraes, M.F.D.; Da Silva, M.X.; Magalhães-Matos, P.C.; Albuquerque, A.C.A.; Tebaldi, J.H.; Mathias, L.A.; Hoppe, E.G.L. Filarial nematodes with zoonotic potential in ring-tailed coatis (*Nasua nasua* Linnaeus, 1766, Carnivora: Procyonidae) and domestic dogs from Iguaçu National Park, Brazil. *Vet. Parasitol. Reg. Stud. Rep.* **2017**, *8*, 1–9, doi:10.1016/j.vprsr.2017.01.003.
62. Orihel, T.C. *Brugia guyanensis* sp. n. (Nematoda: Filarioidea) from the Coatimundi (*Nasua nasua vittata*) in British Guiana. *J. Parasitol.* **1964**, *50*, 115, doi:10.2307/3276043.

Publication N°13

A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France

Younes Laidoudi[1,2], David Ringot[3], Stéphanie Watier-Grillot[4], Marie Varloud[5], Oleg Mediannikov[1,2] and Bernard Davoust[1,2,4*]

[1] Aix Marseille Univ, IRD, APHM, MEPHI, IHU Méditerranée Infection, Marseille, France
[2] IHU Méditerranée Infection, Marseille, France
[3] French military health service, 33e Veterinary Group, Tours, France
[4] French military health service, Animal epidemiology working group, Marseille, France
[5] Ceva Santé Animale, Libourne, France

ARTICLE DE RECHERCHE

Publié dans : Parasite, 2019, 26, 72

(doi.10.1051/parasite/2019073)

Parasite **26**, 72 (2019)

https://doi.org/10.1051/parasite/2019073

PARASITE
Available online at:
www.parasite-journal.org

RESEARCH ARTICLE OPEN ACCESS

A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France

Younes Laidoudi[1,2], David Ringot[3], Stéphanie Watier-Grillot[4,5], Bernard Davoust[1,2,4,*], and Oleg Mediannikov[1,2]

[1] Aix Marseille Univ, IRD, APHM, MEPHI, IHU Méditerranée Infection, 13385, Marseille, France
[2] IHU Méditerranée Infection, 13385 Marseille, France
[3] French Military Health Service, 33e Veterinary Group, 37076 Tours, France
[4] Animal Epidemiology Working Group of the Military Health Service, 13014 Marseille, France
[5] French Armed Forces Centre for Epidemiology and Public Health (CESPA), 13014 Marseille, France

Received 29 November 2019, Accepted 5 December 2019, Published online 16 December 2019

Abstract – Canine dirofilarioses are nematode infections caused by two species of the genus *Dirofilaria: D. immitis* and *D. repens*. We describe here an outbreak of *D. immitis* and *D. repens* infection in military working dogs (MWDs) housed in a kennel in the Indre department (centre of France). Out of a total of 17 dogs, 6 (35.2%) tested positive for *D. immitis*, *D. repens* or both parasites. Infested dogs were treated and prophylactic measures were implemented for the entire kennel staff. To our knowledge, this is the first documented description of an outbreak of canine cardiopulmonary dirofilariasis in the center of France, unlike in the south of this country, where *D. immitis* and *D. repens* dirofilariasis are enzootic. In France, as mosquito vectors expand their territory and new non-native vectors are introduced, it is likely that the distribution area of these two diseases of domestic and wild carnivores will be wider and underestimated.

Key words: *Dirofilaria immitis*, *Dirofilaria repens*, Heartworm disease, PCR, Military working dog, France.

Résumé – Un foyer de dirofilariose canine cardiaque et sous-cutanée dans un chenil du centre de la France. Les dirofilarioses canines sont des infections à nématodes causée par deux espèces du genre *Dirofilaria*, *D. immitis* et *D. repens*. Nous décrivons ici un foyer d'infection à *D. immitis* et *D. repens* chez des chiens militaires hébergés dans un chenil dans le département de l'Indre (centre de la France). Sur un total de 17 chiens, 6 (35, 2 %) ont été testés positifs pour *D. immitis*, pour *D. repens* ou pour les deux parasites. Les chiens infestés ont été traités et des mesures prophylactiques ont été mises en place pour tout le personnel du chenil. À notre connaissance, il s'agit de la première description documentée d'un foyer de dirofilariose cardiopulmonaire canine dans le centre de la France, contrairement au sud du pays, où les dirofilarioses à *D. immitis* et *D. repens* sont enzootiques. En France, à mesure que les moustiques vecteurs élargissent leur territoire et que de nouveaux vecteurs non indigènes sont introduits, il est probable que la zone de distribution de ces deux maladies des carnivores domestiques et sauvages sera plus étendue et sous-estimée.

Introduction

Dirofilaria immitis (Leidy, 1856) [23] and *Dirofilaria repens* Railliet & Henry, 1911 [33] are mosquito-borne filarioids (Nematoda: Onchocercidae) infecting wild and domestic mammals of different orders with canids as the predominant definitive hosts. Adult worms of *D. immitis* with a smooth cuticle (measuring between 12 cm and 30 cm in length) colonize the pulmonary arteries and right heart cavities, whereas adult worms of *D. repens,* with a finely striated cuticle, (measuring between 5 cm and 17 cm in length) are located in the subcutaneous tissues [11, 26, 28]. About 120 days after infection of the mammalian host, the viviparous female can be fertilized and produces mobile embryos called microfilariae. Microfilariae (L1 stage) parasitize the blood until being ingested by the mosquito vector (Culicidae) during a blood meal taken on a microfilaremic host. Inside the vector, microfilariae first develop into larval stage 1 (L1), then molt into larval stage 2 (L2), and finally molt into third-stage larvae (L3), which is the infective stage. Clinically, *D. immitis* infection (canine heartworm disease) can remain unapparent for a long time. Symptoms are mainly dominated by right heart failure, ranging from exercise intolerance and fatigue to cardiac decompensation with swelling and possible acute pulmonary oedema [12, 26, 29]. In contrast, the infestation by *D. repens* often passes unnoticed. Dogs infected with *D. repens* microfilariae

*Corresponding author: bernard.davoust@gmail.com

may sometimes develop subcutaneous nodules where adult worms encyst. Sometimes, the disease manifests with more symptoms, such as pruritus and skin changes [38].

Canine dirofilariosis has been known in France for a long time. It was in 1679 that Panhot highlighted heartworm in a dog native to the Dombes region (near Lyon) [30]. Over the last century, several studies have shown the importance of this parasitosis in the Mediterranean region [8, 18]. At the beginning of the 1980s, the infestation rate of military working dogs (MWDs) was high, particularly in Corsica and the Bouches-du-Rhône department [5, 8]. Out of 180 MWDs tested in 1988, 67 (37%) carried microfilariae in the blood, including 17 cases of *D. immitis*, 2 of *D. repens*, 15 mixed infestations and 3 doubtful ones [5]. A screening test carried out on 85 MWDs from northern France (Brittany and Normandy) was negative [6]. The MWDs with heartworm disease may lose their operational fitness, causing an operational impact for the Armed Forces. In fact, MWDs military dogs are used for the detection of explosive devices as well as to secure military sites. The eradication of canine heartworm disease among military dogs in south-eastern France was made possible by the implementation, for the first time in France, of chemoprophylaxis based on the use of ivermectin, completed by a conclusive trial on the efficacy of melarsomine in treating adult *Dirofilaria* [4]. The first demonstration of the long-term effectiveness of this protocol was made in 2015 by comparing the incidence rates of *D. immitis* infestation in MWDs from a kennel located in Corsica and in co-located civilian dogs (the civilian kennel was less than 15 km from the military kennel). The estimated prevalence in civilian dogs was 40.4% (19/47), while no cases were identified in MWDs [36]. Currently, canine dirofilarioses chemoprophylaxis is applied to all MWDs deployed on missions outside mainland France, as well as to MWDs from mainland France located in south-eastern France, during the period of vector activity (May–November). In the present study, we revealed the existence of an indigenous outbreak of *D. immitis* and *D. repens* infestation in the French department of Indre. To the best of our knowledge, this is the first documented description of an outbreak of canine cardiopulmonary dirofilariosis in this region of France.

Materials and methods

Dogs

In October 2018, we performed blood tests on the 17 apparently healthy dogs in the military kennel located near the city of Rosnay (46°42′47″N, 1°14′39″E), in Indre, central France. The commune is located in the "Parc naturel régional de la Brenne". The MWDs, including 11 Belgian Malinois and 6 German Shepherds, were all males aged 2–10 years, with a median of 5 years. The duration of the dogs' presence in the kennel ranged from 6 months to 8.5 years, with a median of 4 years. MWDs, including dogs from the Rosnay kennel, are purchased abroad, mainly in eastern European countries (Germany and Poland, in particular). They arrived at the kennel at about 2 years of age. They received regular veterinary care. From May to October, they benefit from a treatment against ectoparasites, but which has no repellent effect on mosquitoes. For the purpose of the study, two blood samples of 4–5 mL volume were taken from each dog and collected from a dry tube and a citrated tube, respectively. Serum was collected after centrifugation (10 min, 3000 g). Each animal sampled was examined clinically.

Ethics statement

Blood samples were collected in accordance with the requirements of the Animal Ethics Procedures of French veterinarians and with the consent of the owner of the animals (French Armed Forces).

Direct detection of microfilariae

Modified Knott's staining was chosen to identify canine blood microfilaria [21, 25]. One millilitre of citrated blood was mixed with 9 mL of hemolyzing solution (2% acetic acid) in a 15 mL tube, followed by centrifugation for minutes at 500 rpm. The supernatant was removed; the sediment was stained with methylene-blue, transferred to a slide, and covered with a cover slip.

Heartworm antigen detection

Heartworm antigen detection was performed using two rapid diagnostic serological tests marketed in France and targeting the antigen secreted by the adult female worm: (i) a WITNESS® Dirofilaria test (Zoetis, Lyon, France), based on immunomigration (RIM®) technology, and (ii) a DiroCHEK® test (Zoetis, Lyon, France), based on an enzyme-linked immunosorbent assay method known as a sandwich ELISA. Both tests provide rapid results, as well as high sensitivity (99% for WITNESS® Dirofilaria and 98% for DiroCHEK®) and specificity (94% for WITNESS® Dirofilaria and 96% for DiroCHEK®) [17].

Molecular detection of filaria and the Wolbachia endosymbiont of filaria

DNA was extracted from 100 µL of citrated blood, after 4-hour digestion at 56 °C, using an equal volume of buffer G2 supplemented with 15% proteinase K. The extraction was performed using the Biorobot EZ1 System with the EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France), in line with the manufacturer's instructions. DNA was eluted in a final volume of 100 µL and stored at −20 °C. All samples were screened for filaria and their *Wolbachia* complex, using the fast typing approach which consists in a pan-filarial 28S-based qPCR system, followed by a triplex COI-based system targeting *D. immitis*, *D. repens* and *Acanthocheilonema reconditum,* and the duplex ftsZ-based system, targeting specifically the *Wolbachia* endosymbiont of *D. immitis* and that of *D. repens* (Table 1) [22]. Once the specific filarial DNA has been revealed by the qPCR triplex, the sample is considered positive, and the detection of *Wolbachia* DNA confirms infection by *D. immitis* or *D. repens* related to this strain.

Table 1. Primers and probes used in this study.

System name	Primer & probe name	Sequences 5′–3′	Specificity	References
Pan-fil 28S qPCR-based system	qFil-28S-F	TTGTTTGAGATTGCAGCCCA	Filariae	[22]
	qFil-28S-P	6FAM-5′-CAAGTACCGTGAGGGAAAGT-3′-TAMRA		
	qFil-28S-R	GTTTCCATCTCAGCGGTTTC		
All-Wol 16S qPCR-based system	all.Wol.16S.301-F	TGGAACTGAGATACGGTCCAG	Wolbachieae	
	all.Wol.16S.347-P	6FAM-5′-AATATTGGACAATGGGCGAA-3′-TAMRA		
	all.Wol.16S.478-R	GCACGGAGTTAGCCAGGACT		
Triplex TaqMan COI qPCR-based system	Fil.COI.749-F	CATCCTGAGGTTTATGTTATTATTTT		
	D.imm.COI.777-P	6FAM-CGGTGTTTGGGATTGTTAGTG-TAMRA	*Dirofilaria immitis*	
	D.rep.COI.871-P	6VIC-TGCTGTTTTAGGTACTTCTGTTTGAG-TAMRA	*Dirofilaria repens*	
	A.rec.COI.866-P	Cy5-TGAATTGCTGTACTGGGAACT-BHQ-3	*Acanthocheilonema reconditum*	
	Fil.COI.914-R	CWGTATACATATGATGRCCYCA		
Duplex Wol-Diro ftsZ qPCR-based system	WDiro.ftsZ.490-F	AAGCCATTTRGCTTYGAAGGTG	*Wolbachia* endosymbiont of *D. immitis* and *D. repens*	
	WDimm.ftsZ.523-P	6FAM-CGTATTGCAGAGCTCGGATTA-TAMRA		
	WDrep.ftsZ.525-P	6VIC-CATTGCAGAACTGGGACTGG-TAMRA		
	WDiro.ftsZ.600-R	AAACAAGTTTTGRTTTGGAATAACAAT		
Duplex HWs COI qPCR-based system	Hw.COI.723-F	TCAGCATTTGTTTTGGTTTTT		
	D.imm.COI.777-P	6FAM-CGGTGTTTGGGATTGTTAGTG-TAMRA	*D. immitis*	
	A.vas.COI.813-P	6VIC-TGACTGGGAAGAAGGAGGTG-TAMRA	*Angiostrongylus vasorum*	
	Hw.COI.950-R	GCASTAAAATAAGYACGAGWATC		
Pan-Nematoda primers 18S PCR-based system	Fwd.18S.631	TCGTCATTGCTGCGGTTAAA	Nematoda	This study
	Rwd.18S.1825r	GGTTCAAGCCACTGCGATTAA		

Sequencing analysis and phylogenetic genotyping of filaria

Samples harbouring a single DNA of filaria were subjected to sequencing analysis. The pan-Nematoda primers named Fwd.18S.631 & Rwd.18S.1825r (Table 1) were designed and customized to amplify an 1127–1155-bp fragment from the 18S rRNA gene. PCR reactions were carried out in a total volume of 50 µL, consisting of 25 µL of AmpliTaq Gold master mix, 18 µL of ultra-purified water DNAse-RNAse free, 1 µL of each primer and 5 µL of DNA template. The thermal cycling conditions were: incubation step at 95 °C for 15 min, 40 cycles of 1 min at 95 °C, 30 s at 54 °C for the melting temperature, and one and half minutes for the elongation time at 72 °C, followed by a final extension of 5 min at 72 °C. PCR amplification was performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA). The DNA generated through the PCR reaction was purified by filtration using a NucleoFast® 96 PCR DNA purification plate, and was then amplified using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The BigDye PCR products were purified on the Sephadex G-50 Superfine gel filtration resin prior to sequencing on the ABI Prism 3130XL. Nucleotide sequences were assembled and corrected using ChromasPro 2.0.0, then aligned against close reference sequences of filarioids species, representative members of Onchocercidae available in GenBank. The alignment was performed using the ClustalW application within BioEdit v.7.2.5 [16]. The maximum likelihood phylogenetic tree was inferred on MEGA6 [37], based on the Kimura 3-substitution-type model [20].

Results and discussion

Table 2 presents the results of the nine analyses carried out on the blood of 17 MWDs of the Rosnay kennel. For canine dirofilariosis, we observed 35.2% (6/17) positive dogs. The Knott test conducted on dog No. 3 revealed the presence of *D. immitis* microfilariae. DNA and *Wolbachia* complex qPCRs tests as well as the two serological tests gave positive results. Five dogs had mixed infestation with *D. immitis* and *D. repens* (dogs Nos. 2, 7, 8, 10 and 13). *Dirofilaria repens* DNA was identified in dogs Nos. 2, 10 and 13. Furthermore, both *Wolbachia* genotypes known to be associated with *D. immitis* and *D. repens* were also detected in the same samples. Two samples (Nos. 2 and 13) provided positive results with both serological tests. One dog (No. 7) was positive for both *D. immitis* and *D. repens* microfilariae by the Knott test, the PCR test for both *Dirofilaria* and *Wolbachia* DNA, and by

Table 2. Screening for dirofilariosis in a military kennel in the Indre department (central France).

Dog number (No.)	Breed*	Age (year)	Kennel presence time (year)	Parasitological diagnosis: Knott test	Serological screening		Molecular detection of filarial DNA using the qPCR Pan-Filaria 28S	Genotyping: 18S rRNA gene	Molecular identification of filarial species using a COI Triplex qPCR-based system			Molecular identification of *Wolbachia* genotypes using ftsZ duplex qPCR-based system		Diagnosis
					Witness® Dirofilaria	DiroCHEK®			*Dirofilaria immitis*	*Dirofilaria repens*	*Acanthocheilonema reconditum*	*Wolbachia* endosymbiont of *D. immitis*	*Wolbachia* endosymbiont of *D. repens*	
1	BSM	10	8.5	Neg.	Neg.	Neg.	Neg.	NE**	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
2	BSM	7	6	*D. repens*	Pos.	Pos.	Pos.	*D. repens*	Neg.	Pos.	Neg.	Pos.	Pos.	Occult heartworm and subcutaneous dirofilariosis
3	GS	6	5	*D. immitis*	Pos.	Pos.	Pos.	*D. immitis*	Pos.	Neg.	Neg.	Pos.	Neg.	Heartworm disease
4	BSM	6	5	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
5	BSM	6	5	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
6	BSM	6.5	5	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
7	GS	6	4	*D. immitis* + *D. repens*	Neg.	Pos.	Pos.	NE	Pos.	Pos.	Neg.	Pos.	Pos.	Heartworm and subcutaneous dirofilariosis
8	BSM	4.5	3	Neg.	Neg.	Neg.	Pos.	NE	Pos.	Pos.	Neg.	Pos.	Pos.	Occult heartworm and subcutaneous dirofilariosis
9	GS	3.5	2.5	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
10	BSM	4	2	*D. repens*	Neg.	Neg.	Pos.	*D. repens*	Neg.	Pos.	Neg.	Pos.	Pos.	Occult heartworm and subcutaneous dirofilariosis
11	BSM	3	2	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
12	GS	3	2	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
13	GS	4	1.5	*D. repens*	Pos.	Pos.	Pos.	*D. repens*	Neg.	Pos.	Neg.	Pos.	Pos.	Occult heartworm and subcutaneous dirofilariosis
14	GS	2.5	1.5	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
15	BSM	3.5	1	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
16	BSM	2	0.5	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog
17	BSM	2	0.5	Neg.	Neg.	Neg.	Neg.	NE	Neg.	Neg.	Neg.	Neg.	Neg.	Healthy dog

* BSM: Belgian shepherd malinois, GS: German shepherd.
** NE: Not evaluated.

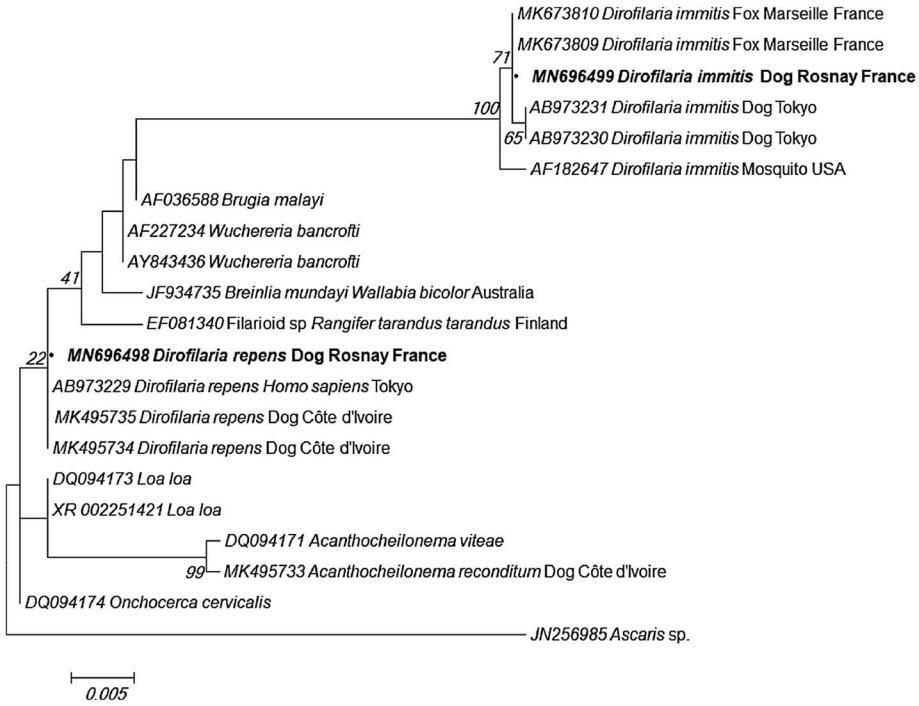


Figure 1. Molecular phylogenetic analysis of the 18S rRNA gene, using the maximum likelihood method based on the Kimura 3-substitution-type model.

ELISA; whereas, it was negative by the immunomigration test. Finally, dog No. 8 was positive for *D. immitis* and *D. repens* and *Wolbachia* DNA; and for both serological tests. All samples were free of *A. reconditum* infection.

Four partial sequences of the 18S rRNA gene have been successfully generated from samples identified as positive for a single DNA of filaria. Phylogenetic analysis revealed that dog No. 3 was infected with *D. immitis,* having 100% identity with *D. immitis* isolated from foxes in France (Marseille) (MK673809, MK673810) and 99.9% with that isolated from dogs in Japan (AB973231). Three *D. repens* sequences were 100% identical both to each other and to *D. repens* isolated from humans in Japan (Fig. 1).

Dog No. 7 died suddenly in November 2018 (1 month after blood sampling for our study) because of a stomach dilation-torsion. The necropsy performed on this dog revealed the presence in the right heart of four females and two males of *D. immitis* (Fig. 2). The heart was not dilated.

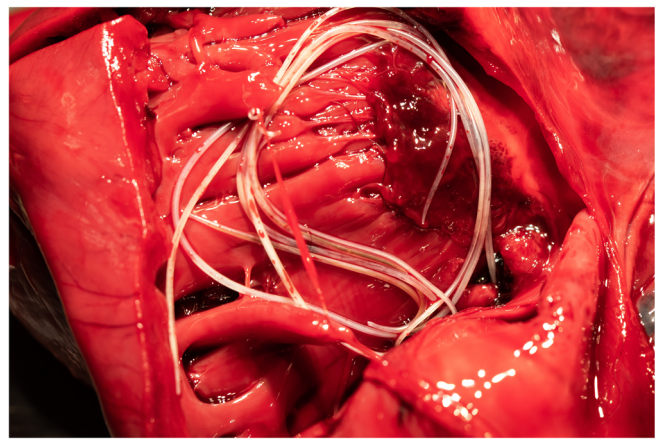


Figure 2. Heartworm (*Dirofilaria immitis*) in the right ventricle of dog No. 7.

It is known that heartworm can occur as an occult infestation, resulting in the presence of at least one mature female (or male) of *D. immitis,* without circulating microfilariae. Occult infestations occur in several situations, including monosexual infestation by male or female worms only, infertility of female worms, low infestation levels and/or destruction of microfilariae due to the host's immune response [35]. In our study, the proportion of occult infestation among dogs infected by *D. immitis* was 66.6% (4/6). The detection of occult infections is usually based on adult worm antigen testing [24]. However, in areas endemic for both *D. immitis* and *D. repens,* the reliability of these tests decreases, due to two main factors: (i) cross-reaction of rapid diagnostic tests between *D. immitis* and other parasites, including *D. repens*, and (ii) the immunosuppressive capacity of *D. repens* over *D. immitis* microfilariae, which hinders diagnosis based on the detection of blood microfilariae [14]. Adulticide treatment of *D. immitis* dirofilariasis, according to the protocol recommended by the American Heartworm Society, was effective against *D. repens* [28]. The approach combining the specific detection of molecular markers of *D. immitis* and *D. repens* showed 100% and 99.3% sensitivity and specificity, respectively. The reliability of the method is not reduced, even in case of occult dirofilariasis possibly accompanied by infestation by other filaroid species like *Acanthocheilonema reconditum*, leading to false positives following cross-reactions of rapid detection tests for *D. immitis* antigens [24]. This is coherent with the results obtained in our study, where occult heartworm

infection associated with circulating microfilariae of *D. repens* was observed in 60% (3/5) of *Dirofilaria* spp. coinfected dogs, of which only 40% (2/5) were positive for *D. immitis* antigen.

Canine filarial infections have increased significantly in recent years [35]. This trend is the consequence of the increase in the range of vectors, as well as the introduction of infected dogs, reservoirs of parasites, into ecosystems favourable to the emergence of secondary indigenous outbreaks. The geographical distribution of heartworm disease and subcutaneous dirofilariosis in France is not precisely known. However, several studies reported that *D. repens* is more endemic and spreads more rapidly than *D. immitis* within northern and eastern Europe [2, 3, 14, 31, 36]. These differences in the epidemiology of the two parasite species could be explained by the fact that in areas where *D. repens* is widespread, the progression of *D. immitis* would be hindered, and *vice versa* [14]. Outbreaks of *D. immitis* have also been reported in western France, they concerned hunting dogs living in kennels near ponds [6, 7]. In our study, the military kennel is located in an infertile swampy area near seven ponds. The local ecosystem is highly favourable to the development of dirofilariosis vectors. *Aedes* (*Stegomyia*) *albopictus*, more commonly known as the "tiger mosquito", has been introduced into metropolitan France from Italy and is now present throughout the south of the country, as well as in regions further north, including the Paris region and the department of Indre since 2017 [27]. This highly invasive and anthropophilic mosquito is known to be a competent vector of dirofilariosis parasites and to be implicated in the transmission of dirofilariosis in the studied area and most likely contributed to the infection of the military dogs considered in this study [36]. In southern Italy, the worrying increase in the proportion of mosquitoes infected by *D. immitis* among *A. albopictus* populations is associated with an increased risk of infestation of dogs with this parasite [15]. An integrated approach to control dirofilariosis vectors and to reduce infection sources and reservoirs for the parasite should be implemented in these areas. In the highly endemic area, the multi-modal prophylactic strategy, consisting in the administration of macrocyclic lactones and the application of repellents effective against mosquitoes, appears to be a tailored strategy.

A wide range of mammalian hosts, including humans and cats, can be infected by both *D. immitis* and *D. repens*, resulting from their low host specificity. Human infestation by *D. immitis* results in a pulmonary form (nodules), the most severe but less frequent form, which is found mainly in southern Europe (Italy, Spain, Greece, etc.) [13]. In France, most cases of human dirofilariasis caused by *D. immitis* are reported in endemic regions, such as Corsica and the Bouches-du-Rhône department [9, 32]. In humans, like in dogs, *D. repens* causes subcutaneous filariasis [1, 19].

We report here for the first time, an outbreak of canine dirofilariosis (*D. repens* and *D. immitis*) in the Rosnay military kennel, with a health risk for military personnel and military dogs in this area. In late 2018, after the detection of several infected dogs in the kennel, the following management measures were implemented: all dogs infected by *D. immitis* and cases of mixed infection ($N = 6$), except one that died before the treatment was administered (dog No. 7), received adulticide (melarsomine), combined with larvicide treatment (ivermectin), doxycycline and glucocorticosteroids (prednisone), according to the treatment protocol recommended by the American Heartworm Society [28]. During treatment, a restriction on physical activity was prescribed. This treatment eliminated *D. repens* and *D. immitis* larvae, as well as *Wolbachia* complex and the existing susceptible larvae. Moreover, all infected dogs were treated with insecticidal repellent effective against mosquitoes during the period of vector activity (from May to November in mainland France). The aim was to prevent secondary cases and outbreaks, in the presence of competent vectors in the area where the military kennel is located.

Conclusions

The epidemiology of dirofilariosis infections is complex and has even been related to the concept of the episystem, represented by the multiple interactions between climate, environment, animals, humans and parasites [34]. As these are zoonoses, physicians and veterinarians must be informed of the risks of transmission in the regions, and especially the local biotopes [10]. We suggest that epidemiological investigations of vectors and canine dirofilariasis be implemented in areas at risk of exposure. Moreover, we suggest implementation of prevention against *D. immitis* and *D. repens* infection in dogs, using macrocyclic lactones [35], in combination with a repellent effective against mosquitoes (permethrin or deltamethrin-based products), as recommended for heartworm prevention in the affected and surrounding areas [28].

Competing interests

The authors declare that they have no competing interests.

Acknowledgements. The authors thank Florian Boisson for his help in dog health surveillance. This study was supported by the French Armed Forces Health Service and the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European FEDER PRIMI funding. We thank Christophe Calleja (Zoetis) for supply of serological kits.

References

1. Benzaquen M, Brajon D, Delord M, Yin N, Bittar F, Toga I, Berbis P, Parola P. 2015. Cutaneous and pulmonary dirofilariasis due to *Dirofilaria repens*. British Journal of Dermatology, 173, 788–791.
2. Capelli G, Genchi C, Baneth G, Bourdeau P, Brianti E, Cardoso L, Danesi P, Fuehrer HP, Giannelli A, Ionică AM, Maia C, Modrý D, Montarsi F, Krücken J, Papadopoulos E, Petrić D, Pfeffer M, Savić S, Otranto D, Poppert S, Silaghi C. 2018. Recent advances on *Dirofilaria repens* in dogs and humans in Europe. Parasites and Vectors, 11(1), 663.
3. Chauve CM. 1997. Importance in France of the infestation by *Dirofilaria* (*Nochtiella*) *repens* in dogs. Parassitologia, 39(4), 393–395.

4. Davoust B, Ducos de Lahitte J. 1986. Utilisation de l'ivermectine dans la prophylaxie de la dirofilariose canine : résultats obtenus après deux ans d'essais chez le chien militaire en zone enzootie. Bulletin de la Société Française de Parasitologie, 4(2), 235–239.
5. Davoust B, Ducos de Lahitte J. 1989. Évolution de l'enzootie de dirofilariose dans les chenils militaires du Sud-Est de la France. Revue de Médecine Vétérinaire, 140(1), 15–19.
6. Doby JM, Couatarmanach A, Aznar C. 1986. Filarioses canines par *Dirofilaria immitis* (Leidy, 1856) et *D. repens* (Railliet et Henry, 1911), dans l'ouest de la France. Bulletin de la Société Française de Parasitologie, 4(2), 229–233.
7. Doby JM, Guiguen C. 1986. Présence de *Dirofilaria immitis* en Bretagne. Bulletin de la Société Française de Parasitologie, 4, 51–54.
8. Ducos de Lahitte J, Davoust B, Dorchies P. 1984. Filariose canine à *Dirofilaria immitis.* Enquête sur la fréquence et la répartition en zone méditerranéenne. Bulletin de la Société Française de Parasitologie, 3, 105–108.
9. Foissac M, Million M, Mary C, Dales JP, Souraud JB, Piarroux R, Parola P. 2013. Subcutaneous infection with *Dirofilaria immitis* nematode in human, France. Emerging Infectious Diseases, 19(1), 171–172.
10. Genchi C, Bowman D, Drake J. 2014. Canine heartworm disease (*Dirofilaria immitis*) in Western Europe: survey of veterinary awareness and perceptions. Parasites and Vectors, 7, 206.
11. Genchi C, Kramer L. 2017. Subcutaneous dirofilariosis (*Dirofilaria repens*): an infection spreading throughout the old world. Parasites and Vectors, 10(Suppl 2), 517.
12. Genchi C, Kramer LH, Rivasi F. 2011. Dirofilarial infections in Europe. Vector-Borne and Zoonotic Diseases, 11(10), 1307–1317.
13. Genchi C, Mortarino M, Rinaldi L, Cringoli G, Traldi G, Genchi M. 2011. Changing climate and changing vector-borne disease distribution: the example of Dirofilaria in Europe. Veterinary Parasitology, 176, 295–299.
14. Genchi C, Rinaldi L, Cascone C, Mortarino M, Cringoli G. 2005. Is heartworm disease really spreading in Europe? Veterinary Parasitology, 133(2–3), 137–148.
15. Giangaspero A, Marangi M, Latrofa MS, Martinelli D, Traversa D, Otranto D, Genchi C. 2013. Evidences of increasing risk of dirofilarioses in southern Italy. Parasitology Research, 112(3), 1357–1361.
16. Hall TA. 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series, 95–98.
17. Henry LG, Brunson KJ, Walden HS, Wenzlow N, Beachboard SE, L Barr K, Long MT. 2018. Comparison of six commercial antigen kits for detection of *Dirofilaria immitis* infections in canines with necropsy-confirmed heartworm status. Veterinary Parasitology, 254, 178–182.
18. Joyeux C, Cabassu J. 1935. Étude sur la filariose des chiens de Camargue. Bulletin de la Société de Pathologie Exotique, 28, 187–193.
19. Kartashev V, Batashova I, Kartashov S, Ermakov A, Mironova A, Kuleshova Y, Ilyasov B, Kolodiy I, Klyuchnikov A, Ryabikina E, Babicheva M, Levchenko Y, Pavlova R, Pantchev N, Morchón R, Simón F. 2011. Canine and human dirofilariosis in the Rostov Region (Southern Russia). Veterinary Medicine International, 2014, 6857 13.
20. Kimura M. 1981. Estimation of evolutionary distances between homologous nucleotide sequences (molecular evolution/comparison of base sequences/base substitution rate/neutral mutation-random drift hypothesis). Genetics, 78, 454–458.
21. Knott J. 1939. A method for making microfilarial surveys on day blood. Transactions of the Royal Society of Tropical Medicine and Hygiene, 33, 191–196.
22. Laidoudi Y, Davoust B, Varloud M, Niang EHA, Fenollar F, Mediannikov O. 2019. Development of a multiplexed qPCRs-based approach for the diagnosis of *Dirofilaria immitis, D. repens, Acanthocheilonema reconditum* and the others filariosis. bioRxiv 842575; https://doi.org/10.1101/842575.
23. Leidy J. 1856. A synopsis of entozoan and some of the other ecto-congeners observed by the author. Proceedings of Academy National of Sciences of Philadelphia, 8, 42–58.
24. Little S, Saleh M, Wohltjen M, Nagamori Y. 2018. Prime detection of *Dirofilaria immitis*: understanding the influence of blocked antigen on heartworm test performance. Parasites and Vectors, 11, 1–10.
25. Magnis J, Lorentz S, Guardone L, Grimm F, Magi M, Naucke TJ, Deplazes P. 2013. Morphometric analyses of canine blood microfilariae isolated by the Knott's test enables *Dirofilaria immitis* and *D. repens* species-specific and *Acanthocheilonema* (syn. *Dipetalonema*) genus-specific diagnosis. Parasites and Vectors, 6, 48.
26. Mccall JW, Genchi C, Kramer LH, Guerrero J, Venco L. 2008. Heartworm disease in animals and humans. Advances in Parasitology, 66, 193–285.
27. Ministère des Solidarités et de la Santé (France). 2019. Cartes de présence du moustique tigre (*Aedes albopictus*) en France métropolitaine. Web site (updated 28.05.19). https://solidarites-sante.gouv.fr/sante-et-environnement/risques-microbiologiques-physiques-et-chimiques/especes-nuisibles-et-parasites/article/cartes-de-presence-du-moustique-tigre-aedes-albopictus-en-france-metropolitaine.
28. Nelson CT, McCall JW, Jones S, Moorhead A. 2018. Current canine guidelines for the prevention, diagnosis, and management of Heartworm (*Dirofilaria immitis*) infection in dogs. Wilmington, DE: American Heartworm Society. p. 1–35.
29. Otranto D, Dantas-Torres F, Brianti E, Traversa D, Petrić D, Genchi C, Capelli G. 2013. Vector-borne helminths of dogs and humans in Europe. Parasites and Vectors, 6, 16.
30. Panthot JB. 1679. Extrait d'une lettre écrite de Lyon à l'auteur du Journal par Monsieur Panthot D. en Méd. & Professeur aggrégé au Collège de Lyon, contenant deux observations remarquables. Journal des Sçavans, 238.
31. Pantchev N, Schaper R, Limousin S, Norden N, Weise M, Lorentzen L. 2009. Occurrence of *Dirofilaria immitis* and tick-borne infections caused by *Anaplasma phagocytophilum, Borrelia burgdorferi sensu lato* and *Ehrlichia canis* in domestic dogs in France: results of a countrywide serologic survey. Parasitology Research, 105(Suppl 1), S101–S114.
32. Raccurt CP. 1999. Les dirofilarioses, des zoonoses émergentes etsous-estimées en France. Médecine Tropicale, 59(4), 389–400.
33. Railliet A, Henry A. 1911. Sur une filaire péritonéale des porcins. Bulletin de la Société de Pathologie Exotique, 4, 386–389.
34. Simón F, González-Miguel J, Diosdado A, Gómez PJ, Morchón R, Kartashev V. 2017. The complexity of zoonotic filariasis episystem and its consequences: a multidisciplinary view. BioMed Research International, 2017, 6436130.
35. Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E, Montoya-Alonso JA. 2012. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clinical Microbiology Reviews, 25(3), 507–544.
36. Tahir D, Bittar F, Barré-Cardi H, Sow D, Dahmani M, Mediannikov O, Raoult D, Davoust B, Parola P. 2017. Molecular survey of *Dirofilaria immitis* and *Dirofilaria*

repens by new real-time TaqMan®PCR assay in dogs and mosquitoes (Diptera: Culicidae) in Corsica (France). Veterinary Parasitology, 235, 1–7.

37. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30(12), 2725–2729.
38. Tarello W. 2011. Clinical aspects of dermatitis associated with Dirofilaria repens in pets: a review of 100 canine and 31 feline cases (1990–2010) and a report of a new clinic case imported from Italy to Dubai. Journal of Parasitology Research, 2011, 578385.

Cite this article as: Laidoudi Y, Ringot D, Watier-Grillot S, Davoust B & Mediannikov O. 2019. A cardiac and subcutaneous canine dirofilariosis outbreak in a kennel in central France. Parasite **26**, 72.

Publication N°14

Molecular investigation of vector-borne pathogens in red foxes (*Vulpes vulpes*) from Southern France

Hacène Medkour,[1,2] **Younes Laidoudi**,[1,2] Jean-Lou Marié,[3] Florence Fenollar,[2,4] Bernard Davoust,[1,2,3] and Oleg Mediannikov[1,2,5]

[1]Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France
[2]IHU-Méditerranée Infection, Marseille, France
[3]French Army Health Service, Working Group on Animal Epidemiology, Marseille, France
[4]Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France
[5]Corresponding author

ARTICLE DE RECHERCHE

Publié dans : Journal of Wildlife Diseases, 2020

(doi.10.7589/2019-09-234)

DOI: 10.7589/2019-09-234

Journal of Wildlife Diseases, 56(4), 2020, pp. 000–000

MOLECULAR INVESTIGATION OF VECTOR-BORNE PATHOGENS IN RED FOXES (*VULPES VULPES*) FROM SOUTHERN FRANCE

Hacène Medkour,[1,2] Younes Laidoudi,[1,2] Jean-Lou Marié,[3] Florence Fenollar,[2,4] Bernard Davoust,[1,2,3] and Oleg Mediannikov[1,2,5]

[1] Aix Marseille University, IRD, AP-HM, SSA, VITROME, Marseille, France
[2] IHU-Méditerranée Infection, Marseille, France
[3] Working Group on Animal Epidemiology, French Army Health Service, Marseille, France
[4] Aix Marseille University, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France
[5] Corresponding author (email: olegusss1@gmail.com)

ABSTRACT: Because of their free-ranging nature, the probability of wild animals being exposed to vector-borne pathogens is likely higher than that of humans and pets. Recently, the red fox (*Vulpes vulpes*) has been suspected as being a reservoir or host of several pathogens of veterinary and public health importance. We conducted a molecular survey on 93 red foxes hunted in 2008–18, in the departments of Bouches-du-Rhône and Var, in southeastern France, for pathogens including *Leishmania infantum*, Piroplasmida, *Hepatozoon* spp., nematodes, *Coxiella burnetii*, *Borrelia* spp., *Rickettsia* spp., and *Anaplasmataceae*. Spleen samples were screened for the presence of vector-borne pathogens by PCR followed by sequencing. Pathogens were detected in 94% (87/93) of red foxes, and coinfections were identified in 24% (22/93) of foxes. We identified DNA from *Hepatozoon canis*, *L. infantum*, and *Babesia vogeli* in 92% (86/93), 15% (14/93), and 3% (3/93) of red foxes, respectively. We also found DNA of nematodes in 3% (3/93) of foxes; *Spirocerca vulpis* was identified in one fox and *Dirofilaria immitis* in the two others. Interestingly, *C. burnetii* genotype 3, previously described in humans from the same region, was identified in 3% (3/93) of foxes and *Anaplasma platys* in 2% (2/93) of foxes. We did not detect DNA of *Borrelia* spp., *Bartonella* spp., or *Rickettsia* spp. In our study, the prevalence of pathogens did not vary by fox origin, sex, or tick carriage. Molecular evidence of *B. vogeli*, *H. canis*, *S. vulpis*, *D. immitis*, *C. burnetii*, and *A. platys* in red foxes has not previously, to our knowledge, been reported from southern France. We propose that red foxes are potential reservoirs for several pathogens, including major zoonotic agents such as *L. infantum*. They could be incidental hosts for pathogens, such *C. burnetii*. The high prevalence for *H. canis* suggests an important role of foxes in domestic dog (*Canis lupus familiaris*) infection. These animals may pose a threat to human and animal health.

Key words: *Coxiella burnetii*, *Dirofilaria immitis*, France, *Hepatozoon canis*, *Leishmania infantum*, Red fox, *Spirocerca vulpis*, vector-borne pathogens.

INTRODUCTION

Vector-borne diseases (VBD) are transmitted to animals and humans by blood-sucking arthropods (Otranto and Dantas-Torres 2010). Their transmission requires an introduced and/or established vector population, a pathogen, and suitable environmental and climatic conditions across the full cycle of VBD transmission in hosts (Randolph and Rogers 2010). The latter affects everything from vector survival and abundance; pathogen growth and survival in hosts, reservoir organisms, and vector organisms; vector activity and biting rates; and host exposures to disease vectors (Aguirre 2009; Semenza and Suk 2018). Wild and domestic carnivores are considered the most important source of human infections with zoonotic agents (Cleaveland et al. 2001). Recently, intensive clinical and epidemiologic studies have been carried out, especially in domestic canids, but data on VBDs in wild canids are still rare in many countries (Deždek et al. 2010; Zanet et al. 2014).

In Europe, foxes are the most widespread and abundant of wild carnivores (Dumitrache et al. 2015; Otranto et al. 2015a,b). The red fox (*Vulpes vulpes*) is widely distributed in the northern hemisphere, including in metropolitan France. This animal is not highly specialized and lives in various habitats (including periurban areas), mainly because of the availability of food, absence of preda-

tors, and human tolerance (Criado-Fornelio et al. 2018). During their migrations into suburban and urban environments, they can create problems, such as predation (chickens and rabbits), scavenging trash cans, and damaging gardens (Torina et al. 2013). The red fox establishes a link between wild and urban environments, and its huge population size and widespread abundance make it an important reservoir of pathogens for both pets and humans (André 2018).

Foxes have long been known to be the main reservoir for sylvatic rabies in Western and Central Europe, which was very common and a threat to human and animal health before the oral fox vaccination campaign (Duscher et al. 2015). Recently, they have been recognized as a potential reservoir for vector-borne pathogens (VBPs), such as *Leishmania infantum* (Verin et al. 2010; Davoust et al. 2014; Karayiannis et al. 2015), *Babesia* spp. (Cardoso et al. 2013; Najm et al. 2014a; Hodžić et al. 2015), *Hepatozoon canis* (Cardoso et al. 2014), *Anaplasmataceae* bacteria family such as *A. phagocytophilum* and *Ehrlichia canis* (Härtwig et al. 2014; André 2018), *Rickettsia* spp. (Ortuño et al. 2018), *Bartonella* spp. (Kaewmongkol et al. 2011; Gerrikagoitia et al. 2012), and filarial nematodes (Tolnai et al. 2014; Otranto et al. 2015a; Hodžić et al. 2016). In addition, they could be an excellent sentinel for pathogens circulating in the environment as well as a possible source of other pet and human VBDs, mainly because of their proximity to urban or agricultural areas and frequent exposure to different arthropod vectors (Aguirre 2009; Torina et al. 2013; Härtwig et al. 2014; Hodžić et al. 2014).

The role of red foxes in VBD epidemiology is poorly investigated in France. Our study aimed to assess the prevalence and diversity of pathogens belonging to *Piroplamida* sp., *Anaplasmataceae* bacteria, *Filarioidea*, *Hepatozoon* spp., *Borrelia* spp., *Bartonella* spp. and *Rickettsia* spp., *Leishmania infantum*, and *Coxiella burnetii*, in spleen samples from free-ranging red foxes from southern France, a region known to be endemic for numerous VBDs.

MATERIALS AND METHODS

Study area and sample collection

The study was conducted in the departments of Bouches-du-Rhône and Var in southeastern France (Fig. 1). Wild boar (*Sus scrofa*), roe deer (*Capreolus capreolus*), and red fox are the main wild large mammalian species in the area. A military hunting society is authorized to regulate the population size of these three species. During the past decade (2008–18), spleen samples were collected from 93 hunted red foxes, including 57 (61%) from the military camp of Carpiagne (43°14′54″N, 5°30′43″E), 33 (35%) from the camp of Canjuers (43°38′49″N, 6°27′56″E), and three (3%) from the city of Hyères. After hunting, the necropsies were performed aseptically in the laboratory by veterinarians, and spleen samples were kept at −20 C until DNA extraction. Gender and tick carriage were registered for each animal.

Sample treatment and DNA extraction

About 25 mg of each spleen sample was crushed and resuspended in 200 µL of G2 lysis buffer. Mechanical lysis used glass powder, for 30 s, on a Fastprep-24 device (MP Biomedicals, Chicago, Illinois, USA). After that, and for maximum yield, we performed an overnight digestion at 56 C with 15 µL of proteinase K. We extracted DNA from 200 µL of mixture employing a commercial DNA extraction kit QIAamp DNA Mini Kit® (Qiagen, Courtaboeuf, France) through the BIOROBOT EZ1 (Qiagen) per the manufacturer's instructions. We concentrated DNA in 200 µL of distilled water and kept the mixture at −20 C until analysis.

PCR protocols and sequencing

Quantitative real-time PCR (qPCR) systems were applied for the detection of *L. infantum*, *Piroplasmida*, and *Anaplasmataceae* bacteria and *Borrelia*, *Bartonella*, and *Rickettsia* spp. of the spotted fever group, targeting, respectively, minicircle kinetoplast DNA, 5.8S ribosomal RNA (rRNA), 23S rRNA, 16S rRNA, internal transcribed spacer (ITS), and *gltA* genes. After that initial screening, quantitative PCR (qPCR)-positive samples were confirmed by conventional PCR and sequencing. Primers, probes, thermal conditions, and references are detailed in Table 1.

For the initial detection of filarial infections, we used a qPCR targeting the 28S rRNA gene (Laidoudi et al. 2019). After that, a triplex qPCR was used to detect Cox-1 DNA from *D. immitis*, *D. repens*, and *Acanthocheilonema reconditum*. To detect the occult infections from *D. immitis*,

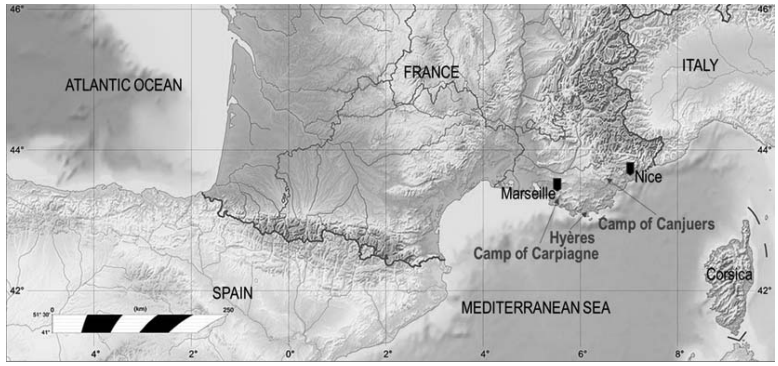


FIGURE 1. Map of southeast France, showing the study areas where the red foxes (*Vulpes vulpes*) were sampled. Samples were screened for vector-borne pathogens. The area is known to be endemic for several vector-borne pathogens. Arrows indicate the sampling sites.

we developed a specific qPCR for the detection of *Wolbachia* endosymbiont of *D. immitis* targeting the *ftsZ* gene, and we screened samples. Simultaneously, all samples were submitted to a PCR with primers able to amplify a 1,230–base pair fragment length of the 18S rRNA gene of Nematoda species, followed by sequencing (Laidoudi et al. 2019).

Screening for *C. burnetii* was performed by two independent qPCR tests (*IS1111* and *IS30A*; Table 1). Samples found positive by the two qPCRs were submitted to the multispacer typing (MST) using primer pairs for Cox2, Cox5, and Cox 37 (Glazunova et al. 2005). The amplicons were sequenced; the sequences obtained were compared with those reported in the *C. burnetii* reference database (IHU Méditerranée Infection 2019). For *Hepatozoon* spp. detection and for species identification, a primer pair able to amplify a 620–base pair fragment length of the 18S rRNA gene was employed (Table 1).

The qPCR reactions were prepared in a final volume of 20 µL containing 10 µL of Master Mix Roche (Eurogentec, Liège, Belgium), 0.5 µL of each forward and reverse primer and Uracil-DNA glycosylase (Eurogentec), 0.5 µL of the labeled probes (Table 1), 3 µL of DNase/RNase-free distilled water, and 5 µL of the DNA sample. We added DNA of the target pathogens as positive controls and master mixtures as negative controls for each test. We performed qPCR amplification under the following thermal conditions: incubation at 50 C for 2 min and an initial denaturation step at 95 C for 5 min, followed by 40 cycles of denaturation at 95 C for 5 s and annealing-extension at 60 C for 30 s, in a CFX96 Real-Time system (Bio-Rad Laboratories, Foster City, California, USA). We considered a result positive when the cycle threshold (Ct) was lower than 35 Ct for all qPCR assays.

The PCR reactions were performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA) and prepared in a 50 µL volume, including 25-µL AmpliTaq Gold Master mix, 1 µL of each primer, 18 µL distilled water, and 5 µL of the DNA sample. The DNA for the target pathogen and master mixture were added as positive and negative controls, respectively, in each assay. Cycler conditions included an initial denaturation step at 95 C for 15 min, followed by 40 cycles of 1 min at 95 C, 30 s annealing at the hybridization temperature for each PCR assays (Table 1), followed by 1 min at 72 C, and a final extension step for 5 min at 72 C. Amplicons were separated and visualized by electrophoresis in 1.5% agarose. All positive samples were purified and directly sequenced using the commercial Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, California, USA) with an ABI automated sequencer (Perkin Elmer). Obtained sequences were edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) and compared using the Blast Local Alignment Search Tool (National Center for Biotechnology Information, 2019) for similarity to sequences available in GenBank. Molecular phylogenetic and evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Statistical analyses

Results were treated and described in XLSTAT Software (version 2018.7, Addinsoft, New York, New York, USA). Prevalence of pathogens was calculated and the chi-squared or Fisher's exact tests compared proportions of positivity according to the region, sex, or tick carriage. A $P<0.05$ was considered as statistically significant.

TABLE 1. Primers and probes used to screen samples from red foxes (*Vulpes vulpes*) from southeast France for vector-borne pathogens.

Targeted microorganisms	Target gene	Name[a]	Primers (5′-3′) and probe	Tm[b] (C)	References
Leishmania infantum	kDNA	RV1	CTTTTCTGGTCCTCCGGGTAGG	—	Mary et al. 2004
		RV2	CCACCCGGCCCTATTTTACACCAA		
		Probe. Leish	FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA		
Leishmania spp.	ITS I	rDNA-10 F	CAATACAGGTGATCGGACAGG	55	Ogado Ceasar Odiwuor et al. 2011
		rDNA-14R	CACGGGGATGACACAATAGAG		
Piroplasmida	5.8S	5.8S-F5	AYYKTYAGCGRTGGATGTC	—	Dahmana et al. 2019
		5.8S-R	TCGCAGRAGTCTKCAAGTC		
		5.8S-S	FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB		
	18S	piro18S-F1	GCGAATGGCTCATTAIAACA	58	
		piro18S-R4	TTTCAGMCTTGCGACCATACT		
Nematoda	28S	Fil-28S-F	TTGTTTGAGATTGCAGCCCA	—	Laidoudi et al. 2019
		Fil-28S-R	GTTTCCATCTCAGCGGTTTC		
		Fil-28S-P	FAM-CAAGTACCGTGAGGGAAAGT-TAMRA		
Dirofilaria immitis, *Dirofilaria repens*, *Acanthocheilonema reconditum*	COI	Fil.COI.749F	CATCCTGAGGTTTATGTTATTATTTT	—	
		Fil.COI.914R	RCWGTATACATATGATGRCCYCA		
		D.imm.COI.777P	FAM-CGGTGTTTGGGATTGTTAGTG-TAMRA		
		D.rep.COI.871P	VIC-TGCTGTTTTAGGTACTTCTGTTTGAG-TAMRA		
		A.rec.COI.866P	Cy5-TGAATTGCTGTACTGGGAACT-BHQ-3		
Nematoda		F.18S.631	TCGTCATTGCTGCGGTTAAA	54	
		R.18S.1825	GGTTCAAGCCACTGCGATTAA		
Wolbachia endosymbiont of *Dirofilaria immitis*	ftsZ	W.imm.ftsZ.784f	GGAGGGGAAAATAGGGCAAT	—	This study
		W.imm.ftsZ.1006r	TACCTTCCATCGCTTGATCG		
		W.imm.ftsZ.886p	FAM-ACGGGTAGTGGGGACATGA-TAMRA		
Hepatozoon spp.	18S	H14HepaF	GAAATAACAATACAAGGCAGTTAAAATGCT	58	Hodžić et al. 2015

Table 1. Continued.

Targeted microorganisms	Target gene	Name[a]	Primers (5′-3′) and probe	Tm[b] (C)	References
H14HepaR			GTGCTGAAGGAGTCGTTTATAAAGA		
Coxiella burnetii	IS1111	F	CAAGAAACGTATCGCTGTGGC	—	Mediannikov et al. 2010
		R	CACAGAGCCACCGTATGAATC		
		S	FAM-CCGAGTTCGAAACAATGAGGGCTG-TAMRA		
	IS30A	F	CGCTGACCTACAGAAATATGTCC	—	
		R	GGGGTAAGTAAATAATACCTTCTGG		
		S	FAM-CATGAAGCGATTTATCAATACGTGTATGC-TAMRA		
	Cox 2	F	CAACCCTGAATACCCAAGGA	59	de Santi et al. 2018
		R	GAAGCTTCTGATAGGCGGGA		
	Cox 5	F	CAGGAGCAAGCTTGAATGCG	59	
		R	TGGTATGACAACCCGTCATG		
	Cox 37	F	GGCTTGTCTGGTGTAACTGT	59	
		R	ATTCCGGGACCTTCGTTAAC		
Anaplasmataceae	23S	TtAna-F	TGACAGCGTACCTTTTGCAT	—	Dahmani et al. 2015
		TtAna-R	GTAACAGGTTCGGTCCTCCA		
		TtAna-S	FAM-CTTGGTTTCGGGTCTAATCC-TAMRA		
		Ana23S-212f	GTTGAAAARACTGATGGTATGCA	55	
		Ana23S-753	TGCAAAAGGTACGCTGTCAC		
Borrelia spp.	16S	Bor-16S-3F	AGCCTTTAAAGCTTCGCTTGTAG	—	Parola et al. 2011
		Bor-16S-3R	GCCTCCCGTAGGAGTCTGG		
		Bor-16S-3P	FAM-CCGGCCTGAGAGGGTGAACGG-TAMRA		
Bartonella spp.	16S–23S internal transcribed spacer	TTB23s F	GGGGCCGTAGCTCAGCTG	—	Raoult et al. 2006
		TTB23s R	TGAATATATCTTCTCTTCACAATTTC		
		TTB23s P	6-FAM-CGATCCCGTCCGGCTCCACCA-TAMRA		
Rickettsia spp.	citrate synthase (gltA)	RKNDO3-F	GTGAATGAAAGATTACACTATTTAT	—	Rolain et al. 2002
		RKNDO3-R	GTATCTTAGCAATCATTCTAATAGC		
		RKNDO3-S	FAM-CTATTATGCTTGCGGCTGTCGGTTC-TAMRA		

[a] F = forward, R = reverse, S = probe.

[b] Annealing temperature, for quantitative PCR, was 60 C.

RESULTS

This study involved 93 red foxes, including 57 from Carpiagne (61%), 33 from Canjuers (35%), and three (3%) from Hyères. Overall, there were 56% (52/93) male and 44% (41/93) female. Among foxes, 49% (46/93) carried ticks identified as *Rhipicephalus turanicus* by keys (Estrada-Pena et al. 2004), including 74% of foxes (n=42) from Carpiagne, 6% (n=2) from Canjuers, and 67% (n=2) from Hyères. Molecular analyses showed that 94% (87/93) of the foxes were infected by at least one pathogen. Remarkably, a high infection rate of 92% (86/93) for *H. canis* was observed (Table 2). Prevalence was 15% (14/93) for *L. infantum* and 3% (3/93) for *B. vogeli* and *C. burnetii*. Two foxes (2%) were infected by *Anaplasma platys*. In addition, 3% (3/93) of foxes had nematode infections, including two cases of *D. immitis*, detected by the triplex qPCR and sequencing, and one infection by *Spirocerca vulpis*, confirmed after sequencing the 18S gene. All foxes tested negative for the DNA of the other filarial pathogens (*D. repens*, *A. reconditum*), the *Wolbachia* endosymbiont of *D. immitis*, and *Borrelia*, *Bartonella*, and *Rickettsia* spp. No statistically significant difference was observed between the prevalence of infections and the foxes' origin, sex, or tick carriage (Table 2).

Monoinfections were detected in 70% (65/93) of the foxes, including 64 cases of *H. canis* and one case of *L. infantum* infection. Coinfections were present in 24% (22/93) of the foxes, all of which were coinfected by *H. canis*. One fox was infected with three pathogens (*L. infantum*, *B. vogeli*, and *H. canis*; Table 3).

The ITS1 sequences obtained with *Leishmania*-specific primers were similar to each other and also showed 99–100% identity with *L. infantum* sequences available in GenBank database (accession nos. KX664454, KX664454, and KX664449) and 96–99% identity with *L. donovani* (accession nos. FN677363 and CP029526; Fig. 2). An obtained sequence for the 18S rRNA of piroplasms showed 98–100% identity with *Babesia vogeli* sequence available in GenBank

TABLE 2. Molecular results and distribution of vector-borne pathogens surveyed in samples from red foxes (*Vulpes vulpes*) from southeast France and shows the prevalence of pathogens and their distribution according to fox origin, gender, and tick carriage.

Pathogen	No. positive (% positive) by risk factor												
	Prevalence			Region				Gender			Tick carriage		
	Negative	Positive	P	Carpiagne	Canjuers	Hyères	P	Male	Female	P	Yes	No	P
Leishmania infantum	79 (85)	14 (15)	<0.0001	5 (9)	8 (24)	1(33)	0.100	9 (17)	5 (12)	0.689	6 (13)	8 (17)	0.805
Babesia spp.	90 (97)	3 (3)	<0.0001	3 (5)	0 (0)	0 (0)	0.381	1 (2)	2 (5)	0.841	2 (4)	1 (2)	0.985
Hepatozoon canis	7 (7)	86 (93)	<0.0001	53 (93)	31 (94)	2 (67)	0.223	48 (92)	38 (93)	1.000	45 (98)	41 (87)	0.113
Nematoda	90 (97)	3 (3)	<0.0001	1 (2)	2 (6)	0 (0)	0.509	1 (2)	2 (5)	0.841	1 (2)	2 (4)	1.000
Coxiella burnetii	90 (97)	3 (3)	<0.0001	3 (5)	0 (0)	0 (0)	0.379	1 (2)	2 (5)	0.841	3 (7)	0 (0)	0.230
Anaplasma platys	91 (98)	2 (2)	<0.0001	2 (3)	0 (0)	0 (0)	0.541	1 (2)	1 (2)	1.000	1 (2)	1 (2)	1.000
Borrelia spp.	93 (100)	0 (0)	<0.0001	0 (0)	0 (0)	0 (0)	–	0 (0)	0 (0)	–	0 (0)	0 (0)	–
Rickettsia spp.	93 (100)	0 (0)	<0.0001	0 (0)	0 (0)	0 (0)	–	0 (0)	0 (0)	–	0 (0)	0 (0)	–

TABLE 3. Mono and mixed infections detected surveyed in samples from red foxes (*Vulpes vulpes*) from southeast France (N=93).

Status	Infections	No. infected	% infected
Monoinfection	*Leishmania infantum*	1	1
	Hepatozoon canis	64	69
	Leishmania infantum + *Hepatozoon canis*	12	13
Coinfection	*Babesia vogeli* + *Hepatozoon canis*	2	2
	Dirofilaria immitis + *Hepatozoon canis*	1	1
	Spirocerca vulpis + *Hepatozoon canis*	1	1
	Coxiella burnetii + *Hepatozoon canis*	3	3
	Anaplasma platys + *Hepatozoon canis*	2	2
	Leishmania infantum + *Babesia vogeli* + *Hepatozoon canis*	1	1
Totals		87	94

(Fig. 3). Forty sequences of 550– to 600–base pairs for the 18S rRNA gene of *Hepatozoon* spp. were obtained, and they showed 99–100% identity with *H. canis* sequences available in GenBank database (Fig. 4). Overall, 36 sequences showed high identity with those previously obtained in wild canids, including nine sequences with close (>99%) identity to those identified in red foxes from Spain (accession no. AY150067.2), golden jackal (*Canis aureus*) from Austria (accession no. KX712123), and pampas fox (*Lycalopex gymnocercus*) from Brazil (accession no. KX816958); 27 sequences were closer (99% similarity) to those found in red foxes from the Czech Republic (accession nos. KU893123 and KU893122) and in golden jackal from Romania (accession no. KX712126). In addition, four sequences were identical to each other and also showed >99% identity with *H. canis* (accession no. LC331054) detected in a dog from Zambia.

The two obtained sequences, of about 1,010 base pairs, from *D. immitis*–positive samples (RC37 from Carpiagne and RC69 from Canjuers) were identical to each other and showed >99% identity with *D. immitis* strains (accession nos. AB973231 and AB973230). Another sequence obtained from another fox was 100% identical with *Spirocerca vulpis* isolate 2017 (accession no. MG957120; Fig. 5).

Genotyping of the three *C. burnetii*–positive samples showed sequences for two of them with a perfect match (100%) with Cox2.5, Cox5.6, and Cox37.7. These foxes were originally from Carpiagne. This combination corresponds to the MST3 genotype currently recognized in the *Coxiella* MST database isolated previously from a human heart valve from Marseille (Glazunova et al. 2005).

Two sequences of 440 and 417 base pairs for the partial 23S rRNA gene of *Anaplasmataceae* were obtained from two foxes from Carpiagne (RCP2 and RCP50). They were similar when compared with each other and showed 98% identity with *A. platys* strain ChieCal05 (accession no. KM021425), strain ChieGuy88 (accession no. KM021414), and strain Gard1 (accession no. KM021412) (Fig. 6).

DISCUSSION

We report the presence of *L. infantum*, *B. vogeli*, *H. canis*, *D. immitis*, *S. vulpis*, *C. burnetii*, and *Anaplasma platys* in red foxes from southern France. A high infection rate was observed. These results supported red foxes as potential reservoirs for pathogens and represent an important sanitary risk for local human and animal (domestic and wild) populations.

Leishmania infantum is the main *Leishmania* species identified in southern France. It has been described since 1968 in red foxes from Cevennes, France, by culturing the

FIGURE 2. Molecular phylogenetic analysis, based on ITS1 partial gene, showed the position of *Leishmania infantum* strains detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–1095.40) is shown. Initial trees for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The analysis involved 35 nucleotide sequences All positions containing gaps and missing data were eliminated. There were 266 positions in the final dataset.

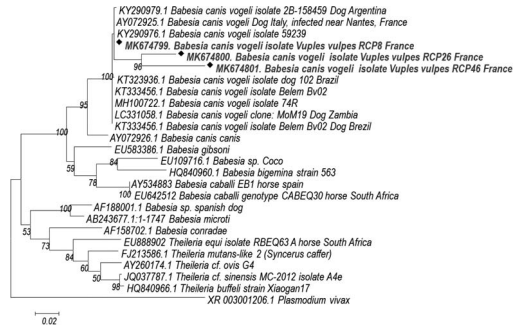


FIGURE 3. Molecular phylogenetic analysis, based on the 18S rRNA partial gene, showed the position of *Babesia vogeli* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–3069.06) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood approach and then selecting the topology with superior log-likelihood value. The analysis involved 26 nucleotide sequences. There were 556 positions in the final dataset.

parasite (Rioux et al. 1968). Our results confirmed the previous studies, which reported a prevalence of 9% (8/92) among red foxes from the Var area, southeastern France, including 7% (6/90) from the camp of Canjuers and two from Hyères (Davoust et al. 2014). An almost similar prevalence of 14% (23/162) was observed in Spain (Sobrino et al. 2008). Molecular studies in central and southern Italy showed prevalences of 52% (48/92) and 40% (20/50), respectively (Dipineto et al. 2007; Verin et al. 2010). Other studies showed an infection rate of 75% (50/67) in red foxes living in central Spain (Criado-Fornelio et al. 2000), and 29% (14/48) in foxes from *L. infantum* periendemic area in the north (del Río et al. 2014). *Leishmania infantum* isolates here were highly identical to isolates 119 and 11PSAZ, isolated from a human and *Phlebotomus sergenti* from Azilal, Morocco (Zouirech et al. 2018). It will be very important to isolate *L. infantum* strains from red foxes and compare them to reference strains that are pathogenic to humans and pets. Domestic dogs were always considered the main reservoir host for *L. infantum*, despite the extension of vaccination in some countries (Otranto et al. 2015b). The presence of *L. infantum*–infected red foxes represents a serious risk to humans, pets, and sylvatic fauna susceptible to leishmaniosis. Further, this risk is insidious, misunderstood, and all the more relevant because domestic dogs, considered the traditional reservoir for leishmaniosis, are more frequently vaccinated or benefit from chemoprophylaxis (e.g., insecticide collars, spot-on; Davoust et al. 2014). For better comprehension of the role of the red fox in the *L. infantum* transmission cycle, other studies are needed, as demonstrated in Brazil for the crab-eating foxes (*Cerdocyon thous*). Gomes

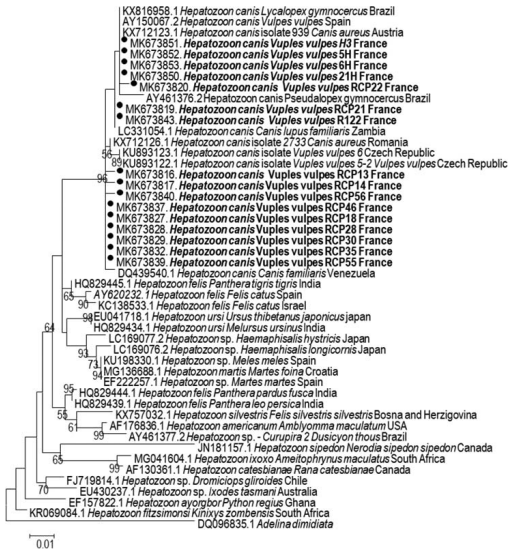


FIGURE 4. Molecular phylogenetic analysis showed the position of *Hepatozoon canis* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history based on the *Hepatozoon* spp. 18S rRNA genes was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–1887.73) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The analysis involved 48 nucleotide sequences. There were 518 positions in the final dataset.

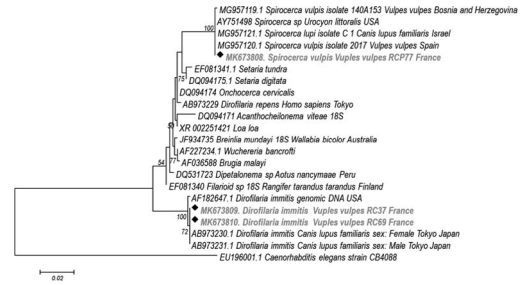


FIGURE 5. Molecular phylogenetic analysis, based on the 18S rRNA partial gene, showed the position of *Spirocerca vulpis* and *Dirofilaria immitis* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–2408.13) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The analysis involved 22 nucleotide sequences. There were 935 positions in the final dataset.

et al. (2007) showed that one of the three healthy *Leishmania chagasi* PCR-positive crab-eating foxes was able to infect 1/12 sandflies, which fed on it, suggesting a sylvatic cycle of *L. chagasi* among crab-eating foxes in Brazil.

We report the presence of Apicomplexa parasites (*B. vogeli* and *H. canis*) in French red foxes. The *B. vogeli*–positive foxes were from Carpiagne, and all of them carried ticks. *Babesia vogeli* is a globally distributed, tick-borne pathogen (Otranto et al. 2009). This parasite has been reported and characterized in 33% dogs and 22% brown dog ticks (*Rhipicephalus sanguineus*) from southern France (René et al. 2012). Several studies in Europe showed the presence of *Babesia* spp. DNA in foxes or their ticks, suggesting a potential role of the red fox in natural endemic cycles of these protozoa (Najm et al. 2014a; Criado-Fornelio et al. 2018). Therefore, the red fox can be regarded as the natural host for these piroplasms. Here, *B. vogeli* isolates detected on foxes were similar to canine isolates, and two new variants showed similar identity with an Italian dog infected near Nantes, France (accession no. aAY072925).

The highest *H. canis*–infection rate in red foxes was registered in the present study. High prevalence, although less than in our study, was reported in Portugal (75%; Cardoso et al. 2014), central Italy (49%; Ebani et al. 2017), Germany (45%; Najm et al. 2014b), and in northern Spain (28%; Gimenez et al. 2009). All these findings suggest that red foxes represent an important reservoir of *H. canis* infection for domestic dogs. The *R. sanguineus* s.l. tick, which benefits from a worldwide distribution, including in the Mediterranean, is the main vector of this parasite (Otranto et al. 2015b). The transmission of *H. canis* in ticks may occur transstadially from larvae to nymphs (Giannelli et al. 2013) and from these

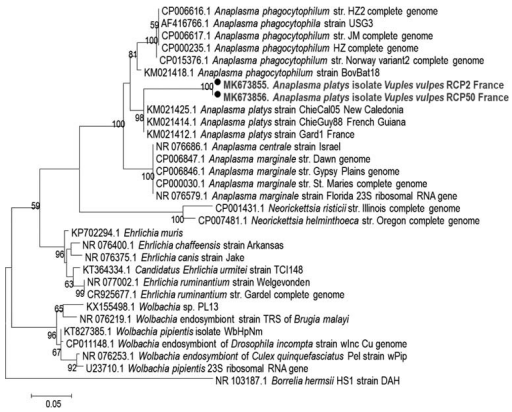


FIGURE 6. Molecular phylogenetic analysis showed the position of *Anaplasma platys* isolates detected in red foxes (*Vulpes vulpes*) from southern France. The evolutionary history based on the 23S rRNA partial gene of the *Anaplasmataceae* bacteria was inferred by using the maximum-likelihood method based on the Tamura-Nei model. The tree with the highest log-likelihood value (–2055.15) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite-likelihood (MCL) approach and then selecting the topology with superior log-likelihood value. The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 370 positions in the final dataset.

to the adult stages (Baneth et al. 2001). In our study, the prevalence varied insignificantly (P=0.113) between animals carrying ticks and animals without ticks. Other transmission routes could be possible and might contribute to the spread of this protozoan and explain this high prevalence in foxes, for example, by vertical transmission or predation of canid hosts on another intermediate hosts, as shown for *H. americanum* (Johnson et al. 2009). The *H. canis* isolates that we detected were highly identical to those detected on wild canids and dogs (Hodžić et al. 2015). Therefore, the epizootiologic importance of possible fox *H. canis* strains needs to be studied further before definitive conclusions can be drawn (Cardoso et al. 2014).

Dirofilaria immitis was identified in two foxes and *S. vulpis* in a third. *Dirofilaria immitis* and *D. repens* are the most important filarial pathogens, mainly affecting domestic carnivores, such as dogs and cats, but also humans and wild carnivores (McCall et al. 2008). They have a large geographic distribution, mainly in southern Europe (Spain, Portugal, Italy, and France), and especially in the Mediterranean (Tahir et al. 2019). In France, *D. immitis* is more frequently found in the south, along the Mediterranean coast: Bouches-du-Rhône (our study area), Vaucluse, and Corsica Island (5–15%), and to a lesser extent, Dordogne and Haute-Garonne (Doby et al. 1986). Low prevalence, <1.5%, of canine dirofilariasis has been reported in France between 1986–89 (Morchón et al. 2012). No data on the infection in foxes are available in France. In Italy, 7% of 132 foxes living in a canine *Dirofilaria*-endemic area were infected (Magi et al. 2008). Studies in Spain showed that prevalence of *D. immitis* infections is higher in foxes from irrigated areas (32%) compared with foxes from semi-arid regions (2%); heartworm was absent in foxes from the mountain (Gortazar et al. 1994). The role of red foxes in *D. immitis* transmission to other canids and/or humans is uncertain and needs further investigation. Strains detected in this study are similar to other *D. immitis* strains from dogs (accession nos. AB973231 and AB973230). Interestingly, we detected *Spirocerca vulpis* on a red fox. This nematode is a new species, recently described from red foxes in Europe (Bosnia and Herzegovina, Italy, and Spain), suggesting a high genetic diversity of the *Spirocerca* spp. infecting canids (Rojas et al. 2018). *Spirocerca vulpis* isolated in this study was similar to isolate 2017 (accession no. MG957120) described in red foxes from Spain. Knowledge on this species is limited, and additional studies are needed to clarify its importance. No experimental model is available to study pathogenesis of this nematode, and its presence in the spleen remains unexplained.

Coxiella burnetii is the etiologic agent of Q fever, with a worldwide distribution (Eldin et al. 2017). We found *C. burnetii* DNA in three foxes from Carpiagne; all of which carried ticks. In recent studies, 2/12 foxes (17%) in Spain (Millán et al. 2016) and 3/153 (2%) in central Italy were PCR positive (Ebani et al.

2017), whereas no foxes (n=105) tested positive in southern Italy (Santoro et al. 2016). Other studies described *C. burnetii* in ticks and fleas collected on red foxes (Psaroulaki et al. 2014a, b). *Coxiella burnetii* can infect a broad range of vertebrate and invertebrate hosts (Meredith et al. 2015). Even if the prevalence of infection is low, our study suggested a possible spillover in the fox population. In our study, the identified genotype, MST3, has been previously isolated on a human heart valve from Marseille, France (Glazunova et al. 2005). This suggests the circulation of a specific *C. burnetii* genotype in southern France.

Anaplasma platys strains had the most similarity to strains detected in domestic dogs from France (accession no. KM021412), French Guiana (accession no. KM021414), and New Caledonia (accession no. KM021425). *Anaplasma platys* has been identified in 14.5% (10/69) of foxes from Portugal, which suggested a possible role of foxes as reservoir of this dog's disease (Cardoso et al. 2015). Red foxes are also suspected to be reservoir for other *Anaplasmataceae* sp., especially *A. phagocytophilum* and *Ehrlichia canis* in Europe (Ebani et al. 2011; Torina et al. 2013; Hodžić et al. 2015; Millán et al. 2016). All foxes tested negative for *Borrelia* spp., *Bartonella* spp., and *Rickettsia* spp. DNA. In a study conducted on ticks and fleas sampled from foxes from the same region (southern France), 45% (33/73) of ticks (*R. turanicus*) and 10.5% (2/19) of fleas (*Archaeopsylla erinacei*) were found to be infected with *Rickettsia massiliae* and *Rickettsia felis*, respectively (Marié et al. 2012). *Bartonella* DNA was not found in any of the ticks or fleas in that study.

ACKNOWLEDGMENTS

We are grateful to the hunters of military hunting companies and Eric Gaba—Wikimedia Commons user. This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program Investissements d'avenir, reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur, and European funding FEDER PRIMI.

LITERATURE CITED

Aguirre AA. 2009. Wild canids as sentinels of ecological health: A conservation medicine perspective. *Parasite Vectors* 2 (Suppl 1):7.

André MR. 2018. Diversity of *Anaplasma* and *Ehrlichia/Neoehrlichia* agents in terrestrial wild carnivores worldwide: Implications for human and domestic animal health and wildlife conservation. *Front Vet Sci* 5:293.

Baneth GAD, Samish M, Alekseev E, Aroch I, Shkap V. 2001. Transmission of *Hepatozoon canis* to dogs by naturally-fed or percutaneously-injected *Rhipicephalus sanguineus* ticks. *J Parasitol* 87:606–611.

Cardoso L, Cortes HCE, Eyal O, Reis A, Lopes AP, Vila-Viçosa MJ, Rodrigues PA, Baneth G. 2014. Molecular and histopathological detection of *Hepatozoon canis* in red foxes (*Vulpes vulpes*) from Portugal. *Parasite Vectors* 7:113.

Cardoso L, Cortes HCE, Reis A, Rodrigues P, Simões M, Lopes AP, Vila-Viçosa MJ, Talmi-Frank D, Eyal O, Solano-Gallego L, Baneth G. 2013. Prevalence *of Babesia microti*-like infection in red foxes (*Vulpes vulpes*) from Portugal. *Vet Parasitol* 196:90–95.

Cardoso L, Gilad M, Cortes HCE, Nachum-Biala Y, Lopes AP, Vila-Viçosa MJ, Simões M, Rodrigues PA, Baneth G. 2015. First report of *Anaplasma platys* infection in red foxes (*Vulpes vulpes*) and molecular detection of *Ehrlichia canis and Leishmania infantum* in foxes from Portugal. *Parasite Vectors* 8: 144.

Cleaveland S, Laurenson MK, Taylor LH. 2001. Diseases of humans and their domestic mammals: Pathogen characteristics, host range and the risk of emergence. *Philos Trans R Soc B Biol Sci* 356:991–999.

Criado-Fornelio A, Gutierrez-Garcia L, Rodriguez-Caabeiro F, Reus-Garcia E, Roldan-Soriano MA, Diaz-Sanchez MA. 2000. A parasitological survey of wild red foxes (*Vulpes vulpes*) from the province of Guadalajara, Spain. *Vet Parasitol* 92:245–251.

Criado-Fornelio A, Martín-Pérez T, Verdú-Expósito C, Reinoso-Ortiz SA, Pérez-Serrano J. 2018. Molecular epidemiology of parasitic protozoa and *Ehrlichia canis* in wildlife in Madrid (central Spain). *Parasitol Res* 117:2291–2298.

Dahmana H, Amanzougaghene N, Davoust B, Normand T, Carette O, Demoncheaux JP, Mulot B, Fabrizy B, Scandola P, Chik M, et al. 2019. Great diversity of *Piroplasmida* in *Equidae* in Africa and Europe, including potential new species. *Vet Parasitol Reg Stud Rep* 18:100332.

Dahmani M, Loudahi A, Mediannikov O, Fenollar F, Raoult D, Davoust B. 2015. Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from Kabylie, Algeria. *Ticks Tick Borne Dis* 6:198–203.

Davoust B, Mary C, Marié JL. 2014. Detection of *Leishmania* in red foxes (*Vulpes vulpes*) from

Southeastern France using real-time quantitative PCR. *J Wildl Dis* 50:130–132.

Del Río L, Chitimia L, Cubas A, Victoriano I, De la Rúa P, Gerrikagoitia X, Barral M, Muñoz-García CI, Goyena E, García-Martínez D, et al. 2014. Evidence for widespread *Leishmania infantum* infection among wild carnivores in *L. infantum* periendemic northern Spain. *Prev Vet Med* 113:430–435.

de Santi VP, Briolant S, Mahamat A, Ilcinkas C, Blanchet D, de Thoisy B, Reynaud Y, Hyvert G, Marié JL, Edouard S, et al. 2018. Q fever epidemic in Cayenne, French Guiana, epidemiologically linked to three-toed sloth. *Comp Immunol Microbiol Infect Dis* 56: 34–38.

Deždek D, Vojta L, Ćurković S, Lipej Z, Mihaljević Ž, Cvetnić Ž, Beck R. 2010. Molecular detection of *Theileria annae* and *Hepatozoon canis* in foxes (*Vulpes vulpes*) in Croatia. *Vet Parasitol* 172:333–336.

Dipineto L, Manna L, Baiano A, Gala M, Fioretti A, Gravino AE, Menna LF. 2007. Presence of *Leishmania infantum* in red foxes (*Vulpes vulpes*) in southern Italy. *J Wildl Dis* 43:518–520.

Doby JM, Couatarmanach A, Aznar C. 1986. Filarioses canines par *Dirofilaria immitis* (Leidy, 1856) et *D. repens* (Railliet et Henry, 1911), dans l'ouest de la France. *Bull Soc Franç Parasitol* 4:229–233. [French]

Dumitrache MO, Matei IA, Ionică AM, Kalmár Z, D'Amico G, Sikó-Barabási S, Ionescu DT, Gherman CM, Mihalca AD. 2015. Molecular detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* sensu lato genospecies in red foxes (*Vulpes vulpes*) from Romania. *Parasite Vectors* 8:514.

Duscher GG, Leschnik M, Fuehrer H-P, Joachim A. 2015. Wildlife reservoirs for vector-borne canine, feline and zoonotic infections in Austria. *Int J Parasitol Parasite Wildl* 4:88–96.

Ebani VV, Verin R, Fratini F, Poli A, Cerri D. 2011. Molecular survey of *Anaplasma phagocytophilum* and *Ehrlichia canis* in red foxes (*Vulpes vulpes*) from central Italy. *J Wildlife Dis* 47:699–703.

Ebani VV, Rocchigiani G, Nardoni S, Bertelloni F, Vasta V, Papini RA, Verin R, Poli A, Mancianti F. 2017. Molecular detection of tick-borne pathogens in wild red foxes (*Vulpes vulpes*) from central Italy. *Acta Trop* 172:197–200.

Eldin C, Mélenotte C, Mediannikov O, Ghigo E, Million M, Edouard S, Mege JL, Maurin M, Raoult D. 2017. From Q fever to *Coxiella burnetii* infection: A paradigm change. *Clin Microbiol Rev* 30:115–190.

Estrada-Peña A, Bouattour A, Camicas J-L, Walker AR. 2004. *Ticks of domestic animals in the Mediterranean region: A guide to identification of species*. University of Zaragoza, Zaragoza, Spain, 131 pp.

Gerrikagoitia X, Gil H, García-Esteban C, Anda P, Juste RA, Barral M. 2012. Presence of *Bartonella* species in wild carnivores of northern Spain. *Appl Environ Microbiol* 78:885–888.

Giannelli A, Ramos RAN, Di Paola G, Mencke N, Dantas-Torres F, Baneth G, Otranto D. 2013. Transstadial transmission of *Hepatozoon canis* from larvae to nymphs of *Rhipicephalus sanguineus*. *Vet Parasitol* 196:1–5.

Gimenez C, Casado N, Criado-Fornelio Á, de Miguel FÁ, Dominguez-Peñafiel G. 2009. A molecular survey of *Piroplasmida* and *Hepatozoon* isolated from domestic and wild animals in Burgos (northern Spain). *Vet Parasitol* 162:147–150.

Glazunova O, Roux V, Freylikman O, Sekeyova Z, Fournous G, Tyczka J, Tokarevich N, Kovacova E, J. Marrie TJ, Raoult D. 2005. *Coxiella burnetii* genotyping. *Emerg Infect Dis* 11:1211–1217.

Gomes RB, Mendonça IL, Silva VC, Ruas J, Silva MB, Cruz MSP, Barral A, Costa CHN. 2007. Antibodies against *Lutzomyia longipalpis* saliva in the fox *Cerdocyon thous* and the sylvatic cycle of *Leishmania chagasi*. *Trans R Soc Trop Med Hyg* 101:127–133.

Gortázar C, Castillo JA, Lucientes J, Blanco JC, Arriolabengoa A, Calvete C. 1994. Factors affecting *Dirofilaria immitis* prevalence in red foxes in northeastern Spain. *J Wildl Dis* 30:545–547.

Härtwig V, Von Loewenich FD, Schulze C, Straubinger RK, Daugschies A, Dyachenko V. 2014. Detection of *Anaplasma phagocytophilum* in red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) from Brandenburg, Germany. *Ticks Tick Borne Dis* 5: 277–280.

Hodžić A, Alić A, Fuehrer HP, Harl J, Wille-Piazzai W, Duscher GG. 2015. A molecular survey of vector-borne pathogens in red foxes (*Vulpes vulpes*) from Bosnia and Herzegovina. *Parasite Vectors* 8:88.

Hodžić A, Alić A, Klebić I, Kadrić M, Brianti E, Duscher GG. 2016. Red fox (*Vulpes vulpes*) as a potential reservoir host of cardiorespiratory parasites in Bosnia and Herzegovina. *Vet Parasitol* 223:63–70.

Hodžić A, Latrofa MS, Annoscia G, Alić A, Beck R, Lia RP, Dantas-Torres F, Otranto D. 2014. The spread of zoonotic *Thelazia callipaeda* in the Balkan area. *Parasit Vectors* 7:352.

IHU Méditerranée Infection. 2019 Multi spacers typing—*Coxiella burnetii*. http://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/blast.html. Accessed January 2019.

Johnson EM, Panciera RJ, Allen KE, Sheets ME, Beal JD, Ewing SA, Little SE. 2009. Alternate pathway of infection with *Hepatozoon americanum* and the epidemiologic importance of predation. *J Vet Intern Med* 23:1315–1318.

Kaewmongkol G, Kaewmongkol S, Fleming PA, Adams PJ, Ryan U, Irwin PJ, Fenwick SG. 2011. Zoonotic *Bartonella* species in fleas and blood from red foxes in Australia. *Vector Borne Zoonotic Dis* 11:1549–1553.

Karayiannis S, Ntais P, Messaritakis I, Tsirigotakis N, Dokianakis E, Antoniou M. 2015. Detection of *Leishmania infantum* in red foxes (*Vulpes vulpes*) in central Greece. *Parasitol* 142:1574–1578.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33:1870–1874.

Laidoudi Y, Ringot D, Watier-Grillot S, Davoust B, and Mediannikov O. 2019. A cardiac and subcutaneous

canine dirofilariosis outbreak in a kennel in central France. *Parasite* 26:72.

Magi M, Calderini P, Gabrielli S, Dell'Omodarme M, Macchioni F, Prati MC, Cancrini G. 2008. *Vulpes vulpes*: A possible wild reservoir for zoonotic filariae. *Vector Borne Zoonot Dis* 8:249–252.

Marié JL, Davoust B, Socolovschi C, Mediannikov O, Roqueplo C, Beaucournu JC, Raoult D, Parola P. 2012. Rickettsiae in arthropods collected from red foxes (*Vulpes vulpes*) in France. *Comp Immunol Microbiol Infect Dis* 35:59–62.

Mary C, Faraut F, Lascombe L, Dumon H. 2004. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. *J Clin Microbiol* 42:5249–5255.

McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L. 2008. Heartworm disease in animals and humans. *Adv Parasitol* 66:193–285.

Mediannikov O, Fenollar F, Socolovschi C, Diatta G, Bassene H, Molez JF, Sokhna C, Trape JF, Raoult D. 2010. *Coxiella burnetii* in humans and ticks in rural Senegal. *PLoS Negl Trop Dis* 4:e654.

Meredith AL, Cleaveland SC, Denwood MJ, Brown JK, Shaw DJ. 2015. *Coxiella burnetii* (Q-fever) seroprevalence in prey and predators in the United Kingdom: Evaluation of infection in wild rodents, foxes and domestic cats using a modified ELISA. *Transbound Emerg Dis* 62:639–649.

Millán J, Proboste T, Fernández de Mera IGF, Chirife AD, de la Fuente J, Altet L. 2016. Molecular detection of vector-borne pathogens in wild and domestic carnivores and their ticks at the human-wildlife interface. *Ticks Tick Borne Dis* 7:284–290.

Morchón R, Carretón E, González-Miguel J, Mellado-Hernández I. 2012. Heartworm disease (*Dirofilaria immitis*) and their vectors in Europe—New distribution trends. *Front Physiol* 3:00196.

Najm NA, Meyer-Kayser E, Hoffmann L, Herb I, Fensterer V, Pfister K, Silaghi C. 2014a. A molecular survey of *Babesia* spp. and *Theileria* spp. in red foxes (*Vulpes vulpes*) and their ticks from Thuringia, Germany. *Ticks Tick Borne Dis* 5:386–391.

Najm NA, Meyer-Kayser E, Hoffmann L, Pfister K, Silaghi C. 2014b. *Hepatozoon canis* in German red foxes (*Vulpes vulpes*) and their ticks: Molecular characterization and the phylogenetic relationship to other *Hepatozoon* spp. *Parasitol Res* 113:2979–2985.

National Center for Biotechnology Information. 2019. Basic alignment search tool (BLAST). https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed January 2019.

Ogado Ceasar Odiwuor S, Ageed Saad A, De Doncker S, Maes I, Laurent T, El Safi S, Mbuchi M, Büscher P, Dujardin JC, Van der Auwera G. 2011. Universal PCR assays for the differential detection of all Old World *Leishmania* species. *Eur J Clin Microbiol Infect Dis* 30:209–218.

Ortuño A, Sanfeliu I, Nogueras MM, Pons I, López-Claessens S, Castellà J, Antóon E, Segura F. 2018. Detection of *Rickettsia massiliae*/Bar29 and *Rickettsia conorii* in red foxes (*Vulpes vulpes*) and their *Rhipicephalus sanguineus* complex ticks. *Ticks Tick Borne Dis* 9:629–631.

Otranto D, Cantacessi C, Dantas-Torres F, Brianti E, Pfeffer M, Genchi C, Genchi C, Guberti V, Deplazes P. 2015a. The role of wild canids and felids in spreading parasites to dogs and cats in Europe, part II: Helminths and arthropods. *Vet Parasitol* 213:24–37.

Otranto D, Cantacessi C, Pfeffer M, Dantas-Torres F, Brianti E, Deplazes P, Genchi C, Guberti V, Capelli G. 2015b. The role of wild canids and felids in spreading parasites to dogs and cats in Europe, part I: Protozoa and tick-borne agents. *Vet Parasitol* 213:12–23.

Otranto D, Dantas-Torres F. 2010. Canine and feline vector-borne diseases in Italy: Current situation and perspectives. *Parasit Vectors* 3:2.

Otranto D, Dantas-Torres F, Breitschwerdt EB. 2009. Managing canine vector-borne diseases of zoonotic concern: part two. *Trends Parasitol* 25:228–235.

Parola P, Diatta G, Socolovschi C, Mediannikov O, Tall A, Bassene H, Trape JF, Raoult D. 2011. Tick-borne relapsing fever borreliosis, rural Senegal. *Emerg Infect Dis* 17:883–885.

Psaroulaki A, Chochlakis D, Angelakis E, Ioannou I, Tselentis Y. 2014a. *Coxiella burnetii* in wildlife and ticks in an endemic area. *Trans R Soc Trop Med Hyg* 108:625–631.

Psaroulaki A, Chochlakis D, Ioannou I, Angelakis E, Tselentis Y. 2014b. Presence of *Coxiella burnetii* in fleas in Cyprus. *Vector Borne Zoonot Dis* 14:685–687.

Randolph SE, Rogers DJ. 2010. The arrival, establishment and spread of exotic diseases: Patterns and predictions. *Nat Rev Microbiol* 8:361–371.

Raoult D, Roblot F, Rolain JM, Besnier JM, Loulergue J, Bastides F, Choutet P. 2006. First isolation of *Bartonella alsatica* from a valve of a patient with endocarditis. *J Clin Microbiol* 44 :278–279

René M, Chêne J, Beaufils JPP, Moro CV, Bourdoiseau G, Mavingui P, Chabanne L. 2012. First evidence and molecular characterization of *Babesia vogeli* in naturally infected dogs and *Rhipicephalus sanguineus* ticks in southern France. *Vet Parasitol* 187:399–407.

Rioux JA, Albaret JL, Houin R, Dedet JP, Lanotte G. 1968. Ecologie des leishmanioses dans de sud de la France, 2.—Les réservoirs selvatiques. *Ann Parasit Hum Comp* 43:421–428. [French]

Rojas A, Sanchis-Monsonís G, Alić A, Hodžić A, Otranto D, Yasur-Landau D, Martínez-Carrasco C, Baneth G. 2018. *Spirocerca vulpis* sp. nov. (Spiruridae: Spirocercidae): Description of a new nematode species of the red fox, *Vulpes vulpes* (Carnivora: Canidae). *Parasitol* 145:1917–1928.

Rolain JM, Stuhl L, Maurin M, Raoult D. 2002. Evaluation of antibiotic susceptibilities of three rickettsial species including *Rickettsia felis* by a quantitative PCR DNA assay. *Antimicrob Agents Chemother* 46:2747–2751.

Santoro M, Veneziano V, D'Alessio N, Di Prisco F, Lucibelli MG, Borriello G, Cerrone A, Dantas-Torres

F, Latrofa MS, Otranto D, et al. 2016. Molecular survey of *Ehrlichia canis* and *Coxiella burnetii* infections in wild mammals of southern Italy. *Parasitol Res* 115:4427–4431.

Semenza JC, Suk JE. 2018. Vector-borne diseases and climate change: A European perspective. *FEMS Microbiol Lett* 365:fnx244.

Sobrino R, Ferroglio E, Oleaga A, Romano A, Millan J, Revilla M, Arnal MC, Trisciuoglio A, Gortázar C. 2008. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. *Vet Parasitol* 155:198–203.

Tahir D, Davoust B, Parola P. 2019. Vector-borne nematode diseases in pets and humans in the Mediterranean Basin: An update. *Vet World* 12: 1630–1643.

Tolnai Z, Széll Z, Sproch Á, Szeredi L, Sréter T. 2014. *Dirofilaria immitis*: An emerging parasite in dogs, red foxes and golden jackals in Hungary. *Vet Parasitol* 203:339–342.

Torina A, Blanda V, Antoci F, Scimeca S, D'Agostino R, Scariano E, Piazza A, Galluzzo P, Giudice E, Caracappa S. 2013. A molecular survey of *Anaplasma* spp., *Rickettsia* spp., *Ehrlichia* canis and *Babesia microti* in foxes and fleas from Sicily. *Trans Emerg Dis* 60(Suppl.2):125–130.

Verin R, Poli A, Ariti G, Nardoni S, Fanucchi MB, Mancianti F. 2010. Detection of *Leishmania infantum* DNA in tissues of free-ranging red foxes (*Vulpes vulpes*) in central Italy. *Eur J Wild Res* 56: 689–692.

Zanet S, Trisciuoglio A, Bottero E, De Mera IGF, Gortazar C, Carpignano MG, Ferroglio E. 2014. Piroplasmosis in wildlife: *Babesia* and *Theileria* affecting free-ranging ungulates and carnivores in the Italian Alps. *Parasit Vectors* 7:70.

Zouirech M, Rhajaoui M, Faraj C, El Guamri Y, Amahmid O, El Hachimi MY, Bouhout S, El Kharrim K, Belghyti D. 2018. Entomological inventory of Phlebotomine sand flies and epidemiological study of leishmaniasis in Afourer, Azilal province, High Atlas, Morocco. *Med Sante Trop* 28:385–389.

Submitted for publication 20 September 2019.
Accepted 14 March 2020.

Publication N°15

Canine vector-borne protozoa: Molecular and serological investigation for *Leishmania* spp., *Trypanosoma* spp., *Babesia* spp., and *Hepatozoon* spp. in dogs from Northern Algeria

Hacène Medkour[1,2,3], **Younes Laidoudi**[1,2], Ismail Lafri[4,5], Bernard Davoust[1], Abdeslam Mekroud[3], Idir Bitam[4,6] and Oleg Mediannikov[1*]

[1]IHU Méditerranée Infection
[2]Microbes, Evolution, Phylogeny and Infection (MEΦI) UMR Aix-Marseille Université, IRD, APHM -19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France
[3]PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, 25100 El Khroub, Algeria
[4]Vectors-Tropical and Mediterranean Infections (VITROME), UMR Aix-Marseille Université, IRD, SSA, APHM -19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France
[5]Institute of Veterinary Sciences, University of Blida 1, Algeria
[6]Superior School of Food Sciences and Food Industries of Algiers, Algeria

ARTICLE DE RECHERCHE

Publié dans : Veterinary Parasitology: Regional Studies and Reports, 2020, 19

(doi.10.1016/j.vprsr.2019.100353)

Veterinary Parasitology: Regional Studies and Reports 19 (2020) 100353

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

Canine vector-borne protozoa: Molecular and serological investigation for Leishmania spp., Trypanosoma spp., Babesia spp., and Hepatozoon spp. in dogs from Northern Algeria

Hacène Medkour[a,b,c], Younes Laidoudi[a,b], Ismail Lafri[d,e], Bernard Davoust[a], Abdeslam Mekroud[c], Idir Bitam[d,f], Oleg Mediannikov[a,*]

[a] IHU Méditerranée Infection - Microbes, Evolution, Phylogeny and Infection (МЕФИ), France
[b] UMR Aix-Marseille Université, IRD, APHM -19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France
[c] PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, 25100, El Khroub, Algeria
[d] IHU Méditerranée Infection - Vectors-Tropical and Mediterranean Infections (VITROME), UMR Aix-Marseille Université, IRD, SSA, APHM -19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France
[e] Institute of Veterinary Sciences, University of Blida 1, Algeria
[f] Superior School of Food Sciences and Food Industries of Algiers, Algeria

ARTICLE INFO

Keywords:
Dog
Algeria
Leishmaniosis
Babesiosis
Trypanosomiasis
Hepatozoon spp.

ABSTRACT

Dogs are competent reservoirs/hosts of several protozoan pathogens transmitted by blood-feeding arthropods. Throughout their long history of domestication, they have served as a link for the exchange of parasites among livestock, wildlife, and humans and therefore remain an important source of emerging and re-emerging diseases. In Algeria, while canine leishmaniosis (CanL) is well known to be endemic, no data are available on other vector-borne protozoans. Here, we investigate the occurrence and diversity of trypanosomes, piroplasms and *Hepatozoon* spp. and update the epidemiological status of CanL in dogs from Kabylia, northern Algeria. A total of 227 dogs from three regions of Kabylia were enrolled, including 77 dogs with clinical signs. Dogs were clinically examined and were tested for L. *infantum* antibodies using a Rapid Immuno-Migration (RIM™) and a quantitative indirect Immunofluorescence Antibody Test (IFAT). PCR screening and sequencing were performed for vector-borne protozoa. Sixty two percent (141/227) of dogs presented at least one infection, whereas 26% (59/227) were co-infected. *L. infantum* antibodies were detected in 35.7% (81/227) of dogs including 88.7% (68/77) of sick dogs. Molecular investigation revealed prevalence of: 6.6% (15/227), 13.2% (30/227), 41% (93/227) for *Trypanosoma* spp., *B. vogeli* and *H. canis,* respectively. *T. evansi* (3.1%) and potential new subspecies of *T. congolense* had been identified. Dog"s clinical status correlates positively with L. *infantum* antibody titers and the presence of co-infections. Susceptibility to CanL varied according to the dog's aptitude and guard dogs were more infected (51%) *(P-value = .001)*. *B. vogeli* infection was more frequent in juveniles than adults (32% vs 9%, *P-value < .001*) and in females than males (21% vs 10%, *P-value = .02*). To the authors' knowledge, this is the first report on vector-borne protozoa infected dogs in Algeria. Current results are important not only for animal health, but also to avoid serious public health and livestock problems.

1. Introduction

Parasitic protozoa are protists, unicellular eukaryotes. They share the common traits of being heterotrophic and host dependent for survival. They can cause significant morbidity and mortality in domestic animals, thus causing substantial economic losses related to animal production or a major concern for animal owners (Dantas-Torres, 2008a) (Schnittger and Florin-Christensen, 2018). Domestic dogs live in close association with humans and livestock, participating in the transmission of diseases of zoonotic, veterinary and conservation interest (Millán et al., 2013). Dogs can therefore be infected by a large number of these parasites, haemoparasites belong the genus *Leishmania*, *Trypanosoma*, *Babesia* and *Hepatozoon* (Otranto et al., 2009) (Díaz-Regañón et al., 2017). They are causative agents of emerging or re-emerging vector-borne diseases with a major impact on animal and human health and an economic burden (Harrus and Baneth, 2005)

* Corresponding author.
E-mail address: olegusss1@gmail.com (O. Mediannikov).

https://doi.org/10.1016/j.vprsr.2019.100353
Received 13 April 2019; Received in revised form 16 November 2019; Accepted 16 November 2019
Available online 18 November 2019
2405-9390/

(Otranto et al., 2010).

Canine leishmaniosis (CanL) is the most common vector-borne protozoa disease caused by *Leishmania infantum* (Kinetoplastida: Trypanosomatidae). The parasite is mainly transmitted by the infected bite of females of *Phlebotomus* in the Old World and *Lutzomyia* in the New World. It is a severe systemic disease characterized by a wide range of overlapping clinical signs (e.g., lymph node enlargement, weight loss, and splenomegaly). Dogs with CanL may also present skin alterations (alopecia, furfuraceous dermatitis, ulcers, and nodular lesions) (De Tommasi et al., 2013).

Trypanosomes found in mammals (including humans) are blood and sometimes tissue parasites of the order Kinetoplastida, family of Trypanosomatidae, genus *Trypanosoma*, principally transmitted by biting insects, in which most of them undergo a biological cycle (Desquesnes et al., 2013). Some species have a veterinary and economic interest and can infect domestic dogs. This includes *Trypanosoma brucei brucei* and *T. congolense*, the causative agents of Nagana or a similar disease in Africa and Asia, as well as *T. evansi*, the etiological agent of surra and the so-called "mal de cadeiras" outside Africa. Domestic dogs are also important reservoirs of *T. cruzi* in the Americas (Eloy and Lucheis, 2009).

Canine babesiosis and hepatozoonosis, caused by *Apicomplexa* protozoa of the genus *Babesia* and *Hepatozoon*, respectively, are protozoal tick-borne diseases. *Babesia canis, B. vogeli, B. rossi* and *B. gibsoni* are the main causative agents of babesiosis (Otranto et al., 2009). Their geographical distribution and therefore the presence of babesiosis depends largely on the habitat of the relevant vector tick species, with the exception of *B. gibsoni* where evidence of dog-to-dog transmission indicates that infection can be transmitted between fighting dog breeds regardless of the limits of the vector tick infestation (Bilić et al., 2018). Clinical characteristics can vary considerably depending on the different species and strains involved and the factors that determine the host's response to infection such as immune status, age and the presence of co-infections. One of the main clinical manifestations is hemolytic anemia with systemic inflammatory responses observed in severe canine babesiosis (Schnittger and Florin-Christensen, 2018).

Hepatozoon canis and *H. americanum* are the agents of canine hepatozoonoses that range from being asymptomatic with low levels of parasitemia to a severe life-threatening illness characterized by high levels of parasitemia, fever, anemia, and lethargy (Baneth, 2011). These two *Hepatozoon* species are genetically and geographically distinct. *H. canis* is distributed in Africa, southern Europe, the Middle East and Asia, and is mainly transmitted by *Rhipicephalus sanguineus* s.l. and *Haemaphysalis longicornis*. *H. americanum* is found in the Americas and is transmitted by *Amblyomma maculatum* (Qiu et al., 2018).

In Algeria, the largest country in Africa, there are three types of climate: (1) the mild Mediterranean climate of the coast; (2) the transitional climate of the northern hills and mountains in the north of the country, which is slightly more continental and moderately rainy, and (3) the desert climate of the vast area occupied by the Sahara, when reaching the Algerian south. The northern climate is conducive to the development of arthropods such as ticks, fleas, lice, mosquitoes and sandflies and therefore to diseases that can be transmitted; especially canine vector-borne diseases (Gage et al., 2008) (Thiébault and Moatti, 2016). However, only limited data are available on canine protozoan vector-borne infections. Although CanL is known to be endemic in northern Algeria (Adel et al., 2015) and other North African countries (Diouani et al., 2014)(Rami et al., 2003), to date, no information is available on *Trypanosoma* spp., *Babesia* spp. or *Hepatozoon* spp. infections in dogs in Algeria. Therefore, the aim of this study is to assess the prevalence and the diversity Kinetoplastida and Apicomplexa parasites in dogs from Kabylia (Northern Algeria) using molecular and serological techniques.

2. Material and methods

2.1. Ethic statement

The study was carried out in accordance with Algerian legislation guidelines. In addition, the samples were collected after obtaining a verbal consent from dog owners by a veterinarian specialist. Risk assessment was submitted to and approved by the ethics committee and decision board of the veterinary practitioners from the wilayas of the North of Algeria. These institutions are affiliated with the Algerian Ministry of Agriculture and Rural Development (Directions des Services Vétérinaires). Protocol of the study was also approved by the scientific college (Procès-Verbal du CSI N°6, 2018) at the Veterinary Science Institute, University Constantine 1, Algeria. To facilitate field work, collaborations were established with veterinary doctors and their assistants working in these establishments.

2.2. Study area and dogs

The study was conducted in Kabylia, a cultural and natural region in northern Algeria. It is part of the Tell Atlas mountain range, and is located on the shores of the Mediterranean Sea. It covers several wilayas (provinces) of Algeria: the whole of Tizi-Ouzou and Bejaia, most of Bouira and parts of the wilayas of Boumerdes, Jijel, Setif and Bordj Bou Arreridj. Recent study showed prevalence of 10% of CanL in dogs from Kabylia (Mouloua et al., 2017). No data on the other canine vector-borne protozoa are available. This last prevalence (10%) was chosen as attended prevalence for this study. By using 5% as margin of error and 95% of confidence level, the sample size must be at least 140 samples for a cross-sectional survey.

In May 2018, blood samples were collected from 227 dogs living in three Kabylian wilayas, that included 97 dogs from Bouira (36°22′29.6"N, 3°54′7.2″E), 98 dogs from Setif (36°11′28.03"N, 5°24′49.43″E) and 32 dogs from Tizi-Ouzou (36°42′42.55"N, 4°2′45.28″E) (Fig. 1). A questionnaire was completed for each dog with information on age, sex, breed, lifestyle and physical examination results. A clinical score (CS) of 0 to 4 was given to each dog according to the number of clinical signs identified with: CS0 for dogs without clinical signs, CS1: for dogs with one or two clinical signs, CS2: if dogs have three or four clinical signs, CS3: five or six signs and CS4 for dogs with more than six clinical signs. In general, registered signs were compatible with CanL and comprised fever, anorexia, dermatological manifestations, lymphadenopathy, body weight loss, lethargy, muscular atrophy, onychogryphosis, ulceration, ocular manifestations, splenomegaly, epistaxis and neurological disorders. Whenever available, ticks were collected.

2.3. Samples

A volume of six ml of blood were collected from the radial vein of each dog. One drop of whole blood from each sample was used for rapid on-site leishmaniasis screening at the time of sampling and the remaining blood was transferred to EDTA and dry tubes for molecular and serological screening, respectively. Sera were recovered from the dry tubes after centrifugation (10 min, 3000 *g*). Once collected, samples were stored at 4 °C overnight and then frozen at −20 °C until further use.

2.4. Rapid Immuno Migration (RIM™) test and quantitative IFAT for the detection of L. infantum specific antibodies

Two serological tests were used: the immunochromatographic based qualitative rapid test WITNESS® Leishmania (Zoetis, France) and the quantitative indirect immunofluorescence antibody test (IFAT). The rapid test was carried out according to the manufacturer's instructions using one drop of 10 μL of whole blood from each dog directly after

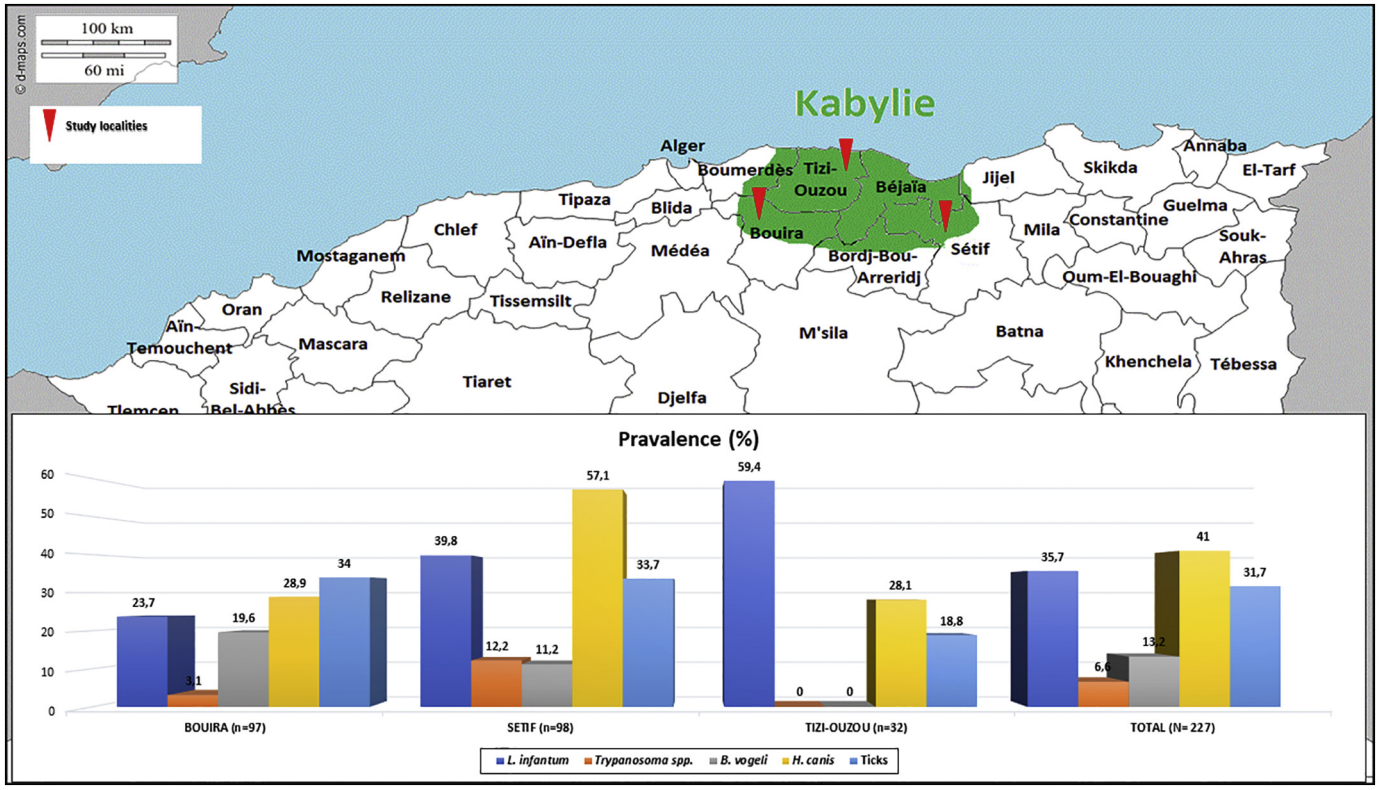


Fig. 1. Carte shows the three localities (Tizi-Ouzou, Bouira and Setif) of the study area (https://upload.wikimedia.org/wikipedia/commons/6/6e/Kabylia.gif) and prevalence of canine vector-borne protozoa in this area.

sampling. IFAT for the detection and the titration of specific immunoglobulins G (IgG) anti-*L. infantum* was performed on sera. Plate wells were sensitized by 20 µL of L. *infantum* commercial antigens (Zoetis, France). The samples were diluted to 1:50 with PBS, 20 µl of every serum dilution was applied per well and plates were incubated for 30 min at 37 °C. They were then washed twice with PBS for 5 min and once with distilled water. After the washing, 20 µL of IgG anti-dog conjugated with fluorescein isothiacyanate (FITC) (Jackson ImmunoResearch Europe Ltd. Cambridge House, UK) were added into each well after dilution at 1:200. The plates were incubated for 30 min at 37 °C in the dark. The washing procedure was repeated as described above. Then, some drops of mounting medium Fluoprep (bioMérieux, France) were added on the cover slips. The reading was performed using a fluorescence microscope and each well was compared with the fluorescence of positive and negative controls added in each plate. Positive control was a known serum of French dog infected by L. *infantum* (at the dilution, 1:1600), and negative control was a known serum of non-infected dog by *Leishmania* form IHU – Méditerranée Infection lab collection. To avoid errors of observation, all samples were examined by two different investigators. All samples negative at 1:50 were considered negative and no further dilutions was performed. All positive results were further investigated using a two-fold serial dilution IFAT at 1:100 and 1:200. If a high positive result was observed, the samples were diluted to both 1:400, 1:800 and 1:1600. At this point, the samples were classified as a high positive for L. *infantum* (> 1:1600).

2.5. Molecular assays

2.5.1. Blood DNA extraction

Total DNA was extracted from 200 µL of blood after digestion with proteinase K at +56 °C overnight using a commercial DNA extraction kit (QIAamp DNA Mini Kit®, [Qiagen, Courtaboeuf, France]) and performed on BIOROBOT EZ1 (Qiagen, Qiagen, Courtaboeuf, France) per the manufacturer's instructions. DNA was eluted in 200 µl of distilled water and stored at −20 °C.

2.5.2. PCR for the detection of Leishmania., Trypanosoma, Babesia and Hepatozoon spp

Blood samples were screened using real-time polymerase chain reaction (qPCR) assays targeting the 18S rRNA gene for *Leishmania* spp., the 5.8S rRNA gene for *Trypanosoma* spp. and the 5.8S rRNA gene for Piroplasmida (Table 1). The specific L. *infantum* qPCR assay based on the amplification of kinetoplast minicircle DNA (kDNA) was performed to quantify the parasite load as previously described (Mary et al., 2004).

qPCR assays were prepared in a final volume of 20 µl with 10 µl of Eurogentec Master Mix Roche, 3 µl of distilled water DNAse and RNAse free, 0.5 µl of each forward and reverse primers (concentrated at 20 µM), 0.5 µl FAM- labeled probe (concentrated at 20 µM) (Table 1), 0.5 µl of UDG and and 5 µl of the DNA template. The amplification was performed in a CFX96 Real-Time system (BioRad Laboratories, Foster City, CA, USA) with the following Roche protocol: a first incubation step at 50 °C for two minutes and an initial denaturation step at 95 °C for five minutes, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing-extension at 60 °C for 30 s. Known DNA of the target protozoa was included as a positive control and master mixtures as a negative control for each test. Samples were considered positive when the cycle threshold (Ct) was lower than 35 Ct for all PCR assays except for the 18S-based *Leishmania* spp. PCR, we considered sample as positive when Ct is lower than 38.

2.5.3. PCR amplification

Positive samples by qPCR were amplified by conventional PCR assays targeting fragment of genes ITS II for *Leishmania* spp., ITS I for *Trypanosoma* spp. and 18S rRNA for Piroplasmida. Screening for *Hepatozoon* spp. was evaluated by conventional PCR using primers that amplify a fragment of approximately 600 bp of the 18S ribosomal DNA gene (Table 1). PCR amplifications were performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA).

Table 1
Sequences of primers set used for detection and species identification in this study.

Targeted microorganisms	Target gene	Name	Primers (5′-3′) and probe	Tm	References
Leishmania spp.	18S	Leish. F	GGTTTAGTGCGTCCGGTG	60 °C	Medkour et al. submitted
		Leish. R	CGGCCCATAAGATCC CCAA		
		Leish. P*	FAM-CGGCCGTAACGCCTTTTCAACTCA -TAMRA		
		Leish. F1	CTGTGACTAAAGAAGCGTGAC	52 °C	
		Leish. R1	AGGCCGAATAGAAAAGATACGT		
	kDNA	RV1	CTTTTCTGGTCCTCCGGGTAGG	60 °C	Mary et al., 2004
		RV2	CCACCCGGCCCTATTTTACACCAA		
		Probe. Leish*	FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA		
	ITS II	LGITSF2	GCATGCCATATTCTCAGTGTC	60 °C	de Almeida et al., 2011
		LGITSR2	GGCCAACGCGAAGTTGAATTC		
Trypanosoma spp.	5.8S	Tryp 5.8SF	CAACGTGTCGCGATGGATGA	60 °C	Medkour et al. submitted
		Tryp 5.8SR	ATTCTGCAATTGATACCACTTATC		
		Tryp 5.8S P*	FAM-GTTGAAGAACGCAGCAAAGGCGAT-TAMRA		
	ITS I	ITS1-CF	CCGGAAGTTCACCGATATTG	58 °C	Njiru et al., 2005
		ITS1-BR	TTGCTGCGTTCTTCAACGAA		
Piroplasmida	5.8S	5.8S-F5	AYYKTYAGCGRTGGATGTC	60 °C	Dahmana et al., 2019
		5.8S-R	TCGCAGRAGTCTKCAAGTC		
		5.8S-S*	FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB		
	18S	piro18S-F1	GCGAATGGCTCATTAIAACA	58 °C	
		piro18S-R4	TTTCAGMCTTGCGACCATACT		
Hepatozoon spp.	18S	H14Hepa18SFw	GAAATAACAATACAAGGCAGTTAAAATGCT	58 °C	Hodžić et al., 2015
		H14Hepa18SRv	GTGCTGAAGGAGTCGTTTATAAAGA		

Abbreviations
Tm: Annealing temperature *: Probe.

Reaction mixtures were prepared in 50 µl volume, including 25 µl Amplitaq gold master mix, 1 µl of each primer, 5 µl of DNA template, and 18 µl of distilled water. Positive and negative controls were added to each reaction. The thermal cycling protocols were: incubation step at 95 °C for 15 min, 40 cycles of one minute at 95 °C, 30 annealing at a different hybridization temperature for each PCR assays and one minute at 72 °C and a final extension step for five minutes at 72 °C (Table 1). All amplicons were visualized in electrophoresis on 1.5% agarose gels.

2.6. Sequencing and phylogenic analysis

All positive amplicons were purified using NucleoFast 96 PCR plates (Macherey Nagel EURL, Hoerdt, France) as per the manufacturer's instructions and sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). The electropherograms obtained were assembled and edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) and compared with those available in the GenBank database by NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences of *Leishmania*, *Trypanosoma*, *Babesia* and *Hepatozoon* species obtained were analyzed using BLAST and compared to those available in the GenBank database. Phylogenetic and molecular evolutionary analysis was inferred using the maximum likelihood method based on the Kimura 2-parameter for nucleotide sequences and used on MEGA software version 6.06 (https://www.megasoftware.net/). Statistical support for the internal branches of the trees was evaluated by bootstrapping with 100 iterations.

2.7. Statistical analysis

IBM SPSS statistics version 23 software was used for the statistical analysis. After set of databases in XL 2010, we realized a descriptive study of the detection of infections in dogs and the number of co-infections detected in each dog, according to the results of the IFAT for leishmaniosis, qPCRs for *Trypanosoma* spp. and Piroplasmida and the standard PCR performed for *Hepatozoon* spp. The association between infections, origin, age, sex, breed, aptitude, clinical conditions of dogs and tick's carriage were evaluated using X^2 test. The exact Fischer test was used when the percentages were low. Statistically significant was consider at *P-value* $< .05$. The comparison between serological tests for CanL diagnosis was performed using the McNemar test in XLSTAT Addinsoft version 2018.7. To investigate the relationship between clinical manifestations and L. *infantum* antibody titers, we transformed qualitive variable of clinical scores (CS0 to CS4) into quantitative variables (from 0 to 4, respectively) and Spearman's test correlation was performed in XLSTAT. Quantitative variables were performed according to the number of infections as follow: 0 for no infection, 1: only one pathogen, 2: infection with two pathogens, 3: infection with three pathogens and 4: four pathogens. Then, we established the relationship between clinical manifestations (clinical scores) and the number of infections (Spearman correlation) in XLSTAT software version 2018.7.

3. Results

3.1. Signalment and clinical status

Age was available for all dogs with a median sampling age of 3.5 years and a range of 6 months to 13 years. Dogs are distributed according to their age as follows: 47 juveniles [6 month −2 years], 108 young dogs [2–4 years], 39 adults [4–6 years] and 33 oldies [> 6 years] dogs. Seventy-two dogs were females and 155 males. 108 dogs were pure bred while the others (119) were mongrel. In addition, 77 (34%) dogs presented at least one clinical sign mentioned above and 150 (66%) were apparently healthy. Dogs were classified according to their clinical signs and a clinical score (CS) was given for each one. One hundred-fifty were asymptomatic (CS0), 32 were classified as CS1 with one or two signs, 31 were classified in CS2 with three or four signs, nine were as CS3 with five of six signs and finally, five dogs were classified as CS4 with many clinical signs (> 6 signs). Among dogs, 32% (72/227) carried ticks.

3.2. Leishmania infantum specific antibodies detection

L. infantum antibodies were detected using Rapid Immuno Migration (RIM™) test and quantitative IFAT, which is considered as gold standard for the diagnosis of CanL (World Organisation for Animal Health (OIE), 2018). Antibodies (IgG) were detected in 36% (81/227) of dogs using IFAT (Table 2). We performed a serial dilution for the positive sera.

Table 2
Prevalence and risk factors associated to canine vector-borne protozoans in this study.

Risk factor		L. infantum									Trypanosoma spp.			B. vogeli			H. canis			Ticks		
		IFAT(No. Pos)		PV	Witness(No. Pos)		PV	qPCR(No. Pos)		PV	qPCR(No. Pos)		PV	qPCR(No. Pos)		PV	qPCR(No. Pos)		PV	Collected(No. Pos)		PV
Prevalence	Positive	35.7	81	–	31.7	72	–	4.8	11	–	6.6	15	–	13.2	30	–	41	93	–	31.7	72	–
	Negative	64.3	146		68.3	155		95.2	216		93.4	212		86.8	197		59	134		68.3	155	
Region	227																					
BOUIRA	97	23.7	23	0.001	18.6	18	0.00	4.1	4	0.71	3.1	3	0.01	19.6	19	0.013	28.9	28	0.00	34.0	33	0.23
SETIF	98	39.8	39		35.7	35		6.1	6		12.2	12		11.2	11		57.1	56		33.7	33	
TIZI-OUZOU	32	59.4	19		59.4	19		3.1	1		0.0	0		0.0	0		28.1	9		18.8	6	
Age	227																					
[0–2]	47	23.4	11	0.13	19.1	9	0.14	6.4	3	0.39	10.6	5	0.20	31.9	15	0.00	42.6	20	0.52	29.8	14	0.74
[2–4]	108	36.1	39		33.3	36		6.5	7		5.6	6		8.3	9		37.0	40		29.6	32	
[4–6]	39	38.5	15		33.3	13		2.6	1		10.3	4		12.8	5		41.0	16		33.3	13	
> 6	33	48.5	16		42.4	14		0.0	0		0.0	0		3.0	1		51.5	17		39.4	13	
Sex	227																					
Female	72	29.2	21	0.16	22.2	16	0.036	2.8	2	0.32	4.2	3	0.4	20.8	15	0.02	40.3	29	0.88	25.0	18	0.14
Male	155	38.7	60		36.1	56		5.8	9		7.7	12		9.7	15		41.3	64		34.8	54	
Breed	227	0.0																				
Defined	108	38.9	42	0.33	34.3	37	0.43	7.4	8	0.08	5.6	6	0.6	12.0	13	0.61	41.7	45	0.83	28.7	31	0.35
Mongrel	119	32.8	39		29.4	35		2.5	3		7.6	9		14.3	17		40.3	48		34.5	41	
Aptitude	227																					
Farm	31	12.9	4	0.001	9.7	3	0.001	3.2	1	0.18	3.2	1	0.34	12.9	4	0.81	41.9	13	0.37	22.6	7	0.34
Guard	75	50.7	38		46.7	35		1.3	1		6.7	5		13.3	10		36.0	27		30.7	23	
Hunting	73	37.0	27		31.5	23		9.6	7		11.0	8		13.7	10		49.3	36		38.4	28	
Pet	9	11.1	1		11.1	1		0.0	0		0.0	0		0.0	0		22.2	2		11.1	1	
Shepherd	39	28.2	11		25.6	10		5.1	2		2.6	1		15.4	6		38.5	15		33.3	13	
Dog's status	227																					
Sick dogs	77	88.3	68	0.00	81.8	63	0.00	13.0	10	0.00	6.5	5	0.60	15.6	12	0.45	53.2	41	0.01	39.0	30	0.1
healthy	150	8.7	13		6.0	9		0.7	1		6.7	10		12.0	18		34.7	52		28.0	42	
Clinical score	227																					
CS0	150	8.7	13	0.00	6.0	9	0.00	0.7	1	0.00	6.7	10	0.46	12.0	18	0.26	34.7	52	0.63	28.0	42	0.13
CS1	32	81.3	26		68.8	22		3.1	1		3.1	1		25.0	8		50.0	16		31.3	10	
CS2	31	90.3	28		87.1	27		19.4	6		12.9	4		9.7	3		51.6	16		48.4	15	
CS3	9	100.0	9		100.0	9		11.1	1		0.0	0		11.1	1		55.6	5		22.2	2	
CS4	5	100.0	5		100.0	5		40.0	2		0.0	0		0.0	0		80.0	4		60.0	3	
Ticks	227																					
Presence	72	44.4	32	0.06	40.3	29	0.06	4.2	3	0.74	9.7	7	0.08	13.9	10	0.83	45.8	33	0.31			
Absence	155	31.6	49		27.7	43		5.2	8		5.2	8		12.9	20		38.7	60		-		

Abbreviations
IFAT: immunofluorescence antibody test PV: P-value CS: clinical score (No. Pos): Number of positive.

Positive dogs had different IgG titers and 12.3% (10/81) were positive at 1:50 dilution. 28.4% (23/81) was positive at 1:100 dilution, 24.7% (20/81) at 1: 200 dilution, 6.2% (5/81) at 1:400 dilution, 20.1% (17/81) at 1:800 dilution and 7.4% (6/81) were positive at dilution above 1:800. The prevalence using the RIM™ test (WITNESS® Leishmania) was 32% (72/227). All but one of the rapid test positive samples were IFAT positive. On the other hand, IFAT was more sensitive and 10 samples were positive while they were negative in the WITNESS® Leishmania test. Statistical differences were observed among the results of these tests (McNemar Test, *p-value = .012*) with 0.88 (0.78–0.94) and 0.99 (0.96–1.00) respectively for the sensitivity and specificity of the rapid test.

3.3. Pathogen DNA detection

Blood samples from a total of 227 dogs from the three different geographical zones were analyzed. Sixty two percent (141/227) of the dogs had at least one infection. qPCR assays showed a prevalence of 4.8% (11/227) for *Leishmania* with an average parasitemia of 7. 10^6 parasite/mL (min: 4; max: 38. 10^6) of blood, 6.6% (15/227) for *Trypanosoma* spp. and 13.2% (30/227) for *Babesia* spp. PCR detects pathogens. DNA for *Hepatozoon canis* was detected in 41% (93/227) of dogs using conventional PCR for the screening (Table 2).

Regarding mono-infection, 36% (82/227) of dogs were infected by one pathogen with: 14% (32/227) being infected with L. *infantum*; 1% (2/227) infected with *Trypanosoma* spp.; 4% (9/227) of dogs by *B. vogeli* and 17.2% (39/227) with *H. canis*. In addition, co-infections by vector-borne protozoa were detected in 26% (59/227) of dogs, with two, three or even all four pathogens. The most prevalent was co-infection L. *infantum-H. canis,* around 16% (36/227) of dogs followed by co-infections with L. *infantum-B.vogeli* and *B. vogeli-H. canis* and association of these three pathogens in 2.2% (5/227) dogs for each type. Co-infections with *Trypanosoma spp. – H. canis* and triple infection combining the latter two *B. vogeli* pathogens with 1% *B. vogeli* were less frequent (2/227). Other co-infections such as: *L. infantum- Trypanosoma* spp.*-B. vogeli, L. infantum-Trypanosoma* spp.*-H. canis* and *Trypanosoma* spp.*- B. vogeli* were rare with only one case (0.4%). Finally, DNA for the four pathogens was detected in one dog (Table 3).

3.4. Pathogen infections versus risk factors

Regarding the prevalence of the protozoan infection according to the region: in Bouira, 24% (23/97) were positive for CanL, 3.1% (3/97) for *Trypanosoma* spp., 19.6% (19/97) for *B. vogeli*, 29% (28/97) for *H. canis*. The prevalence in Setif was as follows: 39.8% (39/98) for L. *infantum*, 12.2% (12/97) for *Trypanosoma* spp., 11.2% (11/97) for *B. vogeli* and 57.1% (56/98) for *H. canis*. For Tizi-Ouzou, 59.4% (19/32) were infected with L. *infantum*, no positive was detected for *Trypanosoma* spp. and *B. vogeli* and 28.1% were infected with *H. canis*. Statistically significant differences were found between the region and the prevalence of pathogens (*P-value* < .01). The dogs were classified into four age classes, as described above. No association could be observed between age and prevalence of all pathogens except *B. vogeli,* whereas prevalence varied with age and juvenile dogs were more sensitive at 31.9% (15/47), (*P-value* < .001). Also, we noted a statistically significant difference between the prevalence of this same pathogen vs sex (*P-value* = .02). Females were found to be more infected (20.8%) (15/72) with *B. vogeli* than males (9.7%) (15/155). In addition, we found that infection rates are the same in dogs whatever their breed (*P-value* > .08). By contrast, *L. infantum* infection varied according to dog's aptitude. Guard dogs were more infected, 50.7% (38/75), followed by hunting dogs, 37% (27/73), shepherd, 28.2% (11/39), farm dogs 12.9% (4/31) and finally pet with 11% (1/9) (*P-value* = .001). A total of 567 ticks were collected from 72 dogs. No link was found between tick infestation and pathogen prevalence (*P-value* > .08).

3.5. Pathogen infection versus clinical signs

We investigated the relationship between the dog's condition (sick or healthy) and pathogenic infections. No link was found between the dog's condition and *Trypanosoma* spp. infections (*P-value* = .6) and *B. vogeli* (*P-value* = .45). By contrast, statistically significant differences were found between dog's condition and L. *infantum* IgG detection (*P-value* < .001). 88% of sick dogs (68/77) had L. *infantum* IgG, whereas these IgGs were detected in 8.7% (13/150) of healthy dogs. The same was true for the relationship between *H. canis* infection and dog status (*P-value* = .01). 53.2% (41/77) of sick dogs were *H. canis* infected while 34.7% (52/150) of healthy dogs were found infected. However, this difference seems to be influenced by the association with other protozoa; and when we considered only dogs mono-infected with *Hepatozoon*, we found 5.2% (4/77) in sick dogs and 23.3% (35/150) in healthy dogs, (*P-value* = .0001) in favor of asymptomatic dogs. *Hepatozoon* infection is therefore much more asymptomatic in dogs.

The clinical signs revealed during the physical examination, described above, are correlated with the clinical manifestations of CanL as described in the LeishVet guide (Luis Cardoso, 2011). Here, we have tried to understand whether there is a correlation between clinical manifestations and antibody titers of L. *infantum* in CanL. We found a good positive correlation (Spearman correlation coefficient: 0.8; min: −1; max: 1), *P-value* = .0001. We explored the hypothesis that clinical

Table 3
Frequency of infections and co-infection on surveyed dogs.

	Bouira	*n* = 97	Setif	*n* = 98	Tizi-Ouzou	*n* = 32	Total	*N* = 227
Infections	No. Positive	%	No. Positive	%	No. Positive	%	No. Positive	%
Mon-infection	33	34,0%	34	34,7%	15	46,9%	82	36,1%
Leishmania infantum	10	10,3%	9	9,2%	13	40,6%	32	14,1%
Trypanosoma spp.	1	1,0%	1	1,0%	0	0,0%	2	0,9%
Babesia canis vogeli	6	6,2%	3	3,1%	0	0,0%	9	4,0%
Hepatozoon canis	16	16,5%	21	21,4%	2	6,3%	39	17,2%
Co-infection	19	19,6%	35	35,7%	5	15,6%	59	26,0%
L + B	4	4,1%	1	1,0%	0	0,0%	5	2,2%
L + H	7	7,2%	24	24,5%	5	15,6%	36	15,9%
L + B + H	2	2,1%	3	3,1%	0	0,0%	5	2,2%
L + T + B	1	1,0%	0	0,0%	0	0,0%	1	0,4%
L + T + H	0	0,0%	1	1,0%	0	0,0%	1	0,4%
L + T + B + H	0	0,0%	1	1,0%	0	0,0%	1	0,4%
T + B	1	1,0%	0	0,0%	0	0,0%	1	0,4%
T + H	0	0,0%	2	2,0%	0	0,0%	2	0,9%
T + B + H	0	0,0%	2	2,0%	0	0,0%	2	0,9%
B + H	4	4,1%	1	1,0%	0	0,0%	5	2,2%
TOTAL	52	53,6%	69	70,4%	20	62,5%	141	62,1%

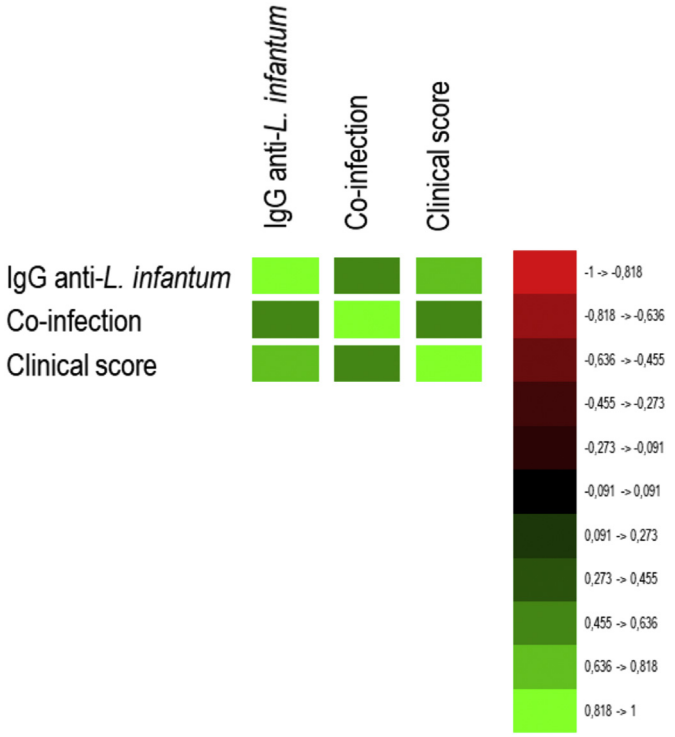


Fig. 2. Correlation matrices showed the relationship between L. *infantum* IgG titers, Clinical score and co-infection in dogs surveyed in this study.

manifestations varied according to the number of pathogen infecting dogs. A positive correlation was found between these last two variables (correlation coefficient: 0.6 and *P-value* = .0001) (Fig. 2).

3.6. Sequencing results and phylogeny

The BLAST comparison of the ten partial sequences obtained for the ITS2 gene of *Leishmania* revealed 97–99% homology with L. *infantum* sequences available in GenBank database (i.e: MH605316; KU680960; KU680959) (Fig. 3. A). Among the nine partial sequences obtained from the ITS1 gene of *Trypanosoma*, seven of them have a 99% identity with *T. evansi* (AB551920; FJ712716; AB551922; etc.) deposed in the GenBank. Obtained sequences from two other dogs when BLASTed showed 92–93% identity to *T. congolense* (FJ712718; MG255204) (Fig. 3. B). These two dogs had moderate clinical status (CS2). In addition, sequencing of 18S rRNA genes of Piroplasmida and *Hepatozoon* spp. allowed news genotypes of *B. vogeli* and *H. canis* to be found (Fig. 3. C and D).

4. Discussion

Dogs are competent reservoirs as host for several pathogens, some of which are zoonotic, and their increased relationship with humans in developing countries as companions poses new public health problems (Otranto et al., 2009). In recent decades, this has considerably increased the number of studies on canine vector-borne diseases (CVBD) in these countries. In contrast, data on CVBD in many African countries, their frequency and diversity, are very insufficient. These are casually absent for some canine vector-borne protozoan diseases in Algeria and

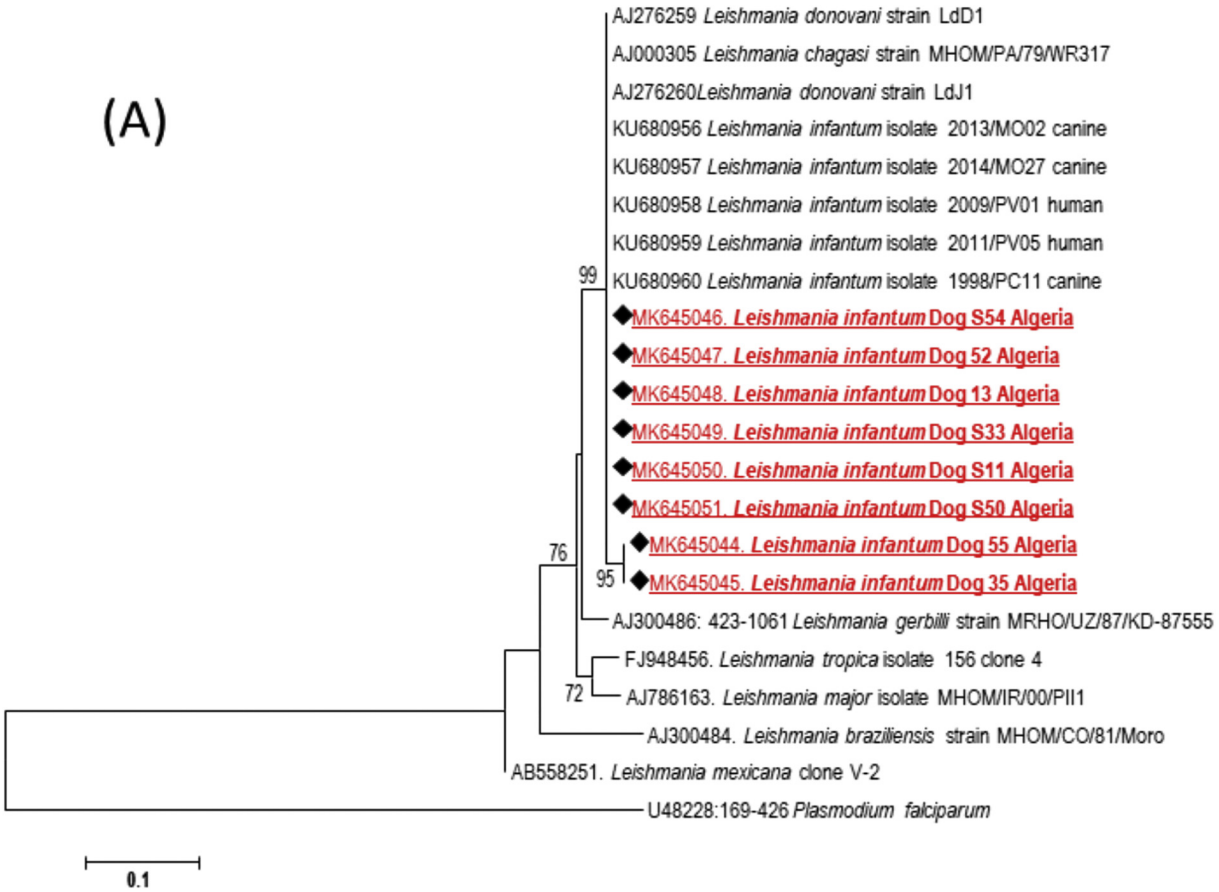


Fig. 3. Molecular Phylogenetic analysis by Maximum Likelihood method on the Tamura-Nei model based on the partial nucleotide sequences (360 pb) of ITS2 rRNA for *Leishmania* spp. (A); (250 pb) of ITS1 for *Trypanosoma* spp. (B); (900 pb) of 18S rRNA for Piroplasmida (C) and (600 pb) of 18S rRNA for *Hepatozoon* spp. (D). Trees highlight the positions of identified pathogens in this study.

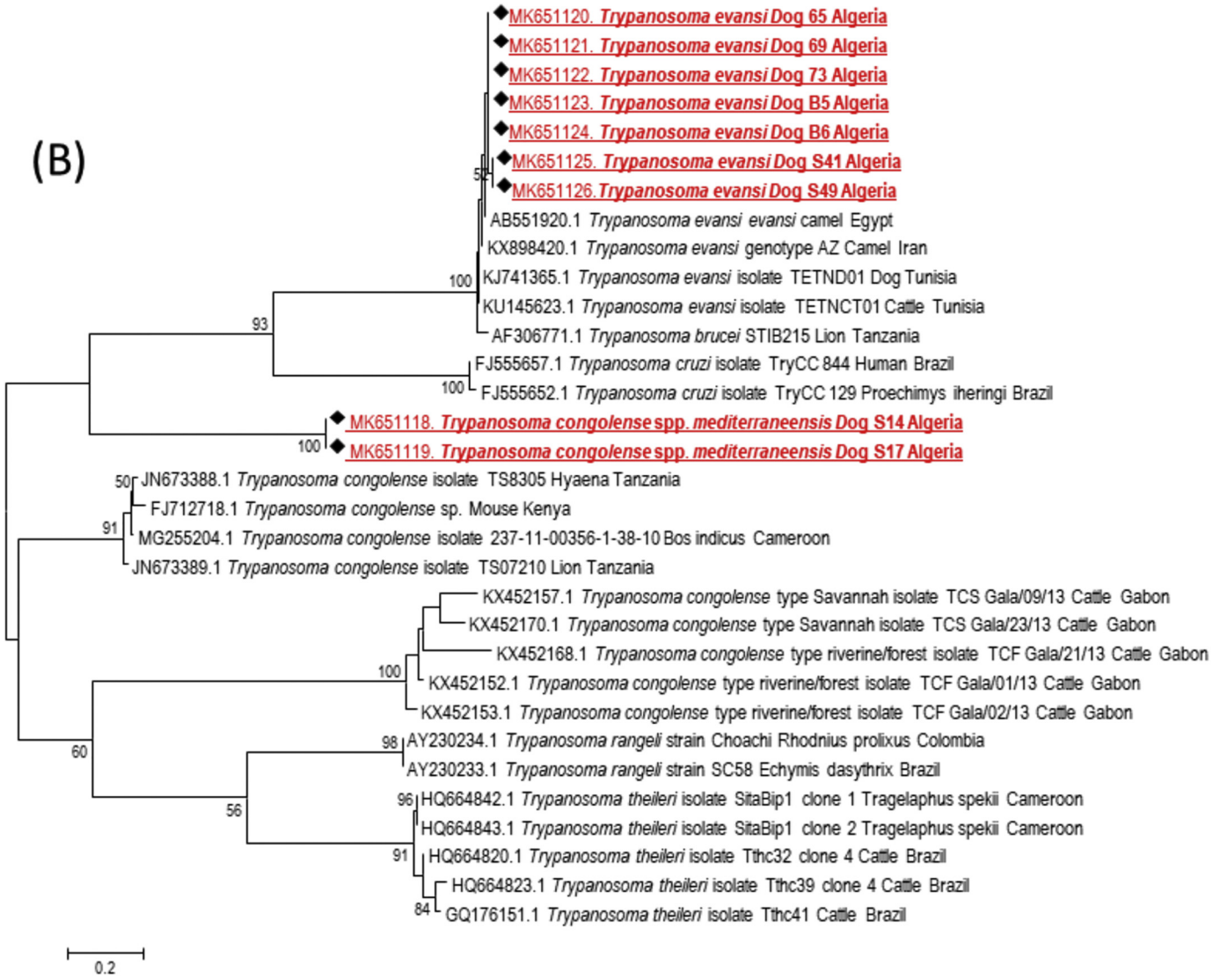


Fig. 3. (*continued*)

all Northern Africa. This study constitutes the first report of *Trypanosoma* spp. *B. vogeli* and *H. canis* infection in dogs from Kabylia (Algeria). In addition, it updates the epidemiological status historical outbreak (Kabylia) of visceral leishmaniasis in Algeria, where a high prevalence of L. *infantum* infection has been detected in dogs. Also, positive correlation was observed between co-infections with these vector-borne pathogens, CanL, and the number of clinical signs identified in dogs. Sixty two percent of dogs presented at least one infection and 26% were found to be co-infected.

We detected L. *infantum* antibodies in 35.7% and 31.7% of dogs using IFAT and WITNESS® Leishmania rapid test, respectively. Sensitivity (88%) and specificity (99%) of the rapid test are good compared to the IFAT test. In a study compared different serological tests, sensitivity and specificity of WITNESS® Leishmania test were 58% and 100%, respectively, (Rodríguez-Cortés et al., 2013). The prevalence of IFAT ranged from 27 to 47% in the study including dogs from six localities in northern Algeria (Adel et al., 2015). Recently, a study conducted on 603 dogs from Kabylia reported an overall seroprevalence of 10% with a significant relationship between the density of phlebotomes, especially *Phlebotomus pernicious* and *P. perfiliewi*, the seroprevalence of CanL as well as the reported human leishmaniasis cases in the region (Mouloua et al., 2017). Sequencing of ITS2 gene showed L. *infantum* identity in all PCR positive dogs. Our results suggest the persistence of a L. *infantum* transmission cycle with possible outbreaks of CanL and/or human visceral leishmaniasis in this focus. Therefore, a national control program against leishmaniosis should be implemented.

Trypanosomes were detected in 6.6% of the Kabylian dogs. No data are available on the presence of these protozoa in Algerian dogs. Our molecular assay by sequencing showed: 1) a high genetic diversity of two sequences obtained on two sick dogs with general clinical signs such as weight body loss, lethargy and hyperthermia, with the closely related species of trypanosomes. These sequences were closely identical and exhibited 92–93% identity with *T. congolense.* We proposed a new entity as sub-species: *T. congolense* spp. *mediterranensis. T. congolense* remains one of the most important agent of canine trypanosomosis (Ogbu et al., 2017) and livestock diseases in Sub-Saharan Africa, particularly affecting cattle (Morrison et al., 2016). The disease is relatively common in Nigeria due to the high prevalence of *Glossina* spp. which is the main factor responsible for the transmission of African trypanosomiasis (Isaac et al., 2016). Recently, 5.3% (5/95) of dogs from Abidjan were found infected with *T. congolense* (Medkour et al., submitted). Seven dogs were infected with *T. evansi*, the agent of surra and a salivarian trypanosome, originating from Africa. It is reported in North Africa, Southern Europe, Latin America and Asia (Pommier de Santi et al., 2018). It is commonly pathogenic in camels, horses, cattle and occasionally in humans (Elhaig et al., 2013) (Joshi et al., 2005) and

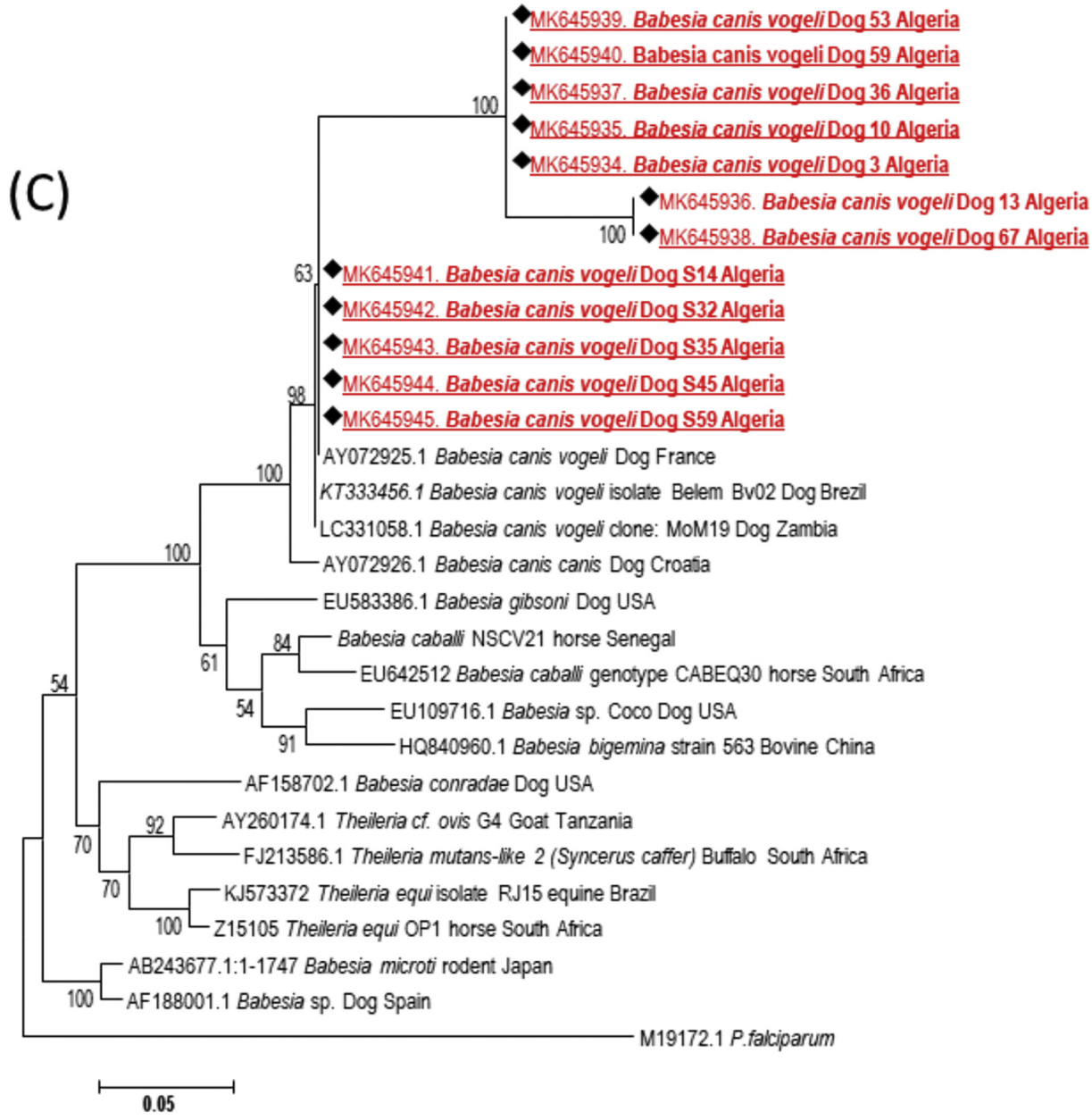


Fig. 3. (*continued*)

dogs (Desquesnes et al., 2013). *T. evansi* has been reported in Sousse (central Tunisia) in a dog showing clinical signs of trypanosomiasis (Rjeibi et al., 2015) and recently in cattle from northern Tunisia (Sallemi et al., 2018). Two other cases of canine trypanosomosis have been reported: one in Germany (Defontis et al., 2012) and another in Afghanistan (Aref et al., 2013). Higher-level *T. evansi* infections have been detected in camels in south-eastern Algeria (Bennoune et al., 2013). On the other side of the Mediterranean, *T. evansi* infection was detected in the province of Alicante, Spain, on a camel and equine farm after the introduction of camels from the Canary Islands (Tamarit et al., 2010). The mode of infection of these dogs is very obscure and further research will be needed to resolve this dilemma. The migration of infected animals, such as camels, sheep and cattle, from south to north can be a source of infection for dogs. The competent vector recognized for African trypanosomes (*Glossina* spp.) is not found in Algeria. The vector remains very questionable and other *Diptera*, such as *Tabanidae*, can be involved in this transmission, which could explain the appearance of new genotypes (*T. congolense* spp. *mediterranensis*) adapted to these vectors.

B. vogeli-infection was found in 13.2% of the sampled dogs. *Babesia canis* is reported in Algeria without conclusive determination of the species in the former subspecies (Matallah et al., 2012). In Tunisia, *B. vogeli* was detected in 6.7% (12/180) of dogs and in 0.6% (1/160) of their ticks *R.sanguineus* (M'ghirbi and Bouattour, 2008). Surveys in Côte d'Ivoire (Medkour et al., submitted), Nigeria (Kamani et al., 2013) and Angola (Luís Cardoso et al., 2016) reported a prevalence of 1.6% (2/123), 0.6% (1/181) and 5.8% (6/103) in dogs, respectively. This species has a global distribution (Dantas-Torres, 2008b) and has been identified in Africa, Asia, Turkey, Australia, North and South America and Costa Rica. In Europe, *B. vogeli* was found in Slovenia, Albania, France, Spain, Portugal and Croatia with a prevalence ranging from 1% to 16.3% (Bilić et al., 2018) (Rojas et al., 2014). The presence of dog's brown ticks the main vector of *B.vogeli* (Otranto, 2018) on surveyed dogs can explain our results.

A high prevalence of *H. canis* was observed in our study (41%). This is the first report in North Africa of this pathogenic protozoan. The

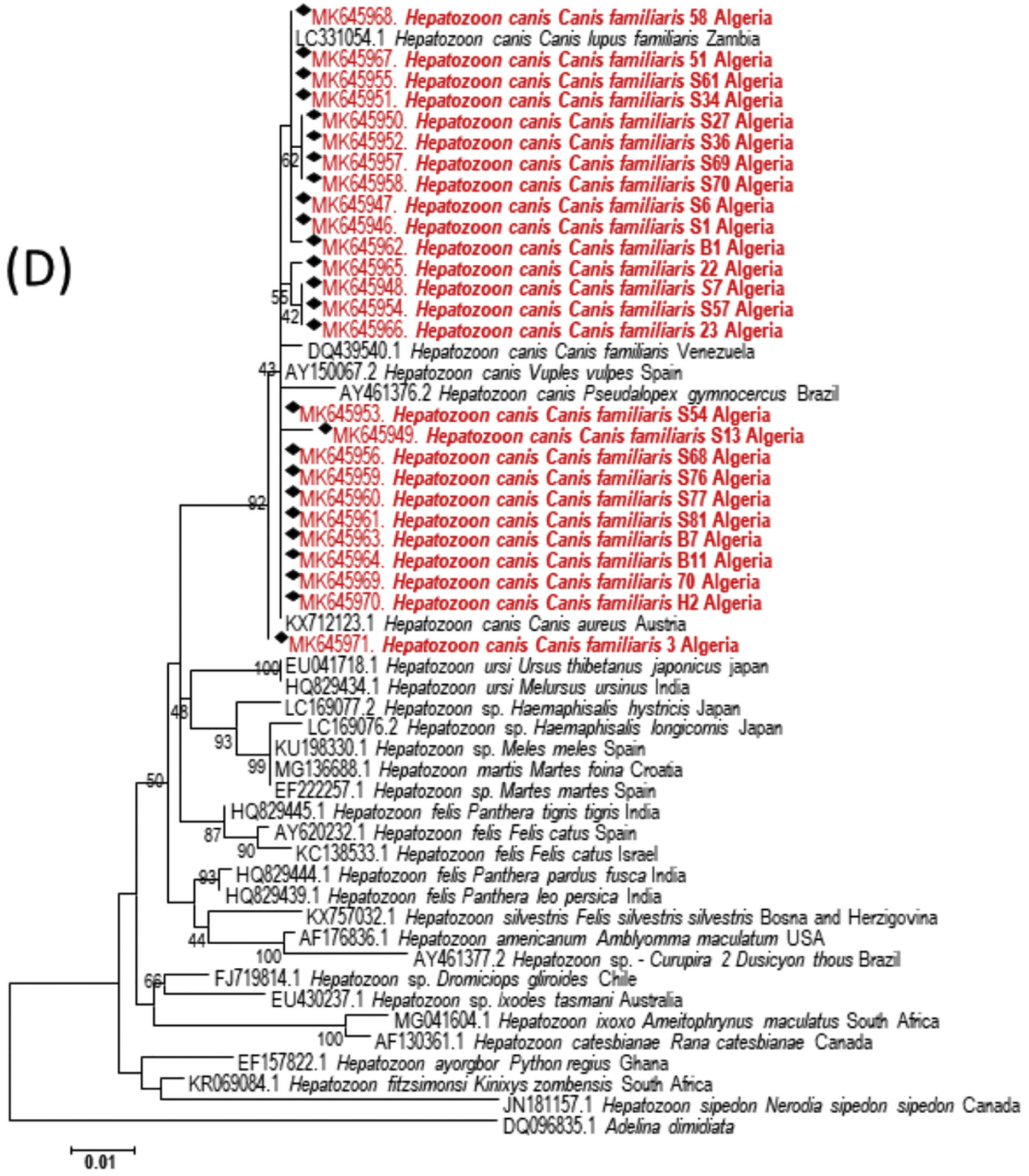


Fig. 3. (*continued*)

same prevalence was detected in dogs from Nigeria and 21% of their ticks were infected (Kamani et al., 2013). 13% of Zambian dogs and 17.5% in Angola were found infected (Williams et al., 2014) (Luís Cardoso et al., 2016). *H. canis* is distributed in Europe, Asia, Africa and in South America (Naouelle and Ghalmi, 2016). As in the case of *B. vogeli*-infection, infection with *H. canis*, can be explained by the widespread prevalence of its main tick vectors in Kabylia.

Amount the distract surveyed, the occurrence of infections varied considerably for all four pathogens. Dogs from Tizi-Ouzou are found to be more infected (59.4%) by L. *infantum,* certainly because the region has been endemic for a long time. *Trypanosoma* spp. infection is higher in Setif (12.2%) than the other distracts (0 and 3%). This may be due to

livestock density and livestock trade in this area, which can be the source of dog's infection. *B. vogeli* and *H. canis* infection are less frequent in dogs from Tizi-Ouzou. The tick infestation rate is lower in this region. Juvenile dogs under two years of age have a significantly higher rate of *Babesia* infection than other age groups. These results are consistent with previous studies that have shown that young dogs are more sensitive to babesiosis because of their underdeveloped immune systems (Taylor et al., 2007). No relationship was found between dog's sex and the occurrence of infections, expect for *B. vogeli* for which males are more infected. Also, guard dogs presented the higher prevalence for CanL, followed by hunting and shepherd dogs. Because they are often tied and/or live outside their homes, they will be more exposed to phlebotomous bites.

With regard to the clinical condition of the dog in relation to the occurrence of infections, sick dogs were more infected with L. *infantum* and *H. canis* than healthy dogs. The clinical signs identified belong to the physical examination correlated with the CanL signs described in the Leishvet guide (Cardoso, 2011). Sick dogs infected with *Hepatozoon* usually had one or more other infections and all but four dogs with *H. canis* mono-infection are asymptomatic. (35/39).

In this study, co-infection was diagnosed in 26% of the dogs surveyed. Co-infection with two protozoa was more frequent than with three protozoa and only one dog was infected with all four pathogens. Co-infections by more than one vector-borne pathogen were observed in many studies on canine vector borne diseases (Rojas et al., 2014) (Medkour et al., submitted). Moreover, a positive correlation was found between the clinical score estimated according to the number of clinical signs and *Leishmania* antibody titers detected by IFAT. Therefore, a dog with an advanced stage of the disease has, in this study, a high rate of antibodies. In addition, a positive relationship was found between the clinical score and the number of pathogens infecting dogs. Therefore, co-infections concur to complicate the clinical manifestations. In a study including *Leishmania* infected dogs, positivity to some vector-borne pathogens was associated with more marked clinicopathological abnormalities as well as disease severity with CanL (Baxarias et al., 2018).

5. Conclusion

Our study demonstrates that dogs from Kabylia (Algeria) are frequent hosts of vector-borne protozoa. *Leishmania* infection and co-infections with more than one pathogen are associated with more clinical manifestations. Molecular evidence of canine *Trypanosoma* spp., *B. vogeli* and *H. canis* as well as the high prevalence of L. *infantum* has been brought to the attention of public and animal health authorities and dog owners. Rigorous surveillance and control plans for these canine diseases are in place in this area to contain them, or at least reduce their spread.

Funding

This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program « Investissements d'avenir », reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All animals sampled in this study were examined with the assistance of their owners. Blood samples were collected by a veterinarian.

Ethical statement

The study was carried out in accordance with Algerian legislation guidelines. In addition, the samples were collected after obtaining a verbal consent from dog owners by a veterinarian specialist. Risk assessment was submitted to and approved by the ethics committee and decision board of the veterinary practitioners from the wilayas of the North of Algeria. These institutions are affiliated with the Algerian Ministry of Agriculture and Rural Development (Directions des Services Vétérinaires). Protocol of the study was also approved by the scientific college (Procès-Verbal du CSI N°6, 2018) at the Veterinary Science Institute, University Constantine 1, Algeria. To facilitate field work, collaborations were established with veterinary doctors and their assistants working in these establishments.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

We are grateful to Azzaz M, Lounas A, Hamidat HK, Chaba A, Amara F, veterinarians and dog owners for their valuable help with the field work.

References

Adel, A., Abatih, E., Speybroeck, N., et al., 2015. Estimation of canine *Leishmania* infection prevalence in six cities of the Algerian littoral zone using a Bayesian approach. PLoS One 10 (3), e0117313. https://doi.org/10.1371/journal.pone.0117313.

Aref, M., Yasin, S.M., Bahear, W., Ghulam, Z., Hastie, L., Dennison, T., Deborggraeve, S., 2013. Canine *Trypanosoma evansi* infection in Afghanistan. Vet. Parasitol. 197 (3–4), 638–641. https://doi.org/10.1016/j.vetpar.2013.07.016.

Baneth, G., 2011. Perspectives on canine and feline hepatozoonosis. Vet. Parasitol. 181 (1), 3–11. https://doi.org/10.1016/j.vetpar.2011.04.015.

Baxarias, M., Álvarez-fernández, A., Martínez-orellana, P., Montserrat-sangrà, S., Ordeix, L., Rojas, A., Solano-gallego, L., 2018. Does co-infection with vector-borne pathogens play a role in clinical canine leishmaniosis ? Parasit. Vectors 1–16.

Bennoune, O., Adili, N., Amri, K., Bennecib, L., Ayachi, A., 2013. Trypanosomiasis of camels (Camelus dromedarius) in Algeria: first report. Veterinary Research Forum : An International Quarterly Journal 4 (4), 273–275. Retrieved from. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4279620&tool=pmcentrez&rendertype=abstract.

Bilić, P., Kuleš, J., Barić Rafaj, R., Mrljak, V., 2018. Canine babesiosis: where do we stand? Acta Veterinaria 68 (2), 127–160. https://doi.org/10.2478/acve-2018-0011.

Cardoso, L., 2011. Parasites & Vectors LeishVet Guidelines for the Practical Management of Canine Leishmaniosis. pp. 1–16. https://doi.org/10.1186/1756-3305-4-86.

Cardoso, L., Oliveira, A.C., Granada, S., Nachum-Biala, Y., Gilad, M., Lopes, A.P., ... Baneth, G., 2016. Molecular investigation of tick-borne pathogens in dogs from Luanda, Angola. Parasit. Vectors 9 (1), 1–6. https://doi.org/10.1186/s13071-016-1536-z.

Dahmana, H., Amanzougaghene, N., Davoust, B., Fenollar, F., Raoult, D., Mediannikov, O., et al., December 2019. Great diversity of *Piroplasmida* in Equidae in Africa and Europe Including New Species. VPRSR 18, 100332. https://doi.org/10.1016/j.vprsr.2019.100332. https://www.sciencedirect.com/science/article/pii/S2405939018302375.

Dantas-Torres, F., 2008a. Canine vector-borne diseases in Brazil. Parasit. Vectors 1 (1), 1–17. https://doi.org/10.1186/1756-3305-1-25.

Dantas-Torres, F., 2008b. The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): from taxonomy to control. Vet. Parasitol. 152 (3–4), 173–185. https://doi.org/10.1016/j.vetpar.2007.12.030.

de Almeida, M.E., Steurer, F.J., Koru, O., Herwaldt, B.L., Pieniazek, N.J., da Silva, A.J., 2011. Identification of *Leishmania* spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2. J. Clin. Microbiol. 49, 3143–3149.

De Tommasi, A.S., Otranto, D., Dantas-Torres, F., Capelli, G., Breitschwerdt, E.B., De Caprariis, D., 2013. Are vector-borne pathogen co-infections complicating the clinical presentation in dogs? Parasit. Vectors 6 (1). https://doi.org/10.1186/1756-3305-6-97.

Defontis, M., Richartz, J., Engelmann, N., Bauer, C., Schwierk, V.M., Büscher, P., Moritz, A., 2012. Canine *Trypanosoma evansi* infection introduced into Germany. Vet. Clin. Pathol. 41 (3), 369–374. https://doi.org/10.1111/j.1939-165X.2012.00454.x.

Desquesnes, M., Holzmuller, P., Lai, D.-H., Dargantes, A., Lun, Z.-R., Jittaplapong, S., 2013. *Trypanosoma evansi* and Surra: a review and perspectives on origin, history, distribution, taxonomy, morphology, hosts, and pathogenic effects. Biomed. Res. Int. 194176. https://doi.org/10.1155/2013/194176.

Díaz-Regañón, D., Villaescusa, A., Ayllón, T., Rodríguez-Franco, F., Baneth, G., Calleja-

Bueno, L., ... Sainz, Á., 2017. Molecular detection of *Hepatozoon* spp. and *Cytauxzoon* sp. in domestic and stray cats from Madrid, Spain. Parasit. Vectors 10 (1), 1–9. https://doi.org/10.1186/s13071-017-2056-1.

Diouani, M.F., Bouafif, N.B.E.N.A., Bettaib, J., Louzir, H., Jedidi, S., Ftaiti, A., ... Salah, A.B.E.N, June 2014. Dogs *L. infantum* infection from an endemic region of the North of Tunisia : a prospective study. Most 85, 55–62.

Elhaig, M.M., Youssef, A.I., El-gayar, A.K., 2013. Veterinary Parasitology molecular and parasitological detection of *Trypanosoma evansi* in camels in Ismailia. Egypt. Vet. Parasitol. 198 (1–2), 214–218. https://doi.org/10.1016/j.vetpar.2013.08.003.

Eloy, L., Lucheis, S., 2009. Canine trypanosomiasis: etiology of infection and implications for public health. J. Venomous Animals Toxins Including Tropical Diseases 15 (4), 589–611. https://doi.org/10.1590/S1678-91992009000400002.

Gage, K.L., Burkot, T.R., Eisen, R.J., Hayes, E.B., 2008. Climate and Vectorborne Diseases. 35(5)https://doi.org/10.1016/j.amepre.2008.08.030.

Harrus, S., Baneth, G., 2005. Drivers for the emergence and re-emergence of vector-borne protozoal and bacterial diseases. Int. J. Parasitol. 35 (11 − 12), 1309–1318. https://doi.org/10.1016/j.ijpara.2005.06.005.

Hodžić, A., Alić, A., Fuehrer, H.P., Harl, J., Wille-Piazzai, W., Duscher, G.G., 2015. A molecular survey of vector-borne pathogens in red foxes (*Vulpes vulpes*) from Bosnia and Herzegovina. Parasit. Vectors 8, 1–7.

Isaac, C., Ciosi, M., Hamilton, A., Scullion, K.M., Dede, P., Igbinosa, I.B., ... Turner, C.M.R., 2016. Molecular identification of different trypanosome species and subspecies in tsetse flies of northern Nigeria. Parasit. Vectors 9 (1), 1–7. https://doi.org/10.1186/s13071-016-1585-3.

Joshi, P.P., Shegokar, V.R., Powar, R.M., Herder, S., Katti, R., Salkar, H.R., ... Truc, P, 2005. Human Trypanosomiasis Caused by *trypanosoma evansi* in India: The First Case Report. 73(3). pp. 491–495.

Kamani, J., Baneth, G., Mumcuoglu, K.Y., Waziri, N.E., Eyal, O., Guthmann, Y., Harrus, S., 2013. Molecular detection and characterization of tick-borne pathogens in dogs and ticks from Nigeria. PLoS Negl. Trop. Dis. 7 (3), 1–7. https://doi.org/10.1371/journal.pntd.0002108.

Mary, C., Faraut, F., Lascombe, L., Dumon, H., 2004. Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity. J. Clin. Microbiol. 42 (11), 5249–5255. https://doi.org/10.1128/JCM.42.11.5249.

Matallah, F., Benakhla, A., Medjouel, L., Matallah, S., 2012. Tick infestation of dogs and prevalence of canine babesiosis in the north-east of Algeria; area of El-Tarf 1. *American-Eurasian*. J. Sustain. Agric. 6 (3), 126–134.

Medkour, H., Laidoudi, Y., Athias, E., Bouam, A., Dizoé, S., Davoust, B. and Mediannikov, O. Molecular and serological detection of animal and human vector-borne pathogens in the blood of dogs from Côte d'Ivoire. Comparative Immunology, Microbiology and Infectious Diseases, under review

M'ghirbi, Y., Bouattour, A., 2008. Detection and molecular characterization of Babesia canis vogeli from naturally infected dogs and *Rhipicephalus sanguineus* ticks in Tunisia. Vet. Parasitol. 152, 1–2, 1–7. https://doi.org/10.1016/j.vetpar.2007.12.018.

Millán, J., Chirife, A.D., Kalema-Zikusoka, G., et al., 2013. Serosurvey of dogs for human, livestock, and wildlife pathogens. Uganda. Emerg. Infect. Dis. 19 (4), 680–682.

Morrison, L.J., Vezza, L., Rowan, T., Hope, J.C., 2016. Animal African trypanosomiasis: time to increase focus on clinically relevant parasite and host species. Trends Parasitol. 32 (8), 599–607. https://doi.org/10.1016/j.pt.2016.04.012.

Mouloua, A., Boubidi, S.C., Bouiba, L., Mezai, G., Madiou, M., Harrat, Z., 2017. Impact environnemental sur la répartition des leishmanioses dans le foyer de Tizi-Ouzou (Algérie). Rev. Med. Vet. 168 (10 − 12), 252–261.

Naouelle, A., Ghalmi, F., 2016. Canine hepatozoonosis a bibliograhic review. Revue Méd. Vét. 167, 7–8 (201-210).

Njiru, Z.K., Constantine, C.C., Guya, S., Crowther, J., Kiragu, J.M., Thompson, R.C.A., et al., 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol. Res. 95, 186–192.

Ogbu, K.I., Anene, B.M., Nweze, N.E., Chukwudi, I.C., Danladi, M.A., 2017. Trypanosomosis in dogs : a review. Int. J. Sci. Appl. Res. Vol. 2 (2) (ISSN 2504–9070).

Otranto, D., 2018. Arthropod-borne pathogens of dogs and cats: from pathways and times of transmission to disease control. Vet. Parasitol. 251, 68–77. https://doi.org/10.1016/j.vetpar.2017.12.021. (November 2017).

Otranto, D., Dantas-Torres, F., Breitschwerdt, E.B., 2009. Managing canine vector-borne diseases of zoonotic concern: part two. Trends Parasitol. 25 (5), 228–235. https://doi.org/10.1016/j.pt.2009.02.005.

Otranto, D., Testini, G., Dantas-Torres, F., Latrofa, M.S., De Paiva Diniz, P.P.V., De Caprariis, D., ... Breitschwerdt, E.B., 2010. Diagnosis of canine vector-borne diseases in young dogs: A longitudinal study. Journal of Clinical Microbiology 48 (9), 3316–3324. https://doi.org/10.1128/JCM.00379-10.

Pommier de Santi, V., Briolant, S., Mahamat, A., Ilcinkas, C., Blanchet, D., de Thoisy, B., ... Raoulti, D. Q, 2018. Fever epidemic in Cayenne, French Guiana, epidemiologically linked to three-toed sloth. Comp. Immunol. Microbiol. Infect. Dis. 56, 34–38. https://doi.org/10.1016/j.cimid.2017.12.004. (May 2017).

Qiu, Y., Kaneko, C., Kajihara, M., Ngonda, S., Simulundu, E., Muleya, W., ... Nakao, R., 2018. Tick-borne haemoparasites and *Anaplasmataceae* in domestic dogs in Zambia. Ticks and Tick-Borne Diseases 9 (4), 988–995. https://doi.org/10.1016/j.ttbdis.2018.03.025.

Rami, M., Atarhouch, T., Sabri, M., Cadi, S.M., Benazzou, T., Dakkak, A., 2003. Leishmaniose canine dans le Rif (côte méditerranéenne marocaine) : enquête séro-épidémiologique. Parasite 10 (1), 79–85. https://doi.org/10.1051/parasite/2003101p77.

Rjeibi, M.R., Ben Hamida, T., Dalgatova, Z., Mahjoub, T., Rejeb, A., Dridi, W., Gharbi, M., 2015. First report of Surra (*Trypanosoma evansi* infection) in a Tunisian dog. Parasite 22, 3. https://doi.org/10.1051/parasite/2015004.

Rodríguez-Cortés, A., Ojeda, A., Todolí, F., Alberola, J., 2013. Performance of commercially available serological diagnostic tests to detect Leishmania infantum infection on experimentally infected dogs. Vet. Parasitol. 191 (3–4), 363–366. https://doi.org/10.1016/j.vetpar.2012.09.009.

Rojas, A., Rojas, D., Montenegro, V., Gutiérrez, R., Yasur-Landau, D., Baneth, G., 2014. Vector-borne pathogens in dogs from Costa Rica: first molecular description of Babesia vogeli and Hepatozoon canis infections with a high prevalence of monocytic ehrlichiosis and the manifestations of co-infection. Vet. Parasitol. 199 (3–4), 121–128. https://doi.org/10.1016/j.vetpar.2013.10.027.

Sallemi, S., Rjeibi, M.R., Rouatbi, M., Amairia, S., Ben Said, M., Khamassi Khbou, M., Gharbi, M., 2018. Molecular prevalence and phylogenetic analysis of *theileria annulata* and *trypanosoma evans*i in cattle in northern Tunisia. Vet. Med. Sci. 4 (1), 17–25. https://doi.org/10.1002/vms3.79.

Schnittger, L., Florin-Christensen, M., 2018. Parasitic Protozoa of Farm Animals and Pets. https://doi.org/10.1007/978-3-319-70132-5.

Tamarit, A., Gutierrez, C., Arroyo, R., Jimenez, V., Zagalá, G., Bosch, I., ... Caballero, C., 2010. *Trypanosoma evansi* infection in mainland Spain. Veterinary Parasitology 167 (1), 74–76. https://doi.org/10.1016/j.vetpar.2009.09.050.

Taylor, M.A., Coop, R.L., Wall, R.L., 2007. Parasites of dogs and cats. In: Veterinary Parasitology, 3d edition. Iowa State, Blackwell Publishing, USA, pp. 409–426.

Thiébault, S., Moatti, J.P., 2016. The Mediterranean Region Under Climate Change : A Scientific Update. pp. 391–401. ISBN 978-2-7099-2219-7; Available from. http://horizon.documentation.ird.fr/exl-doc/pleins_textes/divers16-11/010068495.pdf.

Williams, B.M., Berentsen, A., Shock, B.C., Teixiera, M., Dunbar, M.R., Becker, M.S., Yabsley, M.J., 2014. Prevalence and diversity of *Babesia, Hepatozoon, Ehrlichia, and Bartonella* in wild and domestic carnivores from Zambia, Africa. Parasitol. Res. 113 (3), 911–918. https://doi.org/10.1007/s00436-013-3722-7.

World Organisation for Animal Health (OIE), 2018. Chapter 3.1.11. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. http://www.oie.int/fr/normes/manuel-terrestre/acces-en-ligne.

Publication N°16

Molecular and serological detection of animal and human vector-borne pathogens in the blood of dogs from Côte d'Ivoire

Hacène Medkour[1,2], **Younes Laidoudi**[1,2], Eric Athias[3], Amar Bouam[1,2], Sylvestre Dizoé[4], Bernard Davoust[1,2] and Oleg Mediannikov[1,2*]

[1]Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France
[2] IHU-Méditerranée Infection, Marseille, France
[3]Army health service, Abidjan, Côte d'Ivoire
[4] Kongouanou care center, Côte d'Ivoire

ARTICLE DE RECHERCHE

Publié dans : Comparative Immunology, Microbiology and Infectious Diseases, 2020, 69:101412

(doi:10.1016/j.cimid.2019.101412)

Comparative Immunology, Microbiology and Infectious Diseases 69 (2020) 101412

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Molecular and serological detection of animal and human vector-borne pathogens in the blood of dogs from Côte d'Ivoire

Hacène Medkour[a,b], Younes Laidoudi[a,b], Eric Athias[c], Amar Bouam[a,b], Sylvestre Dizoé[d], Bernard Davoust[a,b], Oleg Mediannikov[a,b,*]

[a] Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France
[b] IHU-Méditerranée Infection, Marseille, France
[c] Army Health Service, Abidjan, Côte d'Ivoire
[d] Kongouanou Care Center, Côte d'Ivoire

ARTICLE INFO

Keywords:
Vector-borne pathogens
Côte d'Ivoire
Dog
PCR
Leishmania infantum
Trypanosoma congolense
Babesia vogeli
Filariidae
Ehrlichia canis

ABSTRACT

In Côte d'Ivoire, limited information are available on vector-borne pathogens, their prevalence and distribution. Here, we assess the occurrence and diversity of canine vector-borne diseases (CVBDs) in Abidjan and Yamoussoukro cities. Blood from a total of 123 dogs were tested for *Leishmania infantum* and *Ehrlichia canis* antibodies and screened for *Leishmania* and *Trypanosoma* spp., Piroplasmida, Filariidae and Anaplasmataceae by PCR and sequencing. Among dogs, 39 % were positive for at least one pathogen. Seroprevalences were: 15.4 % and 12.2 % for *L. infantum* and *E. canis*, respectively. DNA of *L. infantum* and *T. congolense* (4.1 %), *Baabesia vogeli* (1.6 %), Filariidae (*Dirofilaria immitis, D. repens* and *Acanthocheilonema reconditum*) (10.6 %) has been detected. Anaplasmataceae were detected in (17.1 %) and *E. canis* was the only identified specie. Co-infections were observed in 13.8 % of dogs: *E. canis-L. infantum* co-infection was the most prevalent (4.9 %). Age, breed and sex of dogs do not seem to influence infections. Village dogs were more susceptible to CVBDs than kennel dogs (PV = 0.0000008). This study reports for the first time the presence of *L. infantum, B. vogeli, A. reconditum, D. immitis* and *D. repens* in dogs from Côte d'Ivoire and determines the prevalence and diversity of CVBD pathogens. The results indicate that human and animal pathogens are abundant in Ivoirian dogs which requires attention of veterinarians, physicians and authorities against these diseases, especially against major zoonosis such as visceral leishmaniasis (*L. infantum*).

1. Introduction

Canine vector-borne diseases (CVBD) are a group of rapidly spreading, globally distributed diseases caused by a range of arthropod-borne pathogens, including ticks, fleas, mosquitoes and phlebotomine sandflies which are the vectors of a rank of bacteria, viruses, protozoa and helminths. In addition to their veterinary importance, some CVBD-causing pathogens are of major zoonotic concern [1]. Dogs are sentinels and reservoirs of diseases that are a risk for human population, therefore, the interest to follow CBVD in dog populations remains of greater importance. *Ehrlichia canis, Hepatozoon* spp., *Anaplasma* spp., *Leishmania infantum*, *Trypanosoma* spp., *Babesia* spp. and filariae are the most vector-borne pathogens detected in dogs in multiple countries [2–6].

Bacteria from the Anaplasmataceae family include numerous dog-associated pathogens vectored by ticks [4]. Canine monocytic ehrlichiosis (CME) caused by *E. canis* and transmitted by the tick *Rhipicephalus sanguineus*, is the most recognized, and is responsible for severe clinical signs and a high mortality rate in infected dogs. The infection is common in Sub-Saharan Africa, such as Côte d'Ivoire and Gabon [5]. Native African dogs are often asymptomatic (immunotolerance). Warm climates seem to be favorable for the infectious agents propagation [6].

Canine leishmaniosis, caused by *L. infantum* and transmitted by a phlebotomine sand fly vector, is the most serious CVBD considering its large distribution (in Central and South America, Asia and several countries of the Mediterranean Basin) and high pathogenicity [7,8]. In Sub-Saharan Africa, data on CanL are rare, except some reports in

Abbreviations: Bp, Base pair; CanL, Canine leishmaniosis; CME, Canine monocytic ehrlichiosis; Ct, Cycle threshold; CVBD, Canine vector-borne diseases; EDTA, Ethylenediaminetetraacetic acid; IFAT, Immunofluorescence antibody test; PCR, Polymerase chain reaction; PV, P-value; qPCR, Quantitative PCR

* Corresponding author at: MEPHI, IHU - Méditerranée Infection, 19-21 Boulevard Jean Moulin, 13385 Marseille cedex 05, France.
E-mail addresses: hacenevet1990@yahoo.fr (H. Medkour), younes.laidoudi@yahoo.com (Y. Laidoudi), ericathias@yahoo.fr (E. Athias), amarbouam@yahoo.fr (A. Bouam), drdizoe@yahoo.fr (S. Dizoé), bernard.davoust@gmail.com (B. Davoust), olegusss1@gmail.com (O. Mediannikov).

https://doi.org/10.1016/j.cimid.2019.101412
Received 21 June 2019; Received in revised form 17 December 2019; Accepted 19 December 2019

Senegal [9], Burkina-Faso [10]. Anecdotal *L. infantum* infections in human were also reported in Côte d'Ivoire [11] and Gambia [12].

Canine trypanosomosis is a disease caused by haemoprotozoan parasites *Trypanosoma brucei brucei* and *T. congolense* in Africa [13]. This disease can also be caused by *T. cruzi,* the agent of American trypanosomosis known as the Chagas disease in humans. Dogs are also infected by *T. b. rhodesiense* and *T. b. gambiense* pathogens for humans [14].

Babesiosis is a potentially serious illness caused by intraerythrocytic protozoan parasites of the genus *Babesia*. *B. vogeli*, *B. gibsoni* and *B. rossi* are the main causative agents of the canine babesiosis vectored by ticks. They may also be transmitted vertically [15] or by blood exchange in fighting dogs [16]. *Babesia* species have a global distribution and have been identified in African dogs [17].

Among the vector-borne pathogenic helminths belonging to the order Spiruridae, the suborder *Spirurina* and the families Filariidae and Onchocercidae: *Dirofilaria immitis* and *D. repens* are probably the best known to cause infestations in dogs and humans [1,18], followed by *Acanthocheilonema reconditum* (*Spirurida*, Onchocercidae) often infects dogs [19]. Differently from other filarioids transmitted by mosquitoes (e.g., *D. immitis* and *D. repens*) or ticks (e.g., *Cercopithifilaria* spp.) to dogs, *A. reconditum* is vectored by fleas (i.e., *Ctenocephalides canis, C. felis, Pulex irritans, P. simulans, Echidnophaga gallinae*) or lice (i.e., *Heterodoxus spiniger, Linognathus setosus*) with a rate of infestation in fleas of about 5 % [19]. Globally, vector-borne diseases account for about 17 % of the burden of all infectious diseases and cause millions of dollars in losses to the livestock industry each year, with implications for human and animal health and the global economy [20]. Information on filarial infections in dogs in sub-Saharan Africa is scarce. From a review of available literature by Schwan (2009) it appeared that all the above mentioned species have been reported from various places on the continent [21]. survey for filariae in dogs in northern Kenya and in Zambia have later been documented [22,23].

In Côte d'Ivoire, few information is available on the prevalence and the distribution of CVBD. The purpose of this study was to evaluate the occurrence of these pathogens of veterinary and public (for some of them) health importance and their diversity in dogs from the city of Abidjan (kennel dogs) and in a city near Yamoussoukro (village dogs) by using serological and molecular tools including new PCR assays.

2. Methods

2.1. Study sample collection and preparation

In April 2018, blood samples were collected from 123 dogs, living in three kennels in Abidjan (N = 95) and Kongouanou town (N = 28), (7°00′42.0″N 5°22′04.0″W) localized 24 km from Yamoussoukro and 241 km from Abidjan. The largest kennel is located (5°16′54.9″N 3°56′46.0″W) in Koumassi, district of Abidjan and the other two are between five (Cocody) and six (Treichville) kilometers away (Fig. 1). Kennel dogs are working dogs used for protection, security and defense. They belong to adapted breeds (ridgeback, Belgian shepherds, etc.). They receive veterinary care and sometimes external pest control. The village dogs from Kongouanou are medium-sized dogs mostly used for hunting. They live outdoors freely in the village and its surroundings. They do not benefit from preventive veterinary care (32 % carried ticks). Data about sanitary status or possible prophylaxis for kennels dogs were collected. A volume of 4–5 mL of blood was collected from the radial vein of each dog, into dry and EDTA tubes; serum was recovered after centrifugation (10 min, 3000 g) and frozen at −20 °C. EDTA tubes were directly frozen at −20 °C. Each animal sampled was examined and information about gender, breed and age were collected.

The animals were examined and sampled after obtaining the verbal consent of the persons (owners) responsible for the dogs. The blood samples were taken from radial vein by veterinarians (French and Ivorian) with the ethical responsibility to ensure the animal care in accordance with Articles 433–434; Chapter 2 of the Ivoirian Penal Code.

2.2. Serological assay to detect E. canis and L. infantum specific antibodies

The presence of anti-*E. canis* and anti-*L. infantum* promastigote specific antibodies was revealed by indirect fluorescent antibody test (IFAT). Plate wells were sensitized by 10 µL and 20 µL of the commercial antigens of *E. canis* and *L. infantum* (Zoetis, France) respectively and assays were assessed following manufacturer's instructions. Both anti-*Ehrlichia* and anti-*Leishmania* antibodies were detected by secondary antibodies against rabbits, anti-dog immunoglobulins G conjugated with fluorescein isothiacyanate (Sigma-Aldrich, St Louis, MO, USA). Sera were considered positive when antibodies titers were higher than 1:100 and 1:80 dilutions for *Ehrlichia* and *Leishmania* respectively.

2.3. Morphological identification of filariae species

Blood samples were examined for the presence of microfilaria after concentration by a modified Knott test (Knott, 1939) which is the gold standard [24]. Microfilariae were quantified and identified when existed. When co-infections were found, we performed a serial dilution until we obtained 1 microfilariae/ 200 mL of blood which was used to extract the DNA. The objective was to concentrate only one species of microfilariae and extract its DNA for a possible future molecular identification.

2.4. Molecular assays

2.4.1. DNA extraction

DNA was extracted from 200 µL of blood after digestion with proteinase K at +56 °C overnight using a commercial DNA extraction kit (QIAamp DNA Mini Kit®, [Qiagen, Courtaboeuf, France]) and performed on BIOROBOT EZ1 (Qiagen, Qiagen, Courtaboeuf, France) per the manufacturer's instructions. In order to achieve maximum yield, digestion was performed overnight with proteinase K in Qiagen lysis buffer before the QIAamp DNA Mini Kit® was used. DNA was eluted in 200 µl of distilled water and stored at −20 °C.

2.4.2. PCR tools design for Leishmania spp. and Trypanosoma spp. screening

We designed primers and probe using the conserved region of encoding ribosomal RNA genes 18S to detect DNA from *Leishmania* species: a first, qPCR for the screening of all *Leishmania* targeting a conserved region of the 18S gene; a second, conventional PCR targeting the same gene that produce the 550-bp amplicon for the following confirmation and species identification. In order to detect DNA from trypanosomes, we designed a qPCR system targeting the ribosomal RNA genes 5.8S. To design the group-specific primers and probes, the corresponding genetic sequences of all Leishmania/Trypanosoma species available in the GenBank were multiple aligned. All conserved motifs were visualized using BioEdit software version 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The free web-based Primer3 software, version 4.0 (http://frodo.wi.mit.edu/primer3/) was used to design the primers and probes based on chosen conserved motifs (Table 1).

The specificity and sensitivity of all PCR assays were tested *in silico* using primer-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and validated *in vitro* using a DNA panel of cultivated *Leishmania* species: *L. infantum, L. donovani, L. major* and DNA of *L. guyanensis* for *Leishmania* PCR systems, a DNA panel of cultivated *Trypanosoma* species: *T. congolense IL 3000*, *T. evansi*, *T. vivax*, *T. brucei brucei, T. brucei gambiense* and *T. brucei gambiense biyiamina* groupe II for *Trypanosoma* qPCR system, and several arthropods of laboratory-maintained colonies as well as human, monkey, donkey, horse, cattle, mouse and dog DNAs for all the PCR systems (Table S1).

Fig. 1. Location of the study: down Abidjan kennel dogs, up dogs from Kongouanou village (**A.** Côte d'Ivoire map, https://commons.wikimedia.org/wiki/Atlas_of_C%C3 %B4te_d%27Ivoire#/media/File:C%C3 %B4te_d%27Ivoire_Map.jpg; **B.** Copyright pictures: B. Davoust).

2.4.3. Molecular screening for the presence of pathogens DNA

All the DNA samples were screened for *Leishmania* spp. using two qPCR assays: i) the 18S-based qPCR was used for the initial screening and the detection of DNA *Leishmania* spp., ii) the specific *L. infantum* qPCR assay based on the amplification of kinetoplast minicircle DNA (kDNA), recognized for its high sensitivity which allows the quantification of the parasite load [25]. qPCR assays were performed to screen DNA samples using primers and probes for Anaplasmataceae, *Trypanosoma* spp. and *Piroplasmida* (Table 1). Sequences of primers and probes and their respective sources used in this study are presented in Table 1. Concerning Filariidae, we used a triplex qPCR to detect *D. immitis, D. repens* and *A. reconditum* targeting the *Cox1* gene. In order to detect the occult filariosis du to *D. immitis* we screened the samples using a qPCR targeting *ftsZ* gene specific to *Wolbachia* endosymbiont of *D. immitis* (Laidoudi et al. unpublished).

Reaction mix for qPCR assays contained 5 µl of the DNA template, 10 µl of Eurogentec Master Mix Roche, 0.5 µl of each reverse and forward primers and UDG, 0.5 µl of the FAM- labeled probe (Table 1) and 3 µl of distilled water DNAse and RNAse free, for a final volume of 20 µl. The qPCR amplification was performed in a CFX96 Real-Time system (Bio-rad Laboratories, Foster City, CA, USA) with the following thermal profile: The first step is an incubation at 50 °C for two minutes

Table 1
Sequences of primers used for species detection and identification in this study.

Targeted microorganisms	Target gene	Name	Primers (5′-3′) and probe	Tm	References
Anaplasmataceae	23S	TtAna-F	TGACAGCGTACCTTTTGCAT	–	[4]
		TtAna-R	GTAACAGGTTCGGTCCTCCA		
		TtAna-S*	FAM-CTTGGTTTCGGGTCTAATCC-TAMRA		
		Ana23S-212f	GTTGAAAARACTGATGGTATGCA	55 °C	
		Ana23S-753	TGCAAAAGGTACGCTGTCAC		
Leishmania spp.	18S	Leish. F	GGTTTAGTGCGTCCGGTG	–	This study
		Leish. R	CGGCCCATAAGATCC CCAA		
		Leish. P*	FAM-CGGCCGTAACGCCTTTTCAACTCA -TAMRA		
		Leish. F1	CTGTGACTAAAGAAGCGTGAC	52 °C	This study
		Leish. R1	AGGCCGAATAGAAAAGATACGT		
	kDNA	RV1	CTTTTCTGGTCCTCCGGGTAGG	–	[25]
		RV2	CCACCCGGCCCTATTTTACACCAA		
		Probe. Leish*	FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA		
	ITS II	LGITSF2	GCATGCCATATTCTCAGTGTC	60 °C	[52]
		LGITSR2	GGCCAACGCGAAGTTGAATTC		
	kDNA	RV1	CTTTTCTGGTCCTCCGGGTAGG	59 °C	[54]
		RV2	CCACCCGGCCCTATTTTACACCAA		
Trypanosoma spp.	5.8S	Tryp 5.8SF	CAACGTGTCGCGATGGATGA	–	This study
		Tryp 5.8SR	ATTCTGCAATTGATACCACTTATC		
		Tryp 5.8S P*	FAM-GTTGAAGAACGCAGCAAAGGCGAT-TAMRA		
	ITS I	ITS1-CF	CCGGAAGTTCACCGATATTG	58 °C	[51]
		ITS1-BR	TTGCTGCGTTCTTCAACGAA		
Piroplasmida	5.8S	5.8S-F5	AYYKTYAGCGRTGGATGTC		[53]
		5.8S-R	TCGCAGRAGTCTKCAAGTC		
		5.8S-S*	FAM-TTYGCTGCGTCCTTCATCGTTGT-MGB		
	18S	piro18S-F1	GCGAATGGCTCATTAIAACA	58 °C	
		piro18S-R4	TTTCAGMCTTGCGACCATACT		

Abbreviations: Tm: Annealing temperature; *: Probe.

Table 2
Prevalence of the vector-borne pathogens detected by serology or PCR and risk factors.

Risk Factor		No.	*Anaplasmataceae*					*Leishmania* spp.					*Trypanosoma* spp.			*Piroplasma*		*Filariidae*		
			IFAT*Ehrlichia*		*PV*	qPCR 23S		PV	IFAT		*PV*	qPCR 18S and kDNA		qPCR 5.8S	*PV*	qPCR 5.8S	*PV*	qPCR Cox1		*PV*
Region	Abidjan	95	14	14.7	*0.18	18	18.9	*0.4	15	15.8	*1.00	4	4.2	5	5.3	*0.58	1	1.0	*0.4	3	3.2	* 0.0000008
	Kongouanou	28	1	3.6		3	10.7		4	14.3		1	3.6	0	0		1	3.6		10	35.7	
	Total	123	15	12.2		21	17.1		19	15.4		5	4.1	5	4.1		2	1.6		13	10.6	
Age (year)	[0–2]	49	4	8.2	0.47	9	18.4	0.58	7	14.3	0.36	2	4.1	0	0	0.12	2	4.1	0.38	6	12.2	0.72
	[2–4]	33	6	18.2		7	21.2		8	24.2		2	6.1	2	6.1		0	0		3	9.1	
	[4–6]	26	4	15.4		7	26.9		3	11.5		0	0	1	3.8		0	0		3	11.5	
	> 6	15	1	6.7		1	6.7		1	6.7		1	6.7	2	13.3		0	0		1	6.7	
Gender	Female	32	2	6.2	*0.35	9	28.1	0.16	8	25	0.08	0	0	0	0	*0.32	1	3.1	*0.45	4	12.5	* 0.45
	Male	91	13	14.3		15	16.5		11	12.1		5	5.5	5	5.5		1	1.1		9	9.9	
Breed	Berger	33	3	9.1	0.36	4	12.1	0.9	5	15.1	0.86	2	6.1	3	9.1	0.34	0	0	0.82	0	0	0.02
	Beauceron	9	2	22.2		2	22.2		1	11.1		1	11.1	0	0		0	0		0	0	
	Ridgeback	11	2	18.2		2	18.2		1	9.1		1	9.1	2	18.2		0	0		1	9.1	
	Rot	9	3	33.3		2	22.2		3	33.3		0	0	0	0		0	0		0	0	
	Labrador	4	0	0		1	25		1	25		0	0	0	0		0	0		0	0	
	Boer Bull	8	0	0		2	25		1	12.5		0	0	0	0		0	0		0	0	
	Dogue	5	1	20		1	20		1	20		0	0	0	0		0	0		0	0	
	Mongrel	44	4	9.1		10	22.7		6	13.6		1	2.3	0	0		2	4.5		12	27.3	

Abbreviations: IFAT: immunoflurescence antibody test;*PV*: P-value, *PV* > 0.05 means that the observed differences between prevalence is not statically significant; *: Fisher's exact test.

and an initial denaturation step at 95 °C for five minutes, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing-extension at 60 °C for 30 s. We included the DNA of the target parasites or bacteria as positive controls and master mixtures as a negative control for each test. Samples were considered positive when the cycle threshold (Ct) was lower than 35 Ct for all qPCR assays and lower than 38 Ct for *Leishmania* 18S-based qPCR.

2.4.4. PCR amplification, sequencing and phylogenic analysis

Positive samples by qPCR for vector borne pathogens were confirmed by standard PCR assays and sequencing for the fragments of the genes 23S for Anaplasmataceae, kinetoplast DNA, ITS II and 18S for *Leishmania* spp., ITS I for *Trypanosoma* spp. and 18S for *Piroplasmida*. Primer pairs are listed in the (Table 1). Positive samples by the triplex qPCR for Filariidae were also confirmed by standard PCR amplifying a 1230 bp from 18S gene of Filariidae species (Laidoudi et al. unpublished).

The standard PCR assays consisted of a volume of 25 µl, including 12.5 µl of Amplitaq Gold Master Mix, 0.75 µl of each primer, 5 µl of DNA model and water. The thermal cycling conditions were: incubation step at 95 °C for 15 min, 40 cycles of 1 min at 95 °C, 30 annealing at a different hybridization temperature for each PCR assays and one minute at 72 °C followed by a final extension for five minutes at 72 °C (Table 1). PCR amplification was performed in a Peltier PTC-200 model thermal cycler (MJ Research Inc., Watertown, MA, USA). The results of amplification were visualized by electrophoresis on 2 % agarose gel. The purification of PCR products was performed using NucleoFast 96 PCR plates (Macherey Nagel EURL, Hoerdt, France) according to the manufacturer's instructions. The amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). The obtained electropherograms were assembled and edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) and compared with those available in the GenBank database by NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The DNA sequences obtained for each gene, for each pathogen, from positive samples were aligned with those available in the GenBank database for the same gene. A maximum-likelihood method was used to infer the phylogenetic analyses and tree reconstruction was performed using MEGA software version 7 (https://www.megasoftware.net/). Bootstrap analyses were conducted using 100 replicates.

2.5. Statistical analysis

Statistical analysis was conducted using IBM SPSS statistics version 23. After the databases were established, the association between infections and age, sex, breeds and origin (Kennels vs village) of dogs was assessed using the X^2 test. The Fischer's exact test was used when the percentages were low. Statistically significant was consider at P-value < 0.05.

3. Results

3.1. Sensitivity and specificity of PCR assays

Designed PCR assays listed in Table 1 were tested. First, we tested the *Leishmania* genus-specific 18S rRNA-based qPCR system by screening of positive controls (Table S1). As expected *in silico*, we amplified all available positive controls: *L. infantum, L. donovani, L. major* and *L. guyanensis*. These controls were also amplified by the *Leishmania* 18S rRNA-based standard PCR system. In order to validate these news PCR systems and to reproduce the results, *Leishmania*-positive samples were tested simultaneously by *Leishmania* genus-specific 18S, *L. infantum*-specific kDNA qPCR assays and 18S, ITS1, ITS2 standard PCR tools. All of them gave the same result. Secondly, we tested the

Table 3
Single and mixed infection of canine vector-borne pathogens.

Infection	Kennel dogs (Abidjan) No. positive	n = 95 %	Village dogs (Kongouanou) No. positive	n = 28 %	Total No. positive	N = 123 %
Single infection	**20**	**21.1**	**11**	**39.3**	**31**	**25.2**
Ehrlichia canis	10	10.5	2	7.1	12	9.8
Leishmania infantum	7	7.4	4	14.3	11	8.9
Trypanosoma congolense	2	2.1	0	0.0	2	1.6
Acanthocheilonema reconditum	1	10.5	4	14.3	5	4.1
Babesia vogeli	0	0.0	1	3.6	1	0.8
Mixed infection	**11**	**11.6**	**6**	**21.4**	**17**	**13.8**
E. canis + *L. infantum*	6	6.3	0	0.0	6	4.9
E. canis + *B. vogeli*	1	1.1	0	0.0	1	0.8
E. canis + *A. reconditum*	0	0.0	4	14.3	4	3.3
L. infantum + *T. congolense*	2	2.1	0	0.0	2	1.6
L. infantum + *A. reconditum*	0	0.0	2	7.1	2	1.6
A. reconditum + *D. immitis* + *D. repens*	2	2.10	0	0.0	2	1.6
Total positive	**31**	**32.6**	**17**	**60.7**	**48**	**39**
Total negative	**64**	**67.4**	**11**	**39.3**	**75**	**61**

Trypanosoma 5.8S rRNA-based qPCR system by screening the controls. As tested *in silico*, it amplified all positive controls (Table S1). The same results were obtained by using *Trypanosoma* ITS1- based standard PCR. None of the DNA negative controls were amplified by any of the PCR tools.

3.2. Serological and molecular results

A total of 123 dogs were sampled: 91 males and 32 females. Ages were available for all dogs. The average age was three years old [4 month–10 years] with 49 juveniles [4 month -2 years], 33 young dogs [2–4 years], 26 adults [4–6 years] and 15 oldies (> 6 years) dogs. Prevalence of the vector-borne pathogens detected by serology or PCR is presented in the Table 2. Results showed that 39 % (48/123) of dogs were positive for at least one of the pathogens by either PCR or serology (Table 3). Screening using qPCR assays showed a prevalence of 17.1 % (21/123) for Anaplasmataceae infections (*E. canis* was the only species identified), followed by Filariidae infections 10.6 % (13/123) with at least one filaria species, *L. infantum* in 4.1 % (5/123) with 28.9 *Leishmania*/mL of blood (Min: 2.1; Max: 117.8) as an average of the parasite load found by the kDNA qPCR, *Trypanosoma* spp. in 4.1 % (5/123) and finally *Babesia* spp. with 1.6 % (2/123). Prevalence by IFAT was of 15.4 % (19/123) for *L. infantum* and 12.2 % (15/123) for *E. canis* (Table 2).

Regarding mono-infections, *E. canis* infection was the most prevalent with 9.7 % (12/123). We found a prevalence 8.9 % (11/123) for *L. infantum*, *A. reconditum* 4.1 % (5/123). *Trypanosoma* spp. 1.6 % (2/123) and finally *Babesia* spp. with 0.8 % (1/123).

Mixed infections 13.8 % (17/123) were also found in this study (Fig. 2). Mixed infection of *E. canis-L. infantum* 4.9 % (6/123) was more frequent than *E. canis–A. reconditum* with 3.3 % (4/123), *L. infantum-Trypanosoma* spp. and *L. infantum–A. reconditum* with 1.6 % (2/123) for each of them and finally, co-infection with *E. canis* and *B. vogeli* with only one case (0.8 %). Triple infection with *A. reconditim-D. immitis-D. repens* were observed in two dogs. One of them was positive by modified Knott's test and the triplex qPCR for the three species. The other one was positive for *A. reconditum* and *D. repens* but negative for *D. immitis* for both tests. By contrast, it was positive by qPCR *ftsZ* for *Wolbachia* endosymbiont of *D. immitis,* suggesting an occult infection by *D. immitis*.

There was no significant association between sex, age, breed and the presence of vector-borne dog infections in this study (PV > 0.05), except for filariasis for which mongrel dogs were more susceptible (PV = 0.02). By contrast, dogs from Kongouanou (village dogs) were found to be more infected 60.7 % (17/28) than dogs from Abidjan (kennel dogs) 32.6 % (31/95) for at least one pathogen (PV = 0.003). Also, Filariidae infections were more frequent in the village dogs 35.7 % (10/28) than the kennel dogs 3.2 % (3/95) (PV = 0.0000008) (Table 3).

3.3. Phylogenetic analysis results

Positive samples for vector-borne pathogens were sequenced. DNA sequences of all positive PCR products were ≥ 99–100 % homologous to those available in the GenBank database for all CVB pathogens identified, except the obtained sequences for *Leishmania* kDNA. For Anaplasmataceae, after sequencing the 485 bp-long amplicons of the 23S rRNA gene portion, BLAST analysis was conclusive for *E. canis* for all sequences obtained with 99–100 % identity with *E. canis* (KM021429) from Algeria and (NR 076375) from the USA. *L. infantum* ITS 2 obtained sequences (420 bp) were 100 % identical to published sequences (KU680956, KU680957, KU680958, KU680959 and KU680960). *Leishmania* kDNA sequence (126–128 bp) exhibited 96–97 % identity with *L. infantum* isolate MHOM/CN/80/D2 from China (Z35292). In addition, *T. congolense* ITS1 sequences (620–630 bp) exhibited 99 % identity with published sequence MG283145. *B. vogeli* 18S rRNA obtained sequences (865 bp), in this study, were 99 % identical to published sequence (LC331058, MG586234). For Filariidae, 18S sequences for *A. reconditum* is note available in the GenBank database. By contrast, the sequences found for *D. repens* had 100 % of identity with (AB973229) published sequence. We could not sequence the positive sample for *D. immitis* because it was co-infected with other species. Phylogenetic trees are built on the basis of these genes and show the position of the identified species (Fig. 3).

Sequences were submitted in the GenBank database under accession numbers: MK494932-MK494941 for *E. canis*, MK481041- MK481044 for *L. infantum* ITS2 sequences, MK770156- MK770158 for *L. infantum* kDNA sequences, MK495745- MK495747 for *T. congolense,* MK495836-MK495837 for *Babesia vogeli*, MK495727-MK495733 for *A. reconditum*, MK495734 and MK495735 for *D. repens.*

4. Discussion

This study describes canine vector-borne infections in dogs from Côte d'Ivoire. Our results showed that pathogens such as *E. canis, L. infantum, T. congolense, B. canis vogeli*, *A. reconditum, D. repens* and *D. immitis* are endemic in this area.

The Anaplasmataceae family includes several pathogens associated with dogs; they are reported worldwide and transmitted by *Ixodidae* ticks. [1]. In this study, we found a prevalence of 17.1 % (21/123) by qPCR 23S. This is not new, as this region is known to be endemic [5]. Sequencing analysis reported the presence of one species, *E. canis,* the causative agent of monocytic ehrlichiosis. We also noted a seroprevalence of 12.2 % (15/123) for *E. canis* by IFAT. It was not be possible to obtain sequences for all qPCR-positive samples. qPCR-positive samples not sequenced and not positive by IFAT for *E. canis* could be another Anaplasmataceae sp. not *E. canis*. In 2003, about 74 % of the

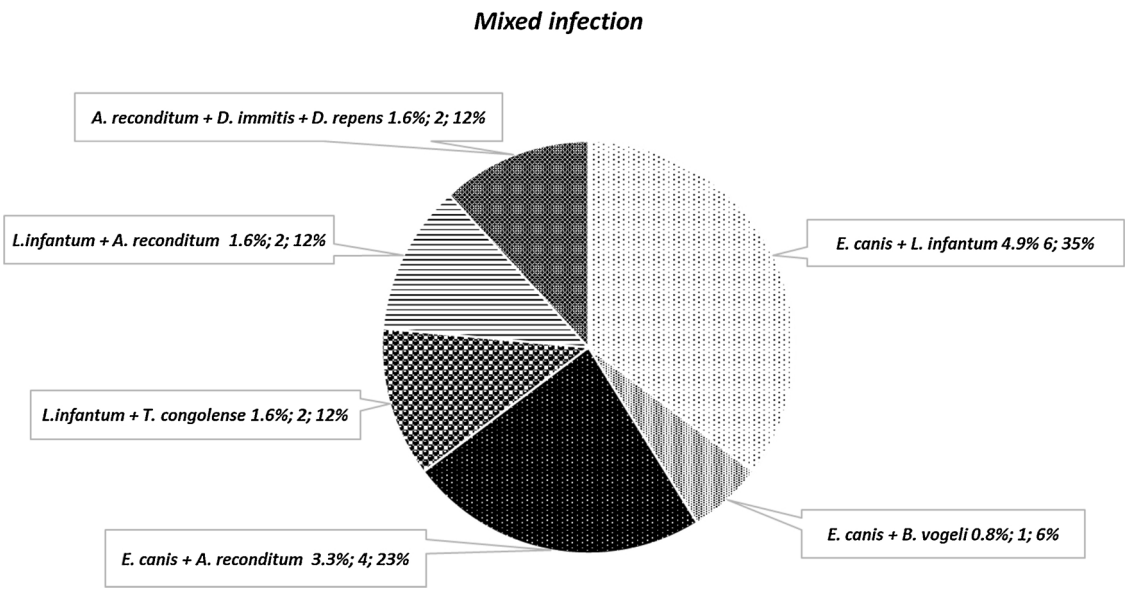


Fig. 2. Mixed infections found in dogs surveyed in the present study. The legends represent: Coinfection and its prevalence; number of dogs presented this coinfection; frequency of the coinfection according to the sum of coinfections (N = 17).

76 dogs in Abidjan and 84 % (93/111) of the dogs in Gabon were seropositive [26]. Also, 27 % of *R. sanguineus* s.l. ticks collected (16/60) from dogs in Abidjan, were positive for *E. canis* using quantitative real-time PCR [27]. Since then, we believe that there is a decreasing in the prevalence due to the awareness of veterinarians and dog owners in the. But the area remains endemic because the dog's state of life promotes transmission and persistence of infection (presence of ticks) and not all dog owners are able to treat and protect their dogs. We did not find *Anaplasma platys,* the agent of canine cyclic thrombocytopenia. However, prevalence of 8.5 and 56.2 % has already been reported in Ivorian dogs and ticks collected on these dogs, respectively [28].

Data about canine leishmaniosis in Côte d'Ivoire are absent. To the best of our knowledge, our study is the first to report *L. infantum* in dogs from this area. The prevalence observed is 15.4 % (19/123) by IFAT. We have developed a qPCR test with a wide specificity (*Leishmania* spp.) capable of detecting the main known pathogens of *Leishmania* species such as *L. donovani, L. infantum, L. major, L. guyanensis* as well as probable new species. Only 4 % (5/123) were positive for PCR, which differs significantly (PV = 0.0026) with serological results. This is not surprising since PCR assays on blood samples often provide less positive results for leishmaniosis than for other sites (bone marrow and lymph node) [27,28], hence the choice of IFAT as gold standard in this study. We achieved species identification by the amplification from the qPCR-positive samples ITS2 gene (Table1) and kinetoplast gene. This was conclusive for *L. infantum*. All positive dogs were apparently healthy. The level of parasite load found is not important (amount 29 parasite/mL blood), which may explain an asymptomatic carriage stage. Cases of human visceral leishmaniasis were reported in sub-Saharan countries such as Côte d'Ivoire and Gambia [29,30], but these reports remain anecdotal with no evidence of an autochthonous transmission cycle. Three human cases of visceral leishmaniasis were observed in a cohort of 528 patients in Abidjan (2001–2002) [11]. The detection of *L. infantum* in local dogs (15.4 %) provides evidence that this region is endemic for CanL and indicates the possible involvement of domestic dogs in the human infection. Classically, sand flies involved in the *Leishmania* transmission belong either to the *Phlebotomus* genus (Old World) or to the *Lutzomyia* genus (New World). The sand fly species involved in *L. infantum* transmission cycle in Côte d'Ivoire are still unknown and need further investigations. The results obtained in Senegal, with the molecular confirmation of *L. infantum* species, high seropositivity in 160 dogs (46.3 %) and 133 humans (33.3 %) [9], the high rate of *L. infantum* on female sand fly species (5.4 % of *Sergentomyia dubia*, 4.2 % of *S. schwetzi* and 3.6 % of *S. magna*) [31] strongly suggests that the *L. infantum* life cycle is well established in this area. In addition, this raises further questions about vectors in sub-Saharan regions and challenges once again the dogma that leishmaniasis is exclusively transmitted by the genus *Phlebotomus* in the Old World. In Burkina-Faso, CanL prevalence is about 5.9 % (5/85) [10]. The disease has mainly been described in the north of the African continent, range of results reported for some countries in the Mediterranean Basin using IFAT: Algeria: 27–45 % [32],Tunisia 18–53 % [33,34], Morocco 9–19 % [35,36].

To perform molecular screening of *Trypanosoma* spp. we have developed a new qPCR system to detect all *Trypanosoma* species. This molecular tool seems to be sensitive and specific and allowed us to found a prevalence of 5.3 % (5/95) in dogs from Abidjan. In 2003, Keck et al. reported a prevalence of 30 % in 123 dogs from kennels of Abidjan by using PCR specific primers for *T. congolense* "forest type" with no difference between age, class, breed and sex [13]. The fact that we found fewer infected dogs may be due to the sensitization of local veterinarians against this disease. *T. congolense* "forest type" has been found in other host animals (sheep, goats, cattle, pigs) in Côte d'Ivoire and other forest areas [37]. However, we did not detect this trypanosome. ITS 1 gene sequencing showed 99 % of identity with *T. congolense* isolate 237-51-00095-1-60-10 (GenBank: MG283145) identified in *Glossina palpalis palpalis* in Cameroon (Fig. 2). In Nigeria, nineteen dogs presenting with canine African trypanosomosis were infected with trypanosomes of the *T. brucei* group [38]. *T. congolense* is cyclically transmitted by tsetse flies (*Diptera: Glossinidae*). Trypanosomosis caused by *T. congolense* is a major threat to livestock production in Sub-Saharan Africa. Infected dogs can revealed pale mucous membranes, weak pulse, enlarged lymph nodes, rough hair coat and loss of skin elasticity [39].

We found a prevalence of 1.6 % (2/123) infected by *B. canis vogeli*. To our knowledge, this is the first detection of this pathogen in Côte d'Ivoire. This species has a global distribution [40] and has been identified in Africa [17,41]. The presence of ticks *Rhipicephalus sanguineus* s.l., the main vector of *B. canis vogeli* [42] on dogs in Côte d'Ivoire [27] may explain our results.

Concerning Filariidae, 10.6 % (13/123) of dogs were infected by at least one species. *A. reconditum* was the most detected by modified Knott's test and the triplex qPCR (13/123). Obtained sequences for 18S rRNA gene showed 99 % identity with *Acanthocheilonema viteae* and *Loa*

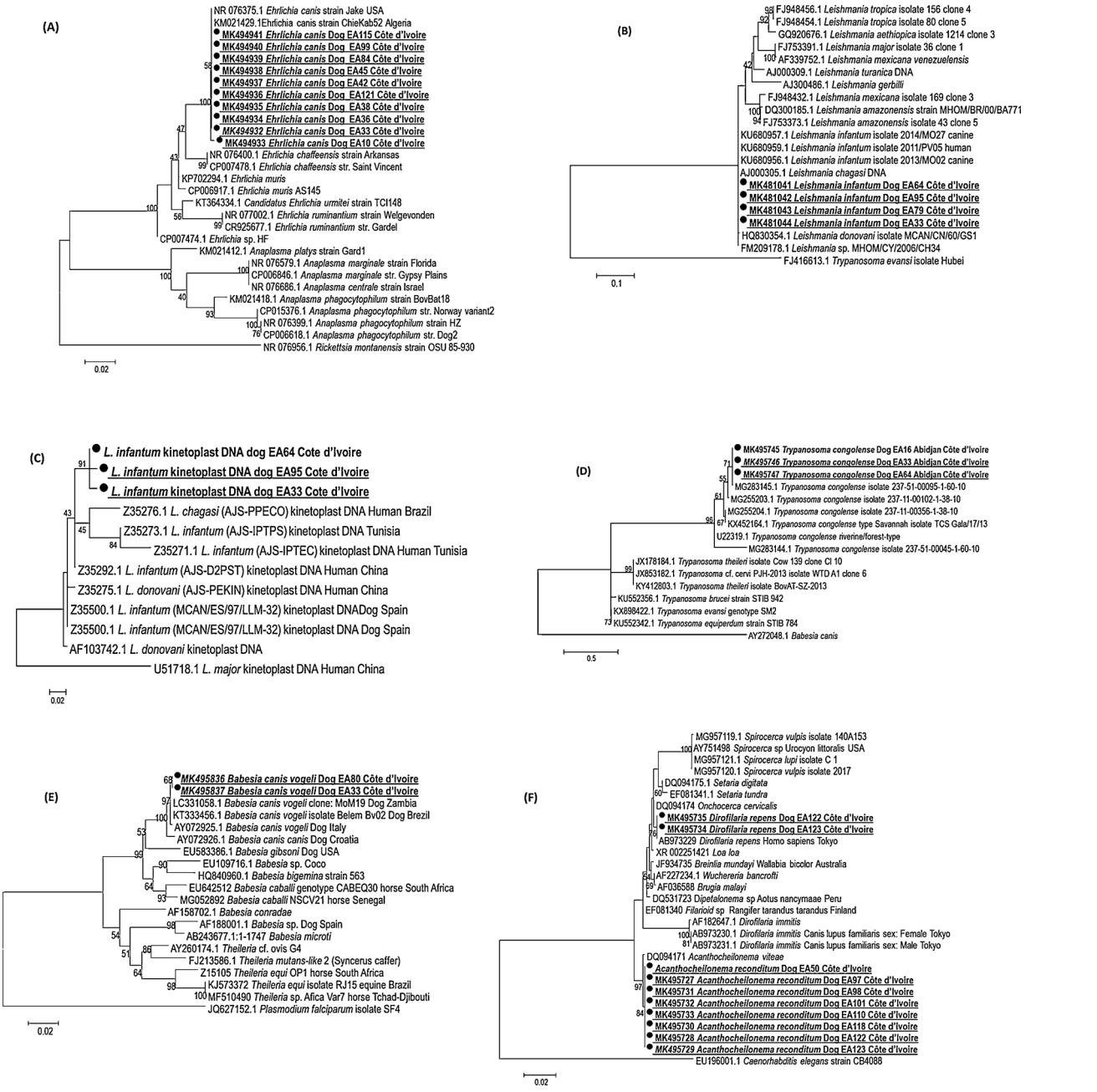


Fig. 3. Phylogenetic trees highlighting the position of *Ehrlichia* spp. (A), *Leishmania* spp. (B and C), *Trypanosoma* spp. (D) and *Babesia* spp. (E) and filaroid species (F) identified in the present study compared to other sequences available on GenBank. The sequences for 23S (A), ITSII and kDNA (B and C), ITSI (D) and 18S (E) and 18S (F) genes, for each of the six trees, were aligned using CLUSTALW, and phylogenetic inferences was conducted in MEGA 7 using the maximum likelihood method based on the Tamura-Nei model for nucleotide sequences.. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. The GenBank accession numbers are indicated at the beginning. Statistical support for the internal branches of the trees was evaluated by bootstrapping with 1,00 iterations. Bootstrapping under 40 were removed.

loa (DQ094171, XR_002251421). No sequence for the 18S rRNA gene of *A. reconditum* is available in GenBank. *A. reconditum* has a worldwide distribution [19][21]. In Liberia, 56 of 137 dogs (40.9 %) and about 9.2 % of Nigerian dogs were found positive for microfilaria of *A. reconditum* from morphological identification, 8.8 % in South Africa and Maputo province, Mozambique [21]. *A. reconditum* is generally considered non-pathogenic for dogs, it lives in the peritoneal cavity and adipose tissue [43]. Recently, it has been reported to induce skin lesions with atopic allergy in a dog [44]. This parasite is transmitted by fleas and chewing lice [22].

We found 13.8 % (17/123) of dogs with two or three pathogens and *E. canis-L. infantum* association was the most observed 4.9 % (6/123) co-infection. Co-infections in dogs is common. It is known that infection of the dog with more than one pathogen is associated with high pathogenicity [45]. The main cases observed are co-infections of dogs with tick-borne pathogens, including *E. canis, B. vogeli, A. platys* and *Hepatozoon canis,* that increase anemia for example, more than mono-infections [46]. Co-infection *L. infantum-A. reconditum* has been found in two dogs (1.6 %). Co-infections *L. infantum* with filariidae are known. In Portugal, 8.3 % of 230 dogs were infected with both *L. infantum* and

D. immitis [47]. This co-infection has also been observed in dogs from Greece [48]. This is relatively observed in dogs living in areas where vectors competent for different pathogens coexist, which can complicate the diagnosis, treatment and prognosis of CVBD [47].

In addition to these classical co-infections, we also noted co-infections of *E.canis-A. reconditum* in 3.3 % (4/123) and *L. infantum–T. congolense* in two dogs. Two dogs had triple infection, one of them was positive for the three filaroid species by modified Knott's test and the triplex Cox1-based qPCR, the other was positive for *A. reconditum* and *D. repens* by these same tools and negative for *D. immitis,* but it was positive on qPCR targeting *ftsZ* gene for *Wolbachia* endosymbiont for *D. immitis,* thus suggesting an occult infection. *D. immitis* has a severe impact on veterinary medicine, because of the heartworm disease threatening dogs and cats, whereas *D. repens*, causing subcutaneous infestation in dogs, is the main agent of the human dirofilariosis [49]. *D. immitis* has been reported from Guinea-Bissau, Nigeria, Senegal, Sierra-Leone and in Central Africa (Angola, Cameroon and Gabon). In addition, it has been reported that *D. repens* is the most common filarial worm in Nigerian dogs and cats. This species is also endemic in Central and Eastern Africa [21]. All these co-infections can lead to overlaps or atypical clinical signs. They should be expected in dogs living in areas that are highly endemic for several vector-borne pathogens, mainly in dogs kept predominantly outdoors and not regularly treated with ectoparacitides [50]. The prevalence of CVBD varied according to the dog's origin and Kongouanou dogs were more infected than those in Abidjan. They also had more filaroid infections. These dogs live freely in the open air in the village and its surroundings. They do not receive preventive veterinary care and most of them were tick carriers, compared to kennel dogs, which receive veterinary care and sometimes external pest control.

5. Conclusion

To the best of our knowledge, this study is the first to report the prevalence and diversity of vector-borne pathogens of humans and animals in dogs from Côte d'Ivoire. Co-infections with more than one pathogen were found to be common. For the first time, we highlighted the presence of *L. infantum, B. vogeli, A. reconditum, D. immitis* and *D. repens* in this region. This last remains endemic for *E. canis* and *T. congolense*. Ivorian veterinarians should be informed of these results in order to be able to detect clinical cases of canine leishmaniasis and make diagnoses using laboratory tests. This epidemiological situation also requires the implementation of a national program to control vector-borne diseases, especially for zoonotic agent such *Leishmania infantum,* the agent of human visceral leishmaniasis.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

We are grateful to Emilien Drancourt, Michel Drancourt, José Gomez, Gaoussou Toure and the dog owners for their valuable help with the field work. We also thank Magali Casanova, Hamadi Karembe, Philippe Vincendeau and Marie Varloud for their help.

This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program « Investissements d'avenir », reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101412.

References

[1] D. Otranto, F. Dantas-Torres, E.B. Breitschwerdt, Managing canine vector-borne diseases of zoonotic concern: part two, Trends Parasitol. 25 (2009) 228–235.
[2] J. Moreno, J. Alvar, Canine leishmaniasis: epidemiological risk and the experimental model, Trends Parasitol. 18 (2002) 399–405.
[3] A. Santamaria, J.E. Calzada, A. Saldaña, M.J. Yabsley, N.L. Gottdenker, Molecular diagnosis and species identification of *Ehrlichia* and *Anaplasma* infections in dogs from Panama, Central America, Vector-Borne Zoonotic Dis. [Internet] 14 (2014) 368–370, https://doi.org/10.1089/vbz.2013.1488 Available from:.
[4] M. Dahmani, B. Davoust, D. Tahir, D. Raoult, F. Fenollar, O. Mediannikov, Molecular investigation and phylogeny of Anaplasmataceae species infecting domestic animals and ticks in Corsica, France, Parasites and Vectors 10 (2017) 1–12.
[5] J.L. Marié, S.E. Shaw, D.A. Langton, O. Bourry, J. Gomez, B. Davoust, Sub-clinical infection of dogs from the Ivory Coast and Gabon with *Ehrlichia*, *Anaplasma*, *Mycoplasma* and *Rickettsia* species, Clin. Microbiol. Infect. 15 (2009) 284–285.
[6] M. Liu, N. Ruttayaporn, V. Saechan, C. Jirapattharasate, P. Vudriko, P.F.A. Moumouni, et al., Molecular survey of canine vector-borne diseases in stray dogs in Thailand, Parasitol. Int. [Internet]. Elsevier Ireland Ltd 65 (2016) 357–361, https://doi.org/10.1016/j.parint.2016.04.011 Available from:.
[7] C. Attipa, L. Solano-gallego, K. Papasouliotis, F. Soutter, D. Morris, C. Helps, et al., Association between canine leishmaniosis and *Ehrlichia canis* co-infection : a prospective case-control study, Parasites Vectors (2018) 1–9.
[8] H. Medkour, Y. Laidoudi, I. Lafri, B. Davoust, A. Mekroud, I. Bitam, et al., Canine vector-borne protozoa: Molecular and serological investigation for *Leishmania* spp., *Trypanosoma* spp., *Babesia* spp., and *Hepatozoon* spp. in dogs from Northern Algeria, Vet. Parasitol. Reg. Stud. Rep. [Internet]. Elsevier 19 (2020) 100353, , https://doi.org/10.1016/j.vprsr.2019.100353 Available from:.
[9] B. Faye, A.L. Bañuls, B. Bucheton, M.M. Dione, O. Bassanganam, M. Hide, et al., Canine visceral leishmaniasis caused by *Leishmania infantum* in Senegal: risk of emergence in humans? Microbes Infect. 12 (2010) 1219–1225.
[10] I. Sangare, A. Djibougou, B. Koudraogo, F. Drabo, A. Diabate, A.- Laure Banu, et al., First detection of *leishmania infantum* in domestic dogs from Burkina Faso (West Africa), Res. J. Parasitol. [Internet] 12 (2016) 27–32. Available from: http://www.scialert.net/abstract/?doi=jp.2017.27.32.
[11] B. Kouassi, K. Horo, V.H. Achi, K.D. Adoubryn, E.S. Kakou, B. Ahui, et al., Leishmaniose viscérale à Abidjan à propos de trois observations (French). Three cases of visceral leishmaniasis in Abidjan, Côte d'Ivoire, Med. Trop. (Mars) 65 (6) (2005) 602–603 30.
[12] P. Desjeux, J.H. Bryan, P. Martin-Saxton, Leishmaniasis in the Gambia. 2. A study of possible vectors and animal reservoirs, with the first report of a case of canine leishmaniasis in the Gambia, Trans. R. Soc. Trop. Med. Hyg. 77 (1983) 143–148.
[13] N. Keck, S. Herder, D. Kaba, P. Solano, J. Gomez, G. Cuny, et al., Epidemiological study of canine trypanosomosis in an urban area of Ivory Coast, Parasite [Internet] 16 (2009) 305–308. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20092062.
[14] L. Eloy, S. Lucheis, Canine trypanosomiasis: etiology of infection and implications for public health, J. Venom Anim. Toxins Incl. Trop. Dis. [Internet] 15 (2009) 589–611. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1678-91992009000400002&lng=en&nrm=iso&tlng=en.
[15] S. Fukumoto, H. Suzuki, I. Igarashi, X. Xuan, Fatal experimental transplacental Babesia gibsoni infections in dogs, Int. J. Parasitol. 35 (2005) 1031–1035.
[16] R. Jefferies, U.M. Ryan, J. Jardine, D.K. Broughton, I.D. Robertson, P.J. Irwin, Blood, Bull Terriers and Babesiosis: further evidence for direct transmission of *Babesia gibsoni* in dogs, Aust. Vet. J. 85 (2007) 459–463.
[17] P.T. Matjila, B.L. Penzhorn, C.P.J. Bekker, A.M. Nijhof, F. Jongejan, Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa, Vet. Parasitol. 122 (2004) 119–125.
[18] D. Otranto, G. Capelli, C. Genchi, Changing distribution patterns of canine vector borne diseases in Italy: leishmaniosis vs. dirofilariosis, Parasites Vectors 2 (Suppl 1) (2009) S2.
[19] E. Brianti, G. Gaglio, E. Napoli, S. Giannetto, F. Dantas-Torres, O. Bain, et al., New insights into the ecology and biology of *Acanthocheilonema reconditum* (Grassi, 1889) causing canine subcutaneous filariosis, Parasitology 139 (2012) 530–536.
[20] WHO, The World Health Report 2004-Changing History 95 World Health Organization, 2004 96p.Available from: http://www.who.int/whr/2004/en/index.html.
[21] E.V. Schwan, Filariosis of Domestic Carnivores in Gauteng, KwaZulu-Natal and Mpumalanga Provinces, South Africa, and Maputo Province, Mozambique, (2009).
[22] K. Albrechtová, K. Sedlák, K.J. PetrŽelková, J. Hlaváč, A.D. Mihalca, A. Lesingirian, et al., Occurrence of filaria in domestic dogs of Samburu pastoralists in Northern Kenya and its associations with canine distemper, Vet. Parasitol. 182 (2011) 230–238.
[23] J. Siwila, E.T. Mwase, P. Nejsum, P.E. Simonsen, Filarial infections in domestic dogs in Lusaka, Zambia, Vet. Parasitol. [Internet]. Elsevier B.V 210 (2015) 250–254, https://doi.org/10.1016/j.vetpar.2015.04.009 Available from:.
[24] J.W. McCall, C. Genchi, L.H. Kramer, J. Guerrero, L. Venco, Chapter 4 heartworm disease in animals and humans, Adv. Parasitol. 66 (2008) 193–285.
[25] C. Mary, F. Faraut, L. Lascombe, H. Dumon, Quantification of *Leishmania infantum* DNA by a real-time PCR assay with high sensitivity, J. Clin. Microbiol. [Internet] 42 (2004) 5249–5255. Available from: http://jcm.asm.org/content/42/11/5249.full.pdf.
[26] C. Roqueplo, V. Cheminel, O. Bourry, J. Gomez, J.M. Prévosto, D. Parzy, et al., Canine ehrlichiosis in the Ivory Coast and Gabon: alteration of biochemical blood parameters based on Ehrlichia canis serology, Clin. Microbiol. Infect. 15 (2009)

41–42.
[27] C. Socolovschi, J. Gomez, J.L. Marié, B. Davoust, P.M. Guigal, D. Raoult, et al., *Ehrlichia canis* in *Rhipicephalus sanguineus* ticks in the Ivory Coast, Ticks Tick Borne Dis. [Internet]. Elsevier GmbH 3 (2012) 411–413, https://doi.org/10.1016/j.ttbdis.2012.10.005 Available from:.
[28] I.A. Matei, G. D'Amico, P.K. Yao, A.M. Ionica, P.W.N. Kanyari, A.A. Daskalaki, et al., Molecular detection of *Anaplasma platys* infection in free-roaming dogs and ticks from Kenya and Ivory Coast, Parasites Vectors 9 (2016) 1–8.
[29] B.L. Travi, F. Dantas-torres, G. Miro, Canine Visceral Leishmaniasis : Diagnosis and Management of the Reservoir Living Among Us, (2018), pp. 1–13.
[30] S. Paltrinieri, L. Gradoni, X. Roura, A. Zatelli, E. Zini, Laboratory tests for diagnosing and monitoring canine leishmaniasis, Vet. Clin. Pathol. 45 (2016) 552–578.
[31] M.W. Senghor, A.A. Niang, J. Depaquit, H. Ferté, M.N. Faye, E. Elguero, et al., Transmission of *Leishmania infantum* in the canine leishmaniasis focus of Mont-Rolland, Senegal: ecological, parasitological and molecular evidence for a possible role of *sergentomyia* sand flies, PLoS Negl. Trop. Dis. 10 (2016) 1–17.
[32] A. Adel, E. Abatih, N. Speybroeck, et al., Estimation of canine *Leishmania* infection prevalence in six cities of the Algerian littoral zone using a Bayesian approach, PLoS One 10 (3) (2015) e0117313.
[33] M.F. Diouani, B. Nbena, J. Bettaib, H. Louzir, S. Jedidi, A. Ftaiti, et al., Dogs . Infantum infection from an endemic region of the north of Tunisia : a prospective study, Arch. Inst. Pasteur Tunis 85 (1–4) (2008) 55–61.
[34] Z. Zoghlami, E. Chouihi, W. Barhoumi, K. Dachraoui, N. Massoudi, H.K. Ben, et al., Interaction between canine and human visceral leishmaniases in a holoendemic focus of Central Tunisia, Acta Trop. [Internet]. Elsevier B.V. 139 (2014) 32–38, https://doi.org/10.1016/j.actatropica.2014.06.012 Available from:.
[35] M. Rami, T. Atarhouch, M. Sabri, S.M. Cadi, T. Benazzou, A. Dakkak, Leishmaniose canine dans le Rif (côte méditerranéenne marocaine) : enquête séro-épidémiologique, Parasite [Internet] 10 (2003) 79–85. Available from: http://www.parasite-journal.org/10.1051/parasite/2003101p77.
[36] R. Nejjar, M. Lemrani, A. Malki, S. Ibrahimy, H. Amarouch, A. Benslimane, Canine leishmaniasis due to *Leishmania infantum* MON-1 in northern Morocco, Parasite [Internet] 5 (1998) 325–330. Available from: http://www.parasite-journal.org/10.1051/parasite/1998054325.
[37] M.K. N'Djetchi, H. Ilboudo, M. Koffi, J. Kaboré, J.W. Kaboré, D. Kaba, et al., The study of trypanosome species circulating in domestic animals in two human African trypanosomiasis foci of Côte d'Ivoire identifies pigs and cattle as potential reservoirs of Trypanosoma brucei gambiense, PLoS Negl. Trop. Dis. 11 (2017) 1–16.
[38] P.U. Umeakuana, W. Gibson, R.C. Ezeokonkwo, B.M. Anene, Identification of *Trypanosoma brucei gambiense* in naturally infected dogs in Nigeria, Parasites Vectors [Internet]. BioMed Central 12 (2019) 1–7, https://doi.org/10.1186/s13071-019-3680-8 Available from:.
[39] P. Ramesh, C.S.R. Chowdary, Y. Chaitanya, Diagnosis and treatment of canine trypanosomiasis – a case study, Int. J. Sci. Environ. Technol. 5 (2016) 3387–3393.
[40] F. Dantas-Torres, The brown dog tick, *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae): From taxonomy to control, Vet. Parasitol. 152 (2008) 173–185.
[41] Y. M'ghirbi, A. Bouattour, Detection and molecular characterization of *Babesia canis vogeli* from naturally infected dogs and *Rhipicephalus sanguineus* ticks in Tunisia, Vet. Parasitol. 152 (2008) 1–7.
[42] D. Otranto, Arthropod-borne pathogens of dogs and cats: from pathways and times of transmission to disease control, Vet. Parasitol. [Internet]. Elsevier 251 (2018) 68–77, https://doi.org/10.1016/j.vetpar.2017.12.021 Available from:.
[43] J. Magnis, S. Lorentz, L. Guardone, F. Grimm, M. Magi, T.J. Naucke, et al., Morphometric analyses of canine blood microfilariae isolated by the Knott's test enables *Dirofilaria immitis* and *D. repens* species-specific and *Acanthocheilonema* (syn. *Dipetalonema*) genus-specific diagnosis, Parasites Vectors 6 (2013) 3–7.
[44] Amilan Sivagurunathan and Asem Mohamed Elsayed Atwa. A case of *acanthocheilonema reconditum* in a dog, Int. J. Med. Sci. 2 (1) (2017) 25–26.
[45] M. Baxarias, A. Álvarez-fernández, P. Martínez-orellana, S. Montserrat-sangrà, L. Ordeix, A. Rojas, et al., Does co-infection with vector-borne pathogens play a role in clinical canine leishmaniosis? Parasites Vectors (2018) 1–16.
[46] A. Rojas, D. Rojas, V. Montenegro, R. Gutiérrez, D. Yasur-Landau, G. Baneth, Vector-borne pathogens in dogs from Costa rica: first molecular description of *Babesia vogeli* and *Hepatozoon canis* infections with a high prevalence of monocytic ehrlichiosis and the manifestations of co-infection, Vet Parasitol [Internet]. Elsevier B.V. 199 (2014) 121–128, https://doi.org/10.1016/j.vetpar.2013.10.027 Available from:.
[47] C. Maia, L. Altet, L. Serrano, J.M. Cristóvão, M.D. Tabar, O. Francino, et al., Molecular detection of *Leishmania infantum*, filariae and Wolbachia spp. in dogs from southern Portugal, Parasites Vectors [Internet] 9 (2016) 16–21, https://doi.org/10.1186/s13071-016-1452-2 Available from:.
[48] P. Ntais, V. Christodoulou, E. Dokianakis, M. Antoniou, *Leishmania infantum* and Dirofilaria immitis coinfection in dogs in Greece, Parasitol. Open [Internet] 2 (2016) e17. Available from: http://www.journals.cambridge.org/abstract_S2055709416000157.
[49] D. Otranto, F. Dantas-Torres, E. Brianti, D. Traversa, D. Petrić, C. Genchi, et al., Vector-borne helminths of dogs and humans in Europe, Parasites Vectors 6 (2013) 1–14.
[50] X. Roura, E. Breitschwerdt, A. Lloret, L. Ferrer, B. Hegarty, Serological evidence of exposure to *Rickettsia*, *Bartonella* and *Ehrlichia* species in healthy or *Leishmania infantum*-infected dogs from Barcelona, Spain, J. Appl. Res. Vet. Med. 3 (2005) 129–137.
[51] Z.K. Njiru, C.C. Constantine, S. Guya, J. Crowther, J.M. Kiragu, R.C.A. Thompson, et al., The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes, Parasitol. Res. 95 (2005) 186–192.
[52] M.E. De Almeida, F.J. Steurer, O. Koru, B.L. Herwaldt, N.J. Pieniazek, A.J. Da Silva, Identification of Leishmania spp. by molecular amplification and DNA sequencing analysis of a fragment of rRNA internal transcribed spacer 2, J. Clin. Microbiol. 49 (2011) 3143–3149.
[53] H. Dahmana, N. Amanzougaghene, B. Davoust, F. Fenollar, D. Raoult, O. Mediannikov, et al., Great diversity of *Piroplasmida* in Equidae in Africa and Europe including new species, Vet. Parasitol. RSR (2019) In press.
[54] L. Lachaud, E. Chabbert, P. Dubessay, J. Dereure, J. Lamothe, J.P. Dedet, P. Bastien, Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers, Parasitology 125 (2002) 197–207.

Publication N°17

Role of reptiles and associated arthropods in the epidemiology of rickettsioses: a One Health paradigm

Jairo Alfonso Mendoza-Roldan[a], Ranju Ravindran Santhakumari Manoj[a], Giada Annoscia[a], Roberta Iatta[a], Piero Lovreglio[b], Angela Stufano[b], Maria Stefania Latrofa[a], Filipe Dantas-Torres[c], Bernard Davoust[d,e], **Younes Laidoudi**[d,e], Oleg Mediannikov[d,e], Domenico Otranto[a,f*]

[a]Department of Veterinary Medicine, Università degli Studi di Bari, 70010, Valenzano, Italy
[b]Interdisciplinary Department of Medicine, Section of Occupational Medicine, University of Bari Aldo Moro, Bari, Italy
[c]Department of Immunology, Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, Pernambuco, 50670-420, Brazil
[d]IRD, AP-HM, MEPHI, Aix Marseille University, 13005 Marseille, France.
[e]IHU-Méditerranée Infection, 13005 Marseille, France
[f]Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran

ARTICLE DE RECHERCHE

Soumis à : PLOS Neglected Tropical Diseases

Running title: Spotted fever group rickettsiae and One Health paradigm

Keywords: Spotted Fever, Lizards, Snakes, Squamata reptiles, Rickettsia monacensis, Rickettsia helvetica, Rickettsia slovaca, Ixodes ricinus, Neotrombicula autumnalis, Coxiella burnetii, Anaplasma phagocytophilum.

Serological and molecular investigation of spotted fever group rickettsiae in humans, lizards and ectoparasites in a natural park in Italy: a cross-sectional study

Jairo Alfonso Mendoza-Roldan, Ranju Ravindran Santhakumari Manoj, Maria Stefania Latrofa, Roberta Iatta, Giada Annoscia, Piero Lovreglio, Angela Stufano, Filipe Dantas-Torres, Bernard Davoust, Younes Laidoudi, Oleg Mediannikov, Domenico Otranto

Author affiliations: Department of Veterinary Medicine, University of Bari, Valenzano, Italy (J. A. Mendoza-Roldan, R. Ravindran Santhakumari Manoj, M. S. Latrofa, G. Annoscia, R. Iatta, D. Otranto), Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran (D. Otranto) Interdisciplinary Department of Medicine, Section of Occupational Medicine, University of Bari, Bari, Italy (P. Lovreglio, A. Stufano), Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, Brazil (F. Dantas-Torres), IRD, AP-HM, MEPHI, Aix Marseille University, Marseille, France (B. Davoust, Y. Laidoudi, O. Mediannikov), IHU-Méditerranée Infection, Marseille, France (B. Davoust, Y. Laidoudi, O. Mediannikov)

Corresponding author: Domenico Otranto, Phone +39 080 4679839, Fax +39 080 4679839, email: domenico.otranto@uniba.it

Summary

Background Tick-borne diseases, including spotted fever rickettsioses, are on the rise in many parts of the world. Anecdotal cases of spotted fever rickettsioses in an important natural park in Basilicata, southern Italy, spurred us to investigate the exposure to *Rickettsia* spp. in forestry workers, local tour guides, farmers and livestock breeders living or working in that area. We also investigated the exposure to *Coxiella burnetti* and *Anaplasma phagocytophilum* in the study participants, considering their likely exposure to these pathogens. Finally, we also assessed the presence of these pathogens in reptiles, their ectoparasites and in questing ticks collected in the same natural park, as well as in a peri-urban area in another region of southern Italy.

Methods Sera from human subjects (n=50) were screened for antibodies against *Rickettsia* spp., *C. burnetti* and *A. phagocytophilum*. The presence of *Rickettsia* spp. was molecularly investigated in questing ticks, reptiles and their ectoparasites captured in the natural park and in a peri-urban area in Puglia.

Findings Antibodies anti-*Rickettsia* spp. (n=4; 8%), *C. burnetti* (n=8; 16%) and *A. phagocytophilum* (n=11; 22%) were detected in human subjects. Ticks collected from forestry workers were positive for spotted fever group (SFG) rickettsiae; *Ixodes ricinus* for *Rickettsia monacensis* and *Dermacentor marginatus* for *Rickettsia slovaca*. *Rickettsia* spp. was detected in lizards (n=4; 3.1%), in *I. ricinus* (n=105; 87.5%) and in *Neotrombicula autumnalis* (n=8; 8.3%) collected from them, as well as in *I. ricinus* (n=71; 28.4%) from the environment. *Rickettsia monacensis* was the most prevalent species sequenced (n=89; 83.2%), followed by *R. helvetica* (n=18; 16.8%). An undescribed member of the family Anaplasmataceae was detected in four lizards and four ectoparasites (1 mite and 3 ticks). Finally, an undescribed *Ehrlichia* sp. was detected in a *I. ricinus* tick collected from a lizard.

Interpretation Forestry workers, local tour guides, farmers and livestock breeders living or working in the natural park investigated herein are exposed to SFG rickettsiae, *C. burnetii* and *A. phagocytophilum*. *Ixodes ricinus* is involved in the transmission of SFG rickettsiae (*R. monacensis* and *R. helvetica*) in southern Europe and lizards could play a role in the sylvatic cycle of *R. monacensis*, as amplifying hosts. *Neotrombicula autumnalis* could be involved in the enzootic cycle of some SFG rickettsiae among these animals.

Funding University of Bari, Italy.

Introduction

Tick-borne diseases are caused by a range of pathogens, including bacteria, viruses and protozoa.[1] These diseases affect humans worldwide and some people are particularly exposed to them. For instance, spotted fever rickettsioses, Q fever, and human granulocytic anaplasmosis (HGA) are often diagnosed in people living or working in the proximities of forested areas (e.g., park rangers, foresters and hunters) and occupational farmers, veterinarians, laboratory technicians, slaughterhouses and cheese factories personnel.[2]

In humans, *Rickettsia* spp. infection causes conditions of various degrees of severity according to different factors such as the bacterial species, the individual susceptibility and immune status.[3–5] For example, the Mediterranean spotted fever (MSF) caused by *Rickettsia conorii* is the most prevalent and severe form of rickettsiosis in Europe, with human patients usually presented with fever, cutaneous maculo-papular rash, and eschar at the place of tick bite.[6] Q fever caused by *Coxiella burnetii* is usually presented as a flu-like disease, but clinically may vary from self-limiting non-specific fever to atypical pneumonia, endocarditis, hepatitis and neurological manifestation. Similarly, HGA by *Anaplasma phagocytophilum* causes nonspecific febrile illness, which could lead to a fatal outcome.[7,8] These unspecific clinical pictures often result in a lack of diagnosis and unreported cases even in patients with a history of tick bites.[9]

Most rickettsiae are hosted and transmitted by ticks.[3,10] *Coxiella burnetii* is primarily, but not exclusively, airborne, with ticks reputed to occasionally transmit the infection.[11] As such, the epidemiology of tick-borne diseases involves a complex array of risk factors, related to the causative agents, hosts and their shared environment. This highlights how pivotal is the adoption of a One Health approach for understanding the epidemiology of tick-borne diseases and for mitigating their burden on affected populations.[1]

Spotted fever rickettsiosis, Q fever and HGA are endemic in Italy. Mediterranean Spotted Fever (MSF) is of public health significance in southern Italy, especially in Calabria and in the islands of Sardinia and Sicily.[6] Nonetheless, there is limited information on the occurrence of MSF and other

spotted fever rickettsiosis in some regions of southern Italy, as it is the case of Basilicata, where tick vectors, such as *Ixodes ricinus*, are abundant.[12] Incidentally, anecdotal cases of spotted fever rickettsioses in an important natural park in Basilicata spurred us to investigate the exposure to *Rickettsia* spp. in forestry workers, local tour guides, farmers and livestock breeders living or working in that area. We also investigated the exposure to *C. burnetii* and *A. phagocytophilum* in the study participants, considering their likely exposure to these pathogens. Finally, we also assessed the presence of these pathogens in reptiles, their ectoparasites and in questing ticks collected in the same natural park and in a peri-urban area in another region of southern Italy.

Methods

Human blood collection and serological testing

This cross-section study was primarily conducted in the Gallipoli Cognato Park (site 1; 40°32'17"N, 16°07'20.17"E), an important natural park located in the Basilicata region, southern Italy. In February 2020, blood samples were collected from forestry workers and tour guides operating within the natural park, and from farmers and livestock breeders living or working in the surrounding areas. In brief, blood samples (10 ml) were collected in a Vacutainer® tube and transported at 4 °C to the laboratory, where serum samples were obtained by centrifugation at 3000 × *g* for 30 min. Serum samples were stored in 2 ml tubes at -20 °C until serological analyses were performed. Participants were asked to fill a questionnaire providing socio-demographic characteristics, working history, history of exposure to ticks as well as medical history with special focus on the past three months. Participants (n=50) were fully informed about the research aims and features and were provided with an informed consent form, before filling the questionnaire, in accordance with the Helsinki Declaration (WMA 2013). The research protocol was approved by the ethics committee of the University Hospital of Bari (n. 6394, prot. n., 0044469-23062020)

Sera were initially screened for antibodies to *Rickettsia* spp., but considering the characteristics of the study population, they were also tested for antibodies to *C. burnetii* and *A. phagocytophilum*,

by indirect immunofluorescence antibody test (IFAT). First, two-fold dilutions (1:50 and 1:100) of sera (including positive and negative sera) were prepared in phosphate buffered saline (PBS), and a dilution of 1:100 was selected as the cut-off value. Briefly, each slide well was sensitized using in-house generated following antigens: *R. conorii* (strain Malish 7, ATCC VR-613), *R. felis* (strain Marseille, ATCC VR-1525), *R. typhi* (strain Wilmington), *R. helvetica* (strain C9P9), phase I and II of *C. burnetii* (Nine Mile RSA493 strain) and *A. phagocytophilum* (Webster strain). The antigens were deposited separately as microdroplets all around the periphery of each well following the same order in all slides. Twenty µL of each serum sample dilution was applied per well and slides were then incubated for 30 min at 37 °C. After incubation, slides were washed twice with PBS for 5 minutes and once with distilled water. 20 µL of mouse anti-human total immunoglobulin (Ig) conjugated with fluorescein isothiacyanate (FITC) (Sigma-Aldrich, St Louis, MO, USA) were added into each well. Slides were immediately incubated at 37° C for 30 minutes and then washed following the same procedure described above. Positive sera at the cut-off of 1:100 were further investigated for IgG and IgM using a five 2-fold serial dilution IFAT at 1:200 to 1:3400.

Reptiles, ticks and mites

Reptiles and ectoparasites molecularly screened in this study for *Rickettsia* spp. were collected under the frame of two previous studies.[12,13] We initially screened 250 questing *I. ricinus* collected in the park area,[12] as well as 128 lizards, four snakes, and their ectoparasites originating from the same environment.[13] Moreover, one adult female of *I. ricinus* and another of *D. marginatus* collected in February 2020 by park workers on themselves were also included in this study. To have comparable data from an area where *I. ricinus* is considered to be absent, we also tested 38 lizards and their ectoparasites (only mites) collected in a peri-urban area in the surroundings of the Department of Veterinary Medicine, University of Bari (site 2; 41°1'31.584"N, 16°54'3.6288"E), Puglia region, southern Italy.[13]

DNA extraction and molecular analysis

Genomic DNA was extracted from ticks and mites using a lysis with guanidine isothiocyanate protocol (GT),[14] and eluted in AE elution buffer (50 µl for mites, ticks and tissues. Whilst, DNA from lizard and snake blood (~20 µl) and from lizard tails (25 mg) was extracted by using a Qiagen Mini kit and Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany), respectively.

DNA samples were tested by PCR using a pair of primers (CS-78F and CS-323R) targeting a fragment (401 bp) of the gene citrate synthase (*gltA*), present in all species of *Rickettsia*.[15] Positive samples were tested by a second PCR using a pair of primers as previously described (Rr190.70F and Rr190.701R)[16] targeting a fragment of the outer membrane protein A (*ompA*) gene (632 bp), present only in spotted fever group (SFG) rickettsiae. Samples were also screened for *C. burnetii* and for species of the Anaplasmataceae family using primer pairs (CAPI-844-F and CAPI-844-R; EHR16SD and EHR16SR) which amplified a 601 bp fragment of the CAPI gene and 345 bp of 16S rRNA gene, respectively. [17,18] In all PCR runs negative (Milli-Q water) and positive controls of the respective pathogens were included.

Amplicons were purified using 10 µl of PCR product mixed with 0.5 µl of *Escherichia coli* exonuclease I (*Exo*I; MBI, Fermentas, Lithuania), 1 µl of shrimp alkaline phosphatase (SAP) and 0.5 µl of SAP reaction buffer (MBI, Fermentas, Lithuania) to remove primers and unincorporated dNTPs. This mix was incubated at 37°C for 20 min, following enzymes inactivation at 85°C for 15 min. PCR purified products were sequenced using the Taq Dye Doxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems, California, USA) in an automated sequencer (ABI-PRISM 377). Sequences were analysed by Geneious version 11.1.4 software and compared with those available in Genbank database by Basic Local Alignment Search Tool (BLAST).[19]

Rickettsial *ompA* and *gltA* as well as 16S rRNA genes from *Anaplasma* spp. were amplified and the sequences were separately aligned against those closely related species available from GenBank database using the ClustalW application within MEGA6 software.[20] The Akaike information criterion (AIC) option in MEGA6[20] was used to establish the best nucleotide substitution model

adapted to each sequence alignment. Tamura 3-parameter model with a discrete Gamma distribution (+G) was used to generate the *ompA*,[21] *gltA* and the 16S rRNA trees. A maximum likelihood (ML) phylogenetic inference was used with 1000 bootstrap replicates to generate the phylogenetic tree in MEGA6.[20] Homologous sequences of *Rickettsia* were used as outgroup to root the trees, including the *glt*A sequences from *R. belli* and *R. canadensis* (JQ664297, AB297809), the *omp*A sequence of *R. felis* (AY727036) and the 16S rRNA sequence of *R. parkeri* (NR118776).

Results

Out of 50 human subjects tested, 18 (36%) were exposed to at least one pathogen, of which 4 patients had an IgG titre for more than one pathogen (i.e., 1 for *C. burnetii*, *R. typhi* and *A. phagocytophilum;* 1 for *R. felis* and *A. phagocytophilum;* 2 for *C. burnetii* and *A. phagocytophilum*). The remaining 14 patients showed IgG against *A. phagocytophilum* (n=7), *C. burnetii* (n=5), *R. felis* (n=1) and *R. conorii* (n=1). Of the 50 human serum samples, four had IgG antibodies titres against *Rickettsia* spp.: two to *R. felis* (titres 1:100 and 1:200); one to *R. conorii* (1:100) and other to *R. typhi* (1:400). The last serum also reacted to *R. helvetica* antigen with, however, lower titre (1:100). Eleven sera contained antibodies against *A. phagocytophilum* (titres 1:100 to 1:800). Eight sera reacted with *C. burnetii* antigens (titres 1:200 to 1:6400), of which two showed high antibody titres against phase I of *C. burnetii* (one of 1:1600 and another of 1:6400). No serum had IgM antibodies to any of the tested pathogens, excluding acute or recent infections. The mean age of tested patients was 41.9 years with a mean number of tick bites of 2.4 per individual and mean time of last exposure 2.2 years.

Of the snakes (n=4) and lizards (n=128) captured in site 1, 123 (93.2%) were infested by *I. ricinus* (Figure 1) and/or *N. autumnalis* (Figure 2). Lizards (n=40) captured in site 2 were infested only by *N. autumnalis* (25%). The *Rickettsia gltA* gene was amplified from tail tissues of four (i.e., 3.1%; 95% CI: 0.6–5.8%) lizards (i.e., one *Podarcis muralis* and two *Podarcis siculus* from site 1; one *P. siculus* from site 2). *Rickettsia gltA* gene sequences from specimens from site 1 displayed 100% of

194 nucleotide identity with that of *R. monacensis* (KU586332), whereas sequence from site 2 was
195 100% identical to that of *R. helvetica* (KU310588). No snakes tested positive for *Rickettsia* spp. In
196 addition, 87.5% (105/120; 95% CI: 80.2–92.8%) of *I. ricinus* collected from reptiles were positive
197 for the *Rickettsia gltA* gene, of which 65.8% (79/120; 95% CI: 56.6–74.3%) also for the *Rickettsia*
198 *ompA* gene. Out of 97 *N. autumnalis* larvae, eight (8.3%; 95% CI: 38.3–56.8%) yielded positive for
199 the *Rickettsia gltA* gene and four (4.2%; 95% CI: 1.1–10.3%) for the *Rickettsia ompA* gene (Table
200 1). *Rickettsia monacensis* was the only species retrieved with the *ompA* gene in both ticks and mites
201 of lacertids.
202 Of the 250 questing *I. ricinus* collected from the environment in site 1, 28.4% (71/250; 95% CI:
203 3.4–7.1%) yielded positive results for the *gltA* gene and 8.8% (22/250; 95% CI: 3.4–7.1%) for the
204 *ompA* gene. Two different species, namely *R. helvetica* (9/71; only for the *gltA* gene) and *R.*
205 *monacensis* (62/71) were identified with 100% nucleotide identity with GenBank reference
206 sequence KU310588 and MK922661, respectively. Two ticks collected from park rangers scored
207 positive for *Rickettsia ompA* gene, with *I. ricinus* for *R. monacensis* (100% nucleotide identity with
208 MK922661) and *D. marginatus* for *R. slovaca* (100% nucleotide identity with MH532257).
209 An undescribed member of the family Anaplasmataceae was detected in four lizards (3 blood
210 samples and 1 tail) and in four ectoparasites (1 mite and 3 ticks) (nucleotide identity ranging from
211 99.8 to 100% with MG924904; originally designated as "*Candidatus* Cryptoplasma sp."). In
212 addition, one tick (from lizard) scored positive for *Ehrlichia* sp. (99.3% nucleotide identity with *E.*
213 *canis*, MN922610) and a *P. siculus* lizard to the same undescribed Anaplasmataceae (99.0%
214 nucleotide identity with MG924904 and GU734325).
215 *Rickettsia gltA* sequences obtained from ticks and reptiles clustered within the same clades of *R.*
216 *monacensis* and *R. helvetica*, respectively, as distinct clades with the exclusion of the other
217 *Rickettsia* spp. (Figure 3a). Similarly, *Rickettsia ompA* sequences clustered with *R. monacensis* and
218 *R. slovaca*, supported by high bootstrap values (i.e., 93%, Figure 3b). The 16S rRNA gene
219 sequences clustered with those from a group of undescribed organisms belonging to the family

220 Anaplasmataceae, with the exception of a sequence that clustered within the clade of *Ehrlichia* spp.
221 (Figure 4). Outgroups were consistently resolved for both trees. Representative sequences herein
222 generated have been deposited in GenBank under accession numbers from MT829281-MT829303.
223
224 **Discussion**
225 This study confirm that forestry workers, local tour guides, farmers and livestock breeders living
226 or working in a natural park in southern Italy are exposed to *Rickettsia* spp., and also to *A.*
227 *phagocytophilum* and *C. burnetii*. In addition, the overall picture of pathogen circulation in that area
228 is confirmed by the molecular detection of at least two tick-associated *Rickettsia* spp. (*R. helvetica*
229 and *R. monacensis*) in lizards, as well as in their ectoparasites (i.e., *I. ricinus* and *N. autumnalis*)
230 and in questing ticks from the environment.
231 Overall, serological analysis revealed antibody titres against *Rickettsia* spp. in study participants,
232 all with a history of tick bites, confirming the risk of human exposure in the study area. In
233 particular, *R. monacensis* (the most frequent species in site 1) is considered to be an emerging
234 human pathogen, along with other rickettsiae (e.g., *R. massiliae*, *R. aeschlimannii* and *R. sibirica*
235 *mongolitimonae*).[3] The occurrence of *Rickettsia* spp. in the study area was previously recorded in
236 ticks collected from humans.[9] However, data on tick-borne rickettsiosis in southern Italy remain
237 scarce and, although spotted fever rickettsioses are mandatory notifiable diseases in Italy since
238 1990, their incidence is still unknown due to the hindrances in a specific clinical and serological
239 diagnosis.[6] Moreover, the seroprevalence levels of antibodies against *A. phagocytophilum* and *C.*
240 *burnetii* indicate a frequent contact of the local population with these bacteria.
241 The detection of *R. helvetica* and *R. monacensis* in *I. ricinus* (free living, from reptiles and from
242 humans), along with the high abundance of this tick in the study area,[12] provides a strong
243 circumstantial evidence of its involvement in the transmission of these rickettsiae in this part of
244 Europe. Given that larval stages of *N. autumnalis* parasitize reptiles, birds and humans,[22] the
245 retrieval of *Rickettsia* spp. in these mites suggests the possibility of the transmission pathways

246 between reptiles and humans. Our data is of interest considering that only *N. autumnalis* mites were
247 collected on lizards in site 2, where *Rickettsia* spp. were detected also in one lizard. This raises the
248 question whether this could represent a potential threat for human health, even in absence of *I.*
249 *ricinus.* Regardless the possible role of *N. autumnalis* in the ecology of *Rickettsia* spp., public
250 awareness about the risk of mite and tick bites is advocated.
251 Though other animal species (e.g., mice) may also act as hosts of *Rickettsia* spp. in this area, the
252 prevalence of *R. helvetica* and *R. monacensis* in lizards (i.e., 3.1%) and in ticks collected on them
253 (i.e., 87.5%) indicate that lizards could be acting as amplifying hosts for these bacteria. Indeed, this
254 is also supported by the detection of rickettsiae in their blood. Findings herein presented agree with
255 other studies in which *R. helvetica* was detected in *P. muralis* tissue (6.2%; 1/16) from the northern
256 Apennine area of Italy.[23] Since most ectoparasites attach and feed around the axillary region of
257 lizards,[24] the detection of *R. monacensis* and *R. helvetica* from the tail tissue may indicate a
258 disseminated infection.[23] Like other small vertebrates, lizards are suitable hosts for immature stages
259 of *I. ricinus* ticks across Europe.[23] Regardless the possible role of reptiles, many wild and domestic
260 mammals could also act as hosts and contribute to the dispersion of *Rickettsia*-infected ticks in
261 different geographical areas.[25]
262 The phylogenetic analyses of the sequences of *R. helvetica* and *R. monacensis* obtained from
263 ticks from humans, questing ticks and reptiles revealed the clustering of these *Rickettsia* spp. with
264 that from humans available in GenBank. The high sequence similarity of *Rickettsia* spp. obtained
265 herein indicates the circulation of these two species among ticks, lizards and humans in the study
266 area.
267 Negative results of PCR from reptiles and their ectoparasites for *C. burnetii* and *A.*
268 *phagocytophilum* could be due to the more predominant role of mammalian hosts in the ecology
269 and maintenance of these bacteria.[26,27] For instance, domestic ruminants are recognized as
270 important reservoirs of *C. burnetii*[28] and rodents have been suspected to participate in the sylvatic
271 cycle of this pathogen in Italy.[29] Furthermore, small mammals have been indicated as main

reservoirs for *A. phagocytophilum*, although large mammals and birds may also serve as a source of infection to tick vectors (*27*).[27]

Finally, the detection of an undescribed member of the Anaplasmataceae in lizards agrees with previous reports from Europe (Slovakia)[30] and USA (*31*).[31] This microorganism is related to genus *Anaplasma*, but represents a lineage distinct from all known *Anaplasma* spp. Our results shed new light on the reptile-tick-Anaplasmataceae interactions in Italy and suggest that lizards of the genus *Podarcis* could act as primary hosts for the maintenance and enzootic circulation of undescribed organisms of unknown pathogenicity and zoonotic potential. In the same way, along with a previous study,[32] our data suggest that the diversity of ehrlichial microorganisms infecting reptiles is presently underestimated.

In conclusion, data presented reveal the exposure of forestry workers, local tour guides, farmers and livestock breeders to *Rickettsia* spp., *C. burnetii* and *A. phagocytophilum* in a natural park in southern Italy. Furthermore, our results suggest that lizards (i.e., *P. muralis* and *P. siculus*) could play a role in the transmission cycle of SFG rickettsiae and that *I. ricinus* is involved in the transmission of these pathogens in southern Europe. Remarkably, lizards could act as amplifying hosts and *N. autumnalis* could be involved in the enzootic cycle of some SFG rickettsiae among these animals.

References

1. Dantas-Torres F, Chomel BB, Otranto D. Ticks and tick-borne diseases: a One Health perspective. *Trends Parasitol* 2012; **28**: 437–46.

2. Fenga C, Gangemi S, De Luca A, et al. Seroprevalence and occupational risk survey for *Coxiella burnetii* among exposed workers in Sicily, Southern Italy. *Int J Occup Environ Health* 2015; **28**: 901.

3. Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clin Microbiol Rev* 2005; **18**: 719–56.

4. Dantas-Torres F. Rocky Mountain spotted fever. *Lancet infect dis*. 200**7**; **7**: 724-32.

5. Sánchez-Montes S, Isaak-Delgado AB, Guzmán-Cornejo C, et al. *Rickettsia* species in ticks that parasitize amphibians and reptiles: Novel report from Mexico and review of the worldwide record. *Ticks Tick Borne Dis* 2019; **10**: 987–94.

6. Gomez-Barroso D, Vescio MF, Bella A, et al. Mediterranean spotted fever rickettsiosis in Italy, 2001–2015: Spatio-temporal distribution based on hospitalization records. *Ticks Tick Borne Dis* 2019; **10**: 43–50.

7. Jin H, Wei F, Liu Q, Qian J. Epidemiology and control of human granulocytic anaplasmosis: a systematic review. *Vector-Borne Zoonot Dis* 2012; **12**: 269–74.

8. González-Barrio D, Jado I, Fernández-de-Mera IG, et al. Genotypes of *Coxiella burnetii* in wildlife: disentangling the molecular epidemiology of a multi-host pathogen. *Environ Microbiol Rep* 2016; **8**: 708–14.

9. Otranto D, Dantas-Torres F, Giannelli A, et al. Ticks infesting humans in Italy and associated pathogens. *Parasit Vectors* 2014; **7**: 328.

10. Dantas-Torres F, Otranto D. Further thoughts on the taxonomy and vector role of *Rhipicephalus sanguineus* group ticks. *Vet Parasitol* 2015; **208**: 9–13.

11. Duron O, Sidi-Boumedine K, Rousset E, Moutailler S, Jourdain E. The importance of ticks in Q fever transmission: what has (and has not) been demonstrated?. *Trends Parasitol* **2015**; **31**: 536–552.

12. Dantas-Torres F, Otranto D. Seasonal dynamics of *Ixodes ricinus* on ground level and higher vegetation in a preserved wooded area in southern Europe. *Vet Parasitol* 2013; **192**: 253–8.

13. Mendoza-Roldan JA, Colella V, Lia RP, et al. *Borrelia burgdorferi* (*sensu lato*) in ectoparasites and reptiles in southern Italy. *Parasit Vectors* 2019; **12**: 35.

14. Chomkzynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 1993; **15**: 532–7.

15. Labruna MB, Whitworth T, Horta MC, et al. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the state of Sao Paulo, Brazil, where Brazilian spotted fever is endemic. *J Clin Microbiol* 2004; **42**: 90–8.

16. Pacheco RC, Horta MC, Moraes-Filho J, Ataliba AC, Pinter A, Labruna MB. Rickettsial infection in capybaras (*Hydrochoerus hydrochaeris*) from São Paulo, Brazil: serological evidence for infection by *Rickettsia bellii* and *Rickettsia parkeri*. *Biomedica* 2007; **27**: 364–71.

17. Reeves WK, Lofits AD, Sanders F, et al. *Borrelia*, *Coxiella* and *Rickettsia* in *Carios capensis* (Acari: Argasidae) from a brown pelican (*Pelecanus accidentalis*) rookery in South Carolina, USA. *Exp Appl Acarol* 2006; **39**: 321–9.

18. Martin AR, Brown GK, Dunstan RH, Roberts TK. *Anaplasma platys* an improved PCR for the detection in dogs. *Exp Parasitol* 2005; **109**: 176–80.

19. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; **215**: 403–10.

20. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 2013; **30**: 2725–9.

21. Tamura K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Mol Biol Evol* 1992; **9**: 678–87.

22. Mendoza-Roldan J, Modry D, Otranto D. Zoonotic parasites of reptiles: a crawling threat. *Trends in Parasitol* 2020; **36**: 677-87.

23. Tomassone L, Ceballos LA, Ragagli C, et al. Importance of common wall lizards in the transmission dynamics of tick-borne pathogens in the northern Apennine Mountains, Italy. *Microb Ecol* 2017; **74**: 961–8

24. Mendoza-Roldan J, Ribeiro SR, Castilho-Onofrio V, et al. Mites and ticks of reptiles and amphibians in Brazil. *Acta Trop* 2020; **208**:105515. doi:10.1016/j.actatropica.2020.105515

374 25. Parola P, Paddock CD, Socolovschi C, et al. Update on tick-borne rickettsioses around the
375 world: a geographic approach. *Clin Microbiol Rev* 2013; **26**: 657–702.
376
377 26. Maurin M, Raoult D. Q fever. *Clin Microbiol Rev* 1999; **12**: 518–53.
378
379 27. Keesing F, Hersh MH, Tibbetts M, et al. Reservoir competence of vertebrate hosts for
380 *Anaplasma phagocytophilum*. *Emerging Infect Dis* 2012; **18**: 2013.
381
382 28. Di Domenico M, Curini V, De Massis F, Di Provvido A, Scacchia M, Camma C. *Coxiella*
383 *burnetii* in Central Italy: novel genotypes are circulating in cattle and goats. *Vector Borne*
384 *Zoonotic Dis* 2014; **14**: 710-15.
385
386 29. Pascucci I, Di Domenico M, Dall'Acqua F, Sozio G, Camma C. Detection of Lyme disease
387 and Q fever agents in wild rodents in central Italy. *Vector Borne Zoonotic Dis* 2015; 7: 404–
388 11.
389
390 30. Kočíková B, Majláth I, Víchová B, et al. Candidatus *Cryptoplasma* Associated with Green
391 Lizards and Ixodes ricinus Ticks, Slovakia, 2004–2011. *Emerging Infect Dis* 2018; **24**: 2348
392
393 31. Eshoo MW, Carolan HE, Massire C, et al. Survey of *Ixodes pacificus* ticks in California
394 reveals a diversity of microorganisms and a novel and widespread Anaplasmataceae species.
395 *PLoS ONE* 2015; **9**: 0135828.
396
397 32. Andoh M, Sakata A, Takano A, et al. Detection of *Rickettsia* and *Ehrlichia* spp. in ticks
398 associated with exotic reptiles and amphibians imported into Japan. *PLoS One* 2015; **10**:
399 e0133700.

Figure legend

Figure 1. *Ixodes ricinus* larvae and nymphs in the axillary region of adult *Podarcis muralis*.

Figure 2. *Neotrombicula autumanlis* larvae in the ventral region of adult *Podarcis siculus.*

Figure 3. Maximum-likelihood phylogenetic trees of *gltA* (a) and *ompA* (b) genes of *Rickettsia* spp. Bootstrap values (>70%) are shown near de nodes. *Rickettsia belli*, *Rickettsia canadensis* (a) and *Rickettsia felis* (b) were used as outgroups. Scale bar indicates nucleotide substitution per site. *Rickettsia* spp. sequenced in this study are in bold. Further details on the origin of samples are in brackets.

Figure 4. Maximum-likelihood phylogenetic tree of 16S rRNA sequences of Anaplasmataceae. Bootstrap values (>70%) are shown near de nodes. *Rickettsia parkeri* was used as outgroup. Scale bar indicates nucleotide substitution per site. Anaplasmataceae sequenced in this study are in bold. Further details on the origin of samples are in brackets.

Table 1. Number and percentage of ectoparasites positive for *Rickettsia* spp. collected on reptile hosts. Percentage of sequence identity for *gltA* and *ompA* genes with sequences available in GenBank.

Host (infested/total)	**Ectoparasite % (n/total)**	***gltA* (n positive/total) sequence identity**	***ompA* (n positive/total) sequence identity**
Podarcis siculus (112/135)	*Ixodes ricinus* 88.3% (99/112)	(64/99) *Rickettsia monacensis* 100% (KU586332) (24/99) *Rickettsia helvetica* 100% (KU310588)	(64/99) *Rickettsia monacensis* 100% (MK922661)
	Neotrombicula autumnalis *77.7%* (87/112)	(5/87) *Rickettsia monacensis*100% (KU586332)	(2/5) *Rickettsia monacensis* 100% (MK922661)
Podarcis muralis (15/16)	*Ixodes ricinus* 100% (15/15)	(8/15) *Rickettsia monacensis*100% (KU586332) (3/15) *Rickettsia helvetica* 100% (KU310588)	(8/15) *Rickettsia monacensis* 100% (MF383610)
	Neotrombicula autumnalis 33.3% (5/15)	(2/15) *Rickettsia monacensis 100% (KU586332)*	(2/15) *Rickettsia monacensis* 100% (MK922661)
Lacerta bilineata (5/5)	*Ixodes ricinus* 100% (5/5)	(5/5) *Rickettsia monacensis* 100% (KU586332)	(5/5) *Rickettsia monacensis* 100% (MK922661)
	Neotrombicula autumnalis 80 (4/5)	(1/4) *Rickettsia monacensis* 100% (KU586332)	
Elaphe quatuorlineata (1/2)	*Ixodes ricinus* 100% (1/1)	(1/1) *Rickettsia Helvetica* 100% (KU310588)	

Figure 1

Click here to access/download;Figure;Fig 1.JPG

Figure 2

Figure 3

Click here to access/download;Figure;Fig 3.tiff

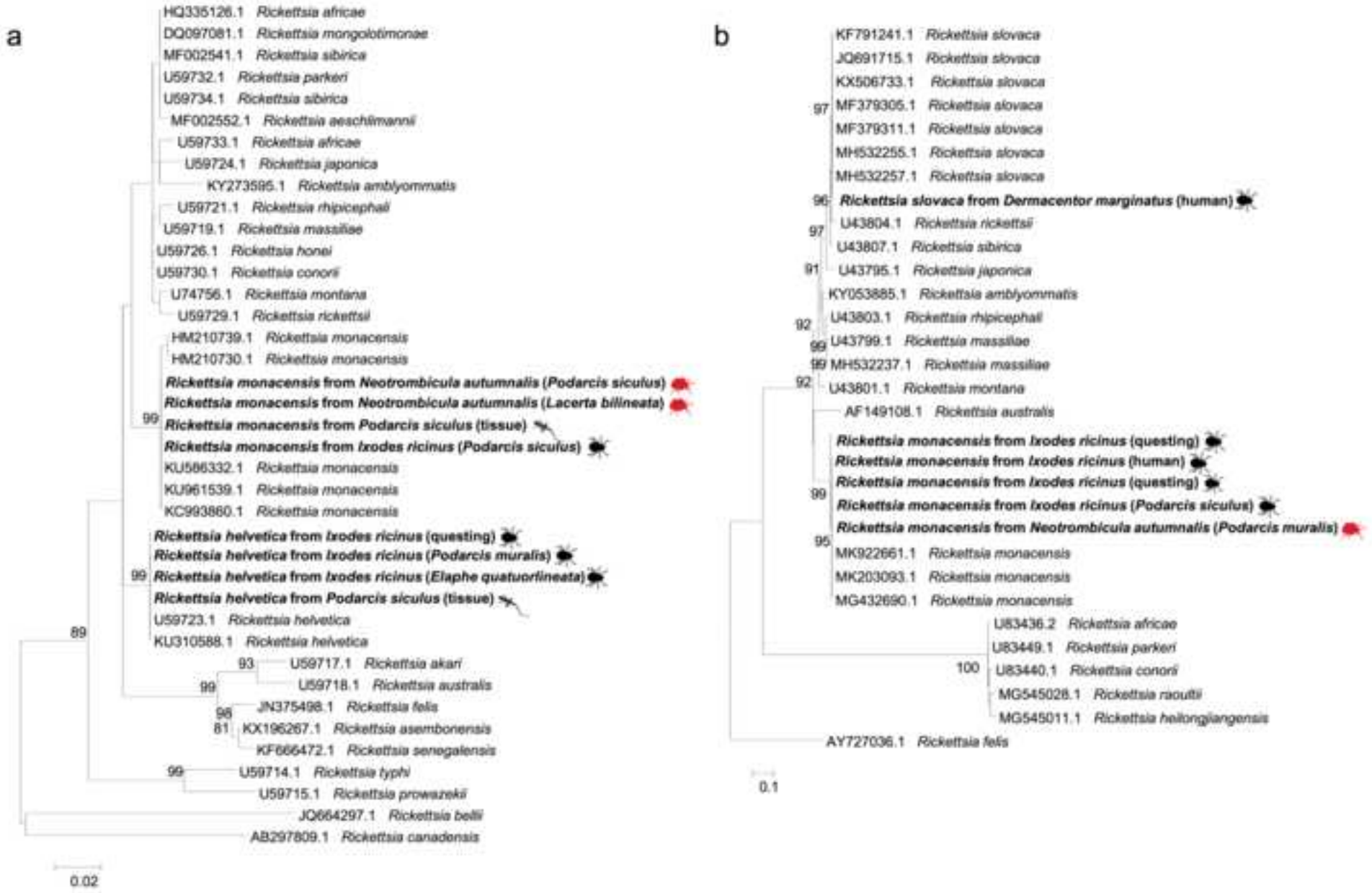
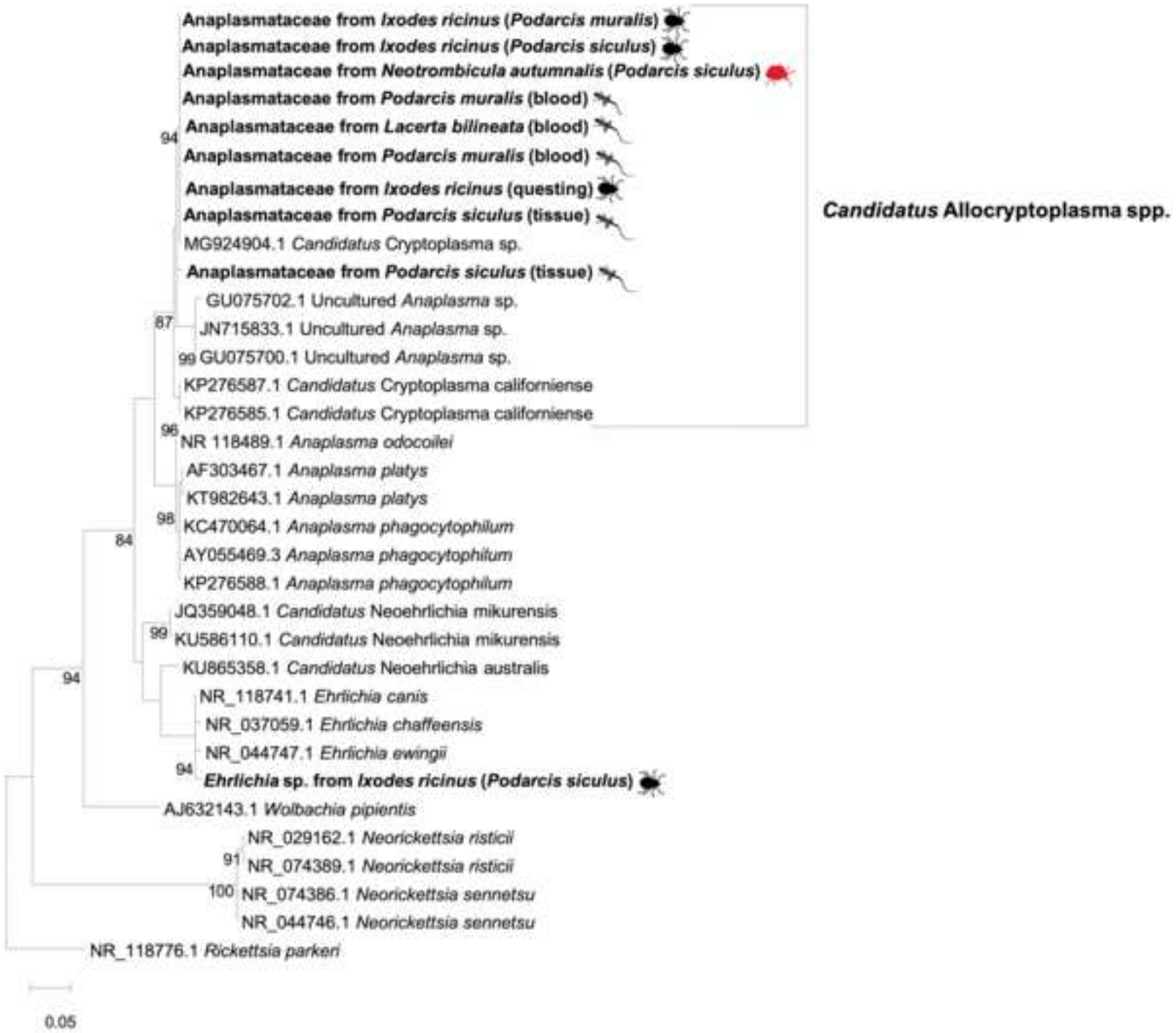


Figure 4

Click here to access/download;Figure;Fig 4.tiff



Conclusions et perspectives

Le présent travail porte essentiellement sur **les maladies infectieuses à transmission vectorielle**. Il apporte quelques nouveautés permettant de mieux comprendre le paradigme des maladies vectorielles. J'ai été amené à relier les techniques de laboratoire de haute performance, à l'expérimentation animale et, parfois, à des études de terrain, auxquelles j'ai, pour certaines, moi-même participé et conçu le projet. Les moyens technologiques modernes de l'IHU, nous ont permis, non seulement, de mieux conduire nos recherches, mais aussi de faire **des avancées dans le domaine de l'étude des maladies vectorielles en général et dans celui des sciences vétérinaires en particulier**. Logiquement, la médecine vétérinaire manque de développement si on la compare avec la médecine humaine. Il y a donc une marge de progrès possible si on investit dans ce domaine, malgré les faiblesses qui existent à l'heure actuelle, comme l'absence d'outils de diagnostic efficaces, le manque de contrôle et de surveillance des maladies vectorielles des animaux. Les études menées ont des répercussions directes sur la santé des animaux mais aussi sur la santé publique. Tout manque de développement dans la recherche retarde l'élimination de certaines maladies, qui suscitent d'énormes dommages.

Dans un premier temps, je me suis consacré à mettre en œuvre **une approche culturomique** permettant l'isolement et la culture de bactéries du genre ***Wolbachia***. En effet, ces bactéries ne sont pas directement impliquées en tant qu'agents infectieux, mais elles peuvent jouer un rôle très important dans le contrôle des maladies infectieuses à transmission vectorielle. Parce qu'elles sont strictement liées à leur hôte, qui peut être le vecteur ou l'agent parasitaire lui-même (filaires), les étudier devient impératif pour mieux contrôler les maladies infectieuses de leur pathogénie à leur épidémiologie, car ce n'est pas toujours le cas. La taxonomie du genre *Wolbachia* est encore incomplète, du fait qu'aucune espèce n'a été caractérisée, jusqu'à maintenant. Cela est dû à l'absence de culture axénique de ces bactéries. Il est important de

noter qu'elles sont considérées comme **exigeantes**, c'est-à-dire qu'elles nécessitent un milieu de culture enrichi et parfaitement adapté à leurs besoins. Au vue de la culturomique des bactéries exigeantes, il apparait que le système **d'isolement et de co-culture cellulaire** que je propose offre une plateforme menant vers le développement de la culture axénique, d'un côté par l'obtention de matériel pur (isolement de souches) et d'un autre côté par l'étude de leurs particularités et exigences. On peut se référer aux bacilles de Whipple (*Tropheryma whipplei*), et à l'agent de la fièvre Q (*Coxiella burnetti*) qui représentent un exemple parfait de succès de la culture axénique et de la caractérisation bactérienne. L'isolement et la co-culture cellulaires ont permis, pour ces cas, d'obtenir la séquence génomique et d'étudier, par conséquent, les besoins et les exigences de ces bactéries (Masselot et al. 2003, Bentley et al. 2003, Ghigo et al. 2002). Ces travaux ont mené à la conception finale de milieux axéniques spécifiques (Omsland et al. 2009, Kersh et al. 2011).

En outre, j'ai pu démontrer par une étude taxo-génomique la nette distinction entre *Wolbachia masseliensis* sp. nov. de *Cimex hemipterius* et la *Wolbachia* de *C. lectularius*, mais aussi par rapport à toutes les autres *Wolbachia* connues à l'heure actuelle. Cela renforce l'idée de **la nécessité de revoir la taxonomie de ce genre bactérien**. Le profil métabolique de cette bactérie est proche de celui des *Wolbachia* du clade D, endosymbionte des filaires lymphatiques humaines, ouvrant de ce fait une nouvelle perspective de recherche à propos de l'interaction hôte-*Wolbachia* et sujet infecté. On espère que cela mènera au développement de nouvelles cibles thérapeutiques et vaccinales. De plus, les différentes interactions existantes entre les *Wolbachia* et leurs hôtes du genre *Cimex* méritent une attention particulière. Des recherches approfondies sont nécessaires afin d'explorer cette piste, qui peut être un bon moyen de contrôle de ces arthropodes, réputés pour être des nuisibles rustiques.

Dans le deuxième chapitre de ma thèse, j'ai d'abord proposé **des outils innovants pour le diagnostic moléculaire des filarioses canines**. L'utilisation de la technologie multiplexe

TaqMan, et la détection des génotypes de *Wolbachia* ont amélioré la qualité du diagnostic des filarioses, et révélé la présence d'infections occultes par biologie moléculaire, ce qui représente une nouveauté. Nous avons ainsi démontré l'utilité des outils développés, pour le diagnostic, la surveillance épidémiologique et l'exploration de la diversité de cette famille de parasites cosmopolites. Un autre aspect intéressant de nos outils, est lié au fait qu'ils utilisent les *Wolbachia* comme cible diagnostic. On rend ainsi possible la détection précoce de l'infection filarienne et on évalue mieux l'efficacité des traitements préventifs. Le diagnostic de ces infections est donc amélioré chez les culs-de-sac épidémiologiques où l'ADN parasitaire n'est pas détectable dans le sang, tel que pour la dirofilariose cardiopulmonaire à *D. immitis* chez le chat et la dirofilariose pulmonaire de l'homme. En médecine humaine, cela permettra d'améliorer la conduite à tenir face à des nodules pulmonaires qui peuvent être confondus avec des métastases conduisant à des thoracotomies. D'autres recherches sont également nécessaires pour comprendre la cinétique des *Wolbachia* dans le sang des sujets infectés, ce qui, probablement, permettra d'éviter les contraintes liées au cycle circadien (microfilarémie nocturne) des filarioses humaines, et donc faciliter le dépistage de ces maladies et accélérer leur éradication.

Par ailleurs, l'identification, la connaissance et la bonne compréhension du cycle de vie des parasites à transmission vectorielle est la clef de voûte de la lutte contre ces agents pathogènes. Les outils du diagnostic moléculaire que j'ai proposés, sont bien adaptés à l'exploration de la présence de parasites, à la fois, chez l'hôte définitif (vertébré) et l'hôte intermédiaire (arthropode). On a ainsi mené à terme l'identification de la filaire *Cercopithifilaria* sp. II, dont l'adulte n'a toujours pas été décrit, tout comme le vecteur inconnu du parasite zoonotique *Onchocerca lupi*. Ainsi, nous avons élargi les connaissances sur les nématodes à transmission vectorielle des PNHs du Nouveau et de l'Ancien Monde. Nous avons fourni, à la fois, des données moléculaires et morphologiques sur des nématodes

gastrointestinaux des PNHs de l'Afrique. Particulièrement, *Abbreviata caucasica*, parasite gastrointestinal détecté chez environ 52% du groupe étudié de chimpanzés du Sénégal. Il y avait, jusqu'à présent, plusieurs lacunes dans les connaissances à propos de ce pathogène, telles que sa description morphologique formelle, son cycle de vie et sa pathogénie. Mes travaux répondent, partiellement, à ces questions. Ils fournissent de nouvelles données morphologiques et moléculaires facilitant l'identification parasitaire. De plus, nous avons tenté de décrire, à l'aide d'un nouvel outil moléculaire (qPCR spécifique), le cycle de vie d'*Abbreviata caucasica* en suspectant les termites comme étant un hôte intermédiaire. Cependant, cette investigation n'a pas fourni suffisamment d'informations du fait que le rôle des termites n'a pas été confirmé. D'autres études, prenant en compte l'effet saison, et le reste des insectes consommés par les chimpanzés, pourront, probablement, aider à la compréhension du cycle de vie de ce parasite. Par ailleurs, nous avons, également, fourni des données préliminaires sur des filaires, non identifiées auparavant, chez les singes hurleurs de l'Amazonie. La caractérisation moléculaire nous a permis d'identifier à deux reprises une filaire du genre *Brugia*, potentiellement zoonotique chez les singes hurleurs et les chiens de Guyane. En outre, nous avons proposé un système de détection moléculaire (qPCR) spécifique au genre *Brugia*, afin de mieux diagnostiquer, surveiller et comprendre le cycle de vie de ce parasite.

Mon travail a aussi porté sur des solutions innovantes dans la lutte contre les maladies à transmission vectorielle du chien. Nous avons démontré la pertinence d'une **stratégie prophylactique mensuelle multimodale** combinant une prévention contre le vecteur et l'agent infectieux lui-même pour le contrôle des principales maladies canines zoonotiques à transmission vectorielle sévissant dans le Bassin méditerranéen, à savoir, les dirofilarioses, la leishmaniose et l'ehrlichiose. La stratégie consiste en l'utilisation, tout au long de l'année, de deux produits (Vectra® 3D associé au Milbactor®) qui a démontré son efficacité contre les maladies transmises par les moustiques, les phlébotomes et les tiques en Corse. En effet, cette

stratégie a été initialement conçue pour protéger contre les dirofilarioses, mais l'utilisation annuelle sans interruption a permis d'obtenir une protection satisfaisante contre les autres maladies vectorielles. Il est donc nécessaire d'appliquer **une prévention annuelle** dans le Bassin méditerranéen afin d'arrêter la propagation de maladies canines à transmission vectorielle et de lutter contre l'extension de zoonoses. D'un autre côté, la stratégie multimodale nécessite certainement un renforcement par d'autres produits préventifs spécifiques à d'autres maladies, par exemple l'association au vaccin anti-leishmanien, d'où la nécessité de mener d'autres essais de ce genre. Parallèlement, nous avons démontré pour la première fois, l'effet d'une protection secondaire qu'on qualifie d'indirecte du Vectra® 3D contre les piqures de moustique, ce qui est également une preuve d'une protection de l'homme suite au traitement des chiens. De plus, le coût abordable des produits préventifs en le comparant à celui des traitements, pourra convaincre d'avantage les propriétaires et les vétérinaires d'élargir la couverture préventive, et, par conséquent, de réduire l'incidence et la propagation des maladies au sein de la population canine.

Toujours dans le cadre de la lutte contre les maladies vectorielles du chien, et afin d'apporter une amélioration de la situation actuelle, nous avons proposé une alternative thérapeutique pour la leishmaniose canine avec un traitement à base d'artésunate. Ce traitement semble être à la fois efficace et abordable en termes de coût, de facilité d'administration et sans effet secondaire, faisant de lui un bon candidat en tant qu'alternative thérapeutique pour la leishmaniose canine.

Il est connu que l'expansion des vecteurs et l'émergence du phénomène de résistance aux produits insecticides ont conduit à l'émergence (ou à la réémergence) de maladies vectorielles, dont la dengue, le chikungunya, le paludisme et les filarioses humaines en Afrique. L'emploi de nouvelles substances insecticides devient alors une nécessité urgente. Dans notre travail, nous avons démontré et caractérisé, partiellement, à partir de la souche bactérienne

Serratia marcescens P400, **une amine** ayant toutes les qualités (solubilité et effet insecticide satisfaisant) avantageuses pour être utilisée dans la lutte antivectorielle. De plus, nous avons participé à la publication de la séquence complète du génome, utilisable pour de futures caractérisations complètes permettant de produire l'amine en question.

D'un autre côté, notre étude exploratoire de l'ADN des **guêpes parasitoïdes** chez des tiques naturellement infectées, représente également une nouvelle piste pour la lutte biologique contre ces vecteurs. Pour autant, des modèles d'infestation expérimentale des tiques par ces entomophages ainsi que la mise en œuvre d'une technique d'élevage des guêpes sont nécessaires pour pouvoir développer cette approche de la **lutte biologique** d'écosystème.

J'ai aussi démontré le rôle joué par **les animaux sentinelles** de la transmission et de la propagation des maladies vectorielles et de l'utilité de la surveillance en amont de l'infection de l'homme. On a rapporté des données originales sur les maladies vectorielles d'origine animale en Guyane, en France métropolitaine, en Algérie tout comme en Côte d'Ivoire. Dans ces zones, on a montré le rôle de sentinelle par excellence joué par l'hôte canidé (particulièrement le chien domestique) dans le maintien et la propagation de maladies vectorielles zoonotiques, à savoir les dirofilarioses, les leishmanioses, la coxiellose et la trypanosomose. On a mis en évidence, en Italie, le rôle joué par des reptiles en tant que sentinelles pour **les rickettsioses**. En République du Congo, les gorilles constituent un réservoir notable de **parasites gastro-intestinaux** (*Necator americanus*, *Ascaris lumbricoides*, *Strongyloides stercoralis* et *Giardia lamblia*), on a montré un lien existant avec les parasitoses humaines. Ces données préliminaires doivent attirer l'attention des autorités sanitaires concernées, pour conduire à la mise en œuvre d'une collaboration entre les services de santé publique et vétérinaires afin de mieux contrôler les infections **en amont de l'homme**. L'histoire naturelle de la pandémie actuelle (Covid-19) est très emblématique de ce besoin de coopération.

Corbin et Strauss ont dit : « *The researcher begins with an area of study and allows the theory to emerge from the data* » (Corbin et Strauss 2009). En se basant sur cette notion, ma thèse montre qu'il reste plusieurs insuffisances pour contrôler les maladies à transmission vectorielle. Elle vise, modestement, à suppléer au manque de mise en œuvre de techniques performantes et donc de connaissances dans la perspective d'un paradigme complet d'étude des phénomènes infectieux dans leur écosystème.

Annexes

Préambule

Lors de la survenue d'une crise sanitaire due à une maladie infectieuse, vectorielle ou non, les taux de morbidité peuvent s'accroître considérablement. La pandémie actuelle due au SARS-CoV-2 en est un bon exemple. Un foyer épidémique peut survenir lorsque plusieurs aspects d'un écosystème (agent pathogène, populations hôtes animales et humaines, environnement) créent une situation idéale de propagation. La surpopulation, la mauvaise conception urbanistique, l'hygiène défectueuse en raison de la pauvreté, l'eau non-potable, les changements climatiques rapides et les catastrophes naturelles, peuvent conduire à des conditions qui facilitent la transmission de ces maladies. Une fois la maladie établie, l'urgence se déclare et les autorités mettent en œuvre une réponse rapide et complète avec la mobilisation de personnel qualifié afin de pouvoir contrôler la maladie. Cependant, et comme nous l'avons montré dans le chapitre précèdent pour des maladies vectorielles, les animaux peuvent être utilisés comme des sentinelles pour agir en amont des épidémies, et par conséquent, pour prévenir la survenue d'une crise sanitaire.

Au cours de ma période de doctorat, j'ai été amené à travailler à d'autres projets liés aux maladies infectieuses chez les animaux dans le cadre de la surveillance épidémiologique de leur rôle de sentinelle. Les travaux auxquels j'ai participé sont les suivants :

I. Dépistages d'agents infectieux d'origine bactérienne dans les fèces de primates non-humains d'Afrique (**Annexe 1**) ;
II. Isolement et identification de l'étiologie d'une pneumonie grave chez un rat des rues (*Rattus norvegicus*) à Marseille (**Annexe 2**) ;
III. Etude d'un cas humain de leptospirose avec identification de la source de l'infection chez des rats (**Annexe 3**).

Annexe 1

African Humans and Nonhuman Primates Bacterial Infections: Expending the Knowledge

Hacène Medkour[1,2,3,†], Inestin Amona[1,4,5,†], Jean Akiana[6], **Younes Laidoudi**[1,2,3], Bernard Davoust[1,2], Idir Bitam[4,7], Ismail Lafri[4,8,9], Anthony Levasseur[1,2], Georges Diatta[4,10], Cheikh Sokhna[1,4,10], R. Adriana Hernandez-Aguilar[11,12], Amanda Barciela[12], Slim Gorsane[13], Henri Banga-Mboko[14], Didier Raoult[1,2], Florence Fenollar[1,4]and Oleg Mediannikov[1,2,*]

[1]IHU Méditerranée Infection,13385, Marseille CEDEX 05, France
[2]Aix Marseille Univ, IRD, AP-HM, Microbes, MEPHI, 13385, Marseille CEDEX 05, France
[3]PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, 25100 El Khroub, Algeria
[4]Aix-Marseille Univ, IRD, AP-HM, SSA, VITROME, 13385, Marseille CEDEX 05, France
[5]Faculté des Sciences et Techniques, Université Marien NGOUABI, Brazzaville, Republic of the Congo
[6]Laboratoire national de santé publique, Brazzaville, Republic of the Congo
[7]Superior School of Food Sciences and Food Industries, 16004, Algiers, Algeria
[8]Institute of Veterinary Sciences, University of Blida 1, Algeria
[9]Laboratory of Biotechnology related to Animal Reproduction (LBRA), University of Blida 1, Blida, Algeria
[10]VITROME IRD 198, Campus IRD/UCAD, Hann Les Maristes, Dakar, Senegal
[11]Department of Social Psychology and Quantitative Psychology, Faculty of Psychology, University of Barcelona, Passeig de la Valld'Hebron 171, 08035, Spain
[12]Jane Goodall Institute Spain and Senegal, Dindefelo Biological Station, Dindefelo, Kedougou, Senegal
[13]Direction interarmées du Service de santé des armées des Forces françaises stationnées à Djibouti
[14]Ecole Nationale d'Agronomie et de Foresterie, Université Marien Ngouabi, Brazzaville, Republic of the Congo
†Those authors contributed equally to this work.

ARTICLE DE RECHERCHE

Soumis à : Yale Journal of Biology and Medecine

Yale J Biol Med

Research Article

Title: African Humans and Nonhuman Primates Bacterial Infections: Expending the Knowledge

Running title: Bacterial Infections of African Primates

Hacène Medkour [1,2,3,†], Inestin Amona [1,4,5,†], Younes Laidoudi [1,2,3], Jean Akiana [6], Bernard Davoust [1,2], Idir Bitam [4,7], Ismail Lafri [4,8,9], Anthony Levasseur [1,2], Georges Diatta [4,10], Cheikh Sokhna [1,4,10], R. Adriana Hernandez-Aguilar [11,12], Amanda Barciela [12], Slim Gorsane [13], Henri Banga-Mboko[14], Didier Raoult [1,2], Florence Fenollar [1,4]and Oleg Mediannikov [1,2,*]

[1] IHU Méditerranée Infection,13385, Marseille CEDEX 05, France; hacenevet1990@yahoo.fr (H.M.); amoninestin@gmail.com (I.A.); younes.laidoudi@yahoo.com (Y.L); bernard.davoust@gmail.com (B.D.); anthony.levasseur@univ-amu.fr (A.L.); cheikh.sokhna@ird.fr (C.S.); didier.raoult@gmail.com (D.R.); florence.fenollar@univ-amu.fr (F.F.)

[2] Aix Marseille Univ, IRD, AP-HM, Microbes, MEPHI, 13385, Marseille CEDEX 05, France

[3] PADESCA Laboratory, Veterinary Science Institute, University Constantine 1, 25100 El Khroub, Algeria

[4] Aix-Marseille Univ, IRD, AP-HM, SSA, VITROME, 13385, Marseille CEDEX 05, France; idirbitam@gmail.com (I.B.); lafrismail@gmail.com (L.I.); georges.diatta@ird.fr (G.D.)

[5] Faculté des Sciences et Techniques, Université Marien NGOUABI, Brazzaville, Republic of the Congo

[6] Laboratoire national de santé publique, Brazzaville, Republic of the Congo; jakiana2000@yahoo.fr (J.A.)

[7] Superior School of Food Sciences and Food Industries, 16004, Algiers, Algeria

[8] Institute of Veterinary Sciences, University of Blida 1, Algeria

[9] Laboratory of Biotechnology related to Animal Reproduction (LBRA), University of Blida 1, Blida, Algeria

[10] VITROME IRD 198, Campus IRD/UCAD, Hann Les Maristes, Dakar, Senegal

[11] Department of Social Psychology and Quantitative Psychology, Faculty of Psychology, University of Barcelona, Passeig de la Valld'Hebron 171, 08035, Spain; r.a.hernandez-aguilar@ibv.uio.no (R.A.H-A.)

[12] Jane Goodall Institute Spain and Senegal, Dindefelo Biological Station, Dindefelo, Kedougou, Senegal; amanda.b@janegoodall.es (A.B.)

[13] Direction interarmées du Service de santé des armées des Forces françaises stationnées à Djibouti; slim.gorsane@intradef.gouv.fr (S.G.)

[14] Ecole Nationale d'Agronomie et de Foresterie, Université Marien Ngouabi, Brazzaville, Republic of the Congo ; hbangamboko@gmail.com (H.B.M.)

* **Corresponding author:** Dr Oleg Mediannikov; IHU Méditerranée Infection, 19-21, Bd Jean Moulin, 13385 Marseille Cedex 05, France ; mail: olegusss1@gmail.com

† **Those authors contributed equally to this work.**

Keywords: Nonhuman primates; humans; infectious diseases; bacteria; Africa; surveillance; emergence.

List of abbreviations

Total of words: 4173

Number of tables, figures and references: 1, 6 and 37

Acknowledgments

We are grateful for all participants in the present study. We thank Rahal Mohamed and Fayçal Zaroual for their valuable help. We thank eco-guards and conservators of Lésio-Louna NP, Odzala- Kokoua NP and Nouabale-Ndoki NP, Agence Congolaise de la Faune et des Aires Protégées (Republic of Congo), Ministry of Health and Population (the Republic of Congo) for their valuable assistance and National Park of Chréa, Blida, Algeria. We thank Paula Alvarez Varona, Daouda Diallo, Mamadou F. Diallo y Mamadou Samba Sylla for help in the field in the Dindéfelo Community Natural Reserve of Senegal.

Author contributions: H.M., I.A., B.D., D.R. and O.M. designed the study. H.M., I.A., B.D., L.Y., J.A., I.B., I.L., A.L., G.D., C.S., S.G., D.R. and O.M. collected the samples. H.M., I.A. performed the lab work. H.M., I.A., F.F. and O.M. carried out the data analysis. H.M. and I.A. drafted the manuscript. H.M., I.A., J.A., B.D., I.B., G.D., R.A.H.-A., A.B., F.F., H.B.M., D.R. and O.M. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Abstract

Close phylogenetic relationship between humans and other primates creates exceptionally high potential for pathogen exchange. The surveillance of pathogens in primates plays an important role in anticipating possible outbreaks. In this study, we conducted a molecular investigation of pathogenic bacteria in feces from African nonhuman primates (NHPs). We also investigated the pathogens shared by the human population and gorillas living in the same territory in the Republic of Congo. In total, 93% of NHPs (n= 176) and 95% (n=38) of humans were found to carry at least one bacterium. Non-*pallidum Treponema* spp. (including *T. succinifaciens*, *T. berlinense* and several potential new species) were recovered from stools of 70% of great apes, 88% monkeys and 79% of humans. Non-*tuberculosis Mycobacterium* spp. were also common in almost all NHP species as well as in humans. In addition, *Acinetobacter* spp., members of the primate gut microbiota, were mainly prevalent in humans and gorillas. Pathogenic *Leptospira* spp. were highly present in humans (82%) and gorillas (66%) stool samples in Congo, but were absent in the other NHPs, therefore suggesting a possible gorillas- humans exchange. Particular attention will be necessary for enteropathogenic bacteria detected in humans such as *Helicobacter pylori*, *Salmonella* spp. (including *S. typhi/ paratyphi*), *Staphyloccocus aureus* and *Tropheryma whipplei*, some of which were also present in gorillas in the same territory (*S. aureus* and *T. whipplei*). This study enhances our knowledge of pathogenic bacteria that threatens African NHPs and humans by using a non-invasive sampling technique. Contact between humans and NHPs results in an exchange of pathogens. Ongoing surveillance, prevention and treatment strategies alone will limit the spread of these infectious agents.

Introduction

More than 60% of known infectious diseases have a zoonotic origin, the majority being caused by pathogens of wild origin [1]. The close phylogenetic relationship between humans and other primates creates high potential for pathogens exchange [2]. As a result, diseases emerge in humans as an unintended consequence of the hunting and butchering of the African great apes and other contacts, responsible for human Ebola outbreaks and the global AIDS pandemic [3][4], as well as the high mortality observed in wild chimpanzee populations (*Pan troglodytes*) associated with the anthropozoonotic transmission of human respiratory viruses [5].

In the tropics, in many rural areas, human population growth and changes in land use are leading to a growing overlap between humans and wild primates [6]. This is mainly the result of large-scale activities, such as extractive industries (i.e., logging, mining); as well as small-scale interfaces, like subsistence use of natural resources, ecotourism and research. These changes increase contact between people and non-human primates (NHPs) and often more intimate contact with wild primates [7][8][9]. By contrast, the increasing fragmentation of habitats is forcing primates to seek resources more widely, including through the active use of human-dominated systems (e.g. crop raiding in agricultural fields and urban occupation) [10][11] . All these scenarios are likely to increase the risk of transmission of zoonosis [12][11] . Some NHPs that persist in anthropogenically modified landscapes, such as monkeys, which are susceptible to many of the same pathogens as humans. As a result, these resilient species have the capacity to act as sentinels for ecosystem health and provide early warning of potential risks to human health [1].

Several bacteria have been reported to be transmitted by direct as well as indirect contacts from NHPs to humans [9]. Further, it had been demonstrated that proximity between wild primates and people can promote transmission of the common gastrointestinal bacterium *Escherichia coli*, as well as other pathogenic microorganisms, such as *Cryptosporidium* [7] and *Shigella*. Other studies stressed that direct contact between species is not mandatory for interspecific disease transmission (i.e., *Shigella*, *Salmonella*, *E. coli*, etc.) [9]. Demonstration of human pathogens negatively impacting wild primates has sparked considerable debate regarding the costs and benefits to endangered primate populations of scientific research, ecotourism and current conservation and management paradigms. Despite the disease-related risks, the consensus is that both research and tourism have contributed in overwhelmingly

positive ways to primate conservation, enhancing their long-term survival by increasing their scientific and economic value. Nevertheless, such activities as well as overlap of humans and NHPs may have unintended consequences for the health and survival of wild primate populations.

The central hypothesis of this work is that key NHPs could carry pathogen agents for both humans and animals. In addition, human behaviors, wildlife behaviors, ecological conditions and landscape features increase the risks of interspecific disease transmission.

Thus, given the deadly epidemics and pandemics that have already occurred related to NHPs (HIV, malaria, Ebola, etc.), it is of utmost importance to study microorganisms common to both NHPs and humans. It is difficult to carry out such studies because, in most cases, we cannot capture them and collect the necessary samples. New approach is possible, using stool samples it is possible to find, not only enteric pathogens, but also blood and urinary pathogens (like filaria, Plasmodia and *Leptospira*). Its collection is absolutely non-invasive. Here, we performed an extensive epidemiological survey for bacteria on NHP feces, using PCR systems known for their specificities and sensitives. Moreover, in a one health context in Congo, we studied a human population sharing the same living area as gorillas in order to study the transmission of pathogens between species.

Material and Methods

Ethical Statement, Animals and Study Area

In Senegal, in August 2016, 48 western chimpanzees (*Pan troglodytes verus*) feces were collected. The study was approved by the Senegalese Ministry of the Environment (Direction of the National Parks, No. 1302, 16 October, 2015). The Direction des Eaux, Forêts, Chasses et Conservation des Sols of the Republic of Senegal gave authorization to collect and export feces samples (No. 1914/DEF/DGF of 5 June 2016) in Collaboration with Jane Goodall Foundation (https://www.janegoodall.org/). Between 2017 and 2019, in the Republic of Congo, 38 gorilla (*Gorilla gorilla*) and 38 human feces were collected as part of a collaborative project carried by the Government of the Republic of Congo and the Aspinall Foundation, which manages a protected area of 170,000 ha located about 140 km north of Brazzaville. In addition, the project was authorized by the Ministry of Health (No 208/MSP/CAB.15 of 20 August 2015) and the Forest Economy and Sustainable Development (No 94/MEFDD/CAB/DGACFAP-DTS of 24 August 2015) of the Republic of Congo. In the Republic of Djibouti, 6 fecal samples of hamadryas baboons (*Papio hamadryas*) were collected in 2017. This collection was carried out in partnership with the Center for Studies and Research of Djibouti. Finally, in Algeria, fecal samples were collected from 69 Barbary macaques (*Macaca sylvanus*), with the authorization of the management of the Chréa National Park (CNP). These primates were synanthropic and lived in close contact with the people who provided them with food **(Figure 1).**

The sampling was non-invasive and did not disturb any wild animal. For NHPs, feces were collected at sleeping sites, feeding sites and places where the primates had been observed. Human stool samples were collected after obtaining the verbal consent of all the participants because of their low level of literacy.

All the humans in our study were apparently healthy. In addition, the fact that the NHP stools were not diarrheal may indicate that they were also in relatively good health. All collected samples were transported to the IHU Méditérranée Infection Laboratory, 13005 Marseille, France, for analysis. They had been identified and stored at either −20°C or −80°C.

DNA Extraction

Initially, 40 mg of stool were mixed with 360µL of G2 lysis buffer from EZ1®DNA Tissue Kit (Qiagen, Hiden, Germany). This was mechanically lysed with tungsten beads (Qiagen, Hiden, Germany) using FastPrep-24TM 5G Grinder for 40s. After 10 min of incubation at 100 °C to allow for complete lysis, tubes were centrifuged at 10,000g for 1 min. Subsequently, 200 µL of supernatant was enzymatically digested using 20 µL of proteinase K (20 mg/mL, Qiagen) and incubated overnight at 56 °C. DNA was extracted from 200µL of sample using the EZ1®DNA Tissue Kit on BIOROBOT EZ1 (Qiagen, Hiden, Germany), according to the manufacturer's instructions. Elution was performed in 200µL volume, then aliquoted in individual tubes of pure extracted DNA, dilutions to 1: 10 and to 1: 100.

The extraction quality and the absence of PCR inhibitors were controlled using the universal eubacterial qPCR targeting the 16S rRNA bacterial genes [14] on pure DNA, dilutions to1: 10 and to 1: 100. By comparison of the Ct values obtained, the dilution to 1: 10 was chosen for the analysis. DNA tubes were stored at -20°C until use.

Molecular screening for bacteria by Real-time PCR assays (qPCR)

The approach consists on screening for bacteria that may be pathogenic for humans and NHPs. We used single target real-time genus or species-specific qPCR assays. We targeted pathogens of medical interest, previously reported in NHPs. At least 12 qPCR assays targeting bacterial genera and 16 species-specific qPCRs, all known for their specificity and sensitivity, were used in multiparallel assays, as shown in **Table S1**. For *Rickettsia* spp., a new qPCR was designed and validated targeting the 16S rRNA gene. qPCR was tested for its specificity using several laboratory-maintained colonies as well as DNA from arthropods, humans, monkeys, donkeys, horses, cattle, mice and dogs as described previously [15].

Assays were carried out in 20 µl final volume containing 10µl of Master Mix Roche (Eurogentec), 0.5µl each primer per reaction at the concentration of 20µM, 0.5 µl UDG, 0.5µl of each probe at the concentration of 5µM and 5µl of the DNA template. The qPCR amplifications were performed in a CFX96 Real-Time system (Biorad Laboratories, Foster City, CA, USA). The thermal conditions included two hold steps at 50°C for 2 minutes, followed by 95°C for 15 minutes and 40 cycles of two steps each (95° C for 30 s and 60° C for 30 s). Each PCR plate contains 96 wells. Known microorganisms' DNAs or plasmids were used as positive controls and master mixtures as a negative control in each reaction.

Genetic amplification by standard PCR, sequencing and phylogeny

In addition to species-specific qPCR screening, we continued identification by PCR/ sequencing for *Treponema* spp., *Acinetobacter* spp., *Rickettsia* spp., *Mycobacterium* spp., and *Leptospira* spp. Positive samples in qPCR assays were subjected to standard PCRs targeting genes and using primer pairs summarized in **Table S1**.

Genetic amplifications were carried out in 50 µl volume consisting of 5µl of DNA template, 25µl of AmpliTaq Gold master mix, 18 µl of ultra-purified water DNAse-RNAse free and 1 µl of primers at 20 µM of concentration. This was performed in a thermocycler (Applied Biosystem, Paris, France). Protocols of amplification were as follows: incubation step for 15 minutes at 95°C, 40 cycles (one minute at 95°C, 30s at the annealing temperature, an elongation step at 72°C) and a final extension step for five minutes at 72°C. Amplicons were visualized on 2% agarose gel and were then purified using NucleoFast 96 PCR plates (Macherey Nagel EURL, Hoerdt, France) as per the manufacturer's instructions. Amplicons were sequenced using the Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an ABI automated sequencer (Applied Biosystems). Generated electropherograms were assembled and edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia) and compared with those available in the GenBank database by NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The fragments obtained were compared with each other and with the related fragments available in the GenBank database. The phylogenetic analyses were inferred using neighbor joining methods and tree reconstructions were performed using MEGA software version 7 (https://www.megasoftware.net/). Bootstrap analyses were conducted using 1000 replicates.

Results

In this study, we were able to collect NHP stool samples from different African counties, including 59 samples from Senegal, 6 from Djibouti and 69 from Algeria. In addition, in Congo, we collected 38 samples from humans and 38 from gorillas shearing the same territory. In total, 93% (160/172) of NHPs tested positive for at least one (min: 1; max: 6) of the bacterial species tested in their feces while 94.7% (36/38) of humans were positive for at least one (min: 1; max: 7) bacterium. Prevalence in NHPs and humans are summarized in the **(Figure 2; Table 1).**

Positive rates for all of *Treponema* (non-*pallidum*) (79.1%), *Mycobacterium* (non-*tuberculosis*) (38.4%), pathogenic *Leptospira* spp. (26.7%) and *Acinetobacter* spp. (9.9%) were high in NHPs as well as in humans, 78.9%,31.6%, 81.6% and 73.7% in humans respectively. Bacteria such as *Rickettsia* (4.7%), *Mycoplasma* and (2.3%) and *Wolbachia* spp. (1.7%) were found only in NHPs. By contrast, *H. pylori* (23.7%), *S. aureus* (10.5%) and *T. whipplei* (10.5%) were more prevalent in humans than in NHPs, for which the prevalence rates were 1.7%, 0.6%, 0.6% respectively. *Salmonella* spp. was detected only in humans, 10.5% (4/38), including two samples (5.2%) positive for *S. typhi/paratyphi.*

No significant differences were observed between great apes (n=86) and monkeys (n=86) for pathogens (great apes %, monkeys %; Z test p-value) such as *Leptospira* spp. (30.2%, 23.3%; 0.388), *Rickettsia* spp. (8.1%, 1.2%; 0.066), *Wolbachia* spp. (3.5%, 0.0%; 0.240), *Mycoplasma* spp. (3.5%, 1.2%; 0.612), *S. aureus* (3.5%, 0.0%; 0.240), *H. pylori* (1.2%, 0.0%; 1.000), *T. whipplei* (1.2%, 0.0%; 1.000). By contrast, significant differences were observed for *Treponema* spp. (69.8%, 88.4%; 0.004), *Mycobacterium* spp. (67.4%, 9.3%; <0.0001) and *Acinetobacter* spp. (16.3%, 3.5%; 0.009). In the Republic of the Congo, there were no differences in bacteria carriage between gorillas (n=38) and humans (n=38) who cohabited the same area as gorillas (gorillas %, humans%; p-value) for *Leptospira* spp. (65.8%, 81.6%; 0.186), *Mycobacterium* spp. (52.6%, 31.6%; 0.096), *Rickettsia* spp. (13.2%, 0.0%; 0.055), *Wolbachia* spp. and *Mycoplasma* spp. (7.9%, 0.0%; 0.229), *S. aureus* (7.9%, 10.5; 1.000), *Salmonella* spp. (0.0%, 10.5%; 1.113) and *T. whipplei* (2.6%, 10.5%; 0.349). Significant differences were observed between gorillas and humans for *Treponema* spp. (100.0%, 78.9%; 0.005), *Acinetobacter* spp. (34.2%, 73.7%;0.010), *H. pylori* (2.6%, 23.7%; 0.012).

All the other bacteria listed in the **Table S1** had not been recovered from both humans and NHPs.

Furthermore, we performed PCR/sequencing for positive samples in qPCR for all of *Mycobacterium* spp., *Leptospira* spp. (pathogenic), *Acinetobacter* spp., *Treponema* spp. and *Rickettsia* spp. Sequencing did not succeed for *Mycobacterium* and *Leptospira* spp. despite specific bands were obtained in standard PCR, and superposed peaks were obtained for the same nucleotide which made it impossible to conclude about sequences. Two good quality sequences of 700 bp length of *gltA* gene of *Rickettsia* were obtained, one on an Algerian macaque and the other on a gorilla from Congo. The sequences were identical in each other and showed 99% identical to *R. africae* isolate QtHyaegR9 (MN306555) detected on a spur-thighed tortoise (*Testudo graeca*) sold in a Qatar live animal market and isolate EgyRickHm-Raas detected on adult, male *Hyalomma marginatum* (camel tick) in Egypt (KX819298) (**Figure 3**).

We obtained 24 sequences of 322-365 bp length for *rpoB* gene of *Acinetobacter* spp. including 3 sequences obtained on macaques, 5 others obtained on gorillas and 16 on humans. One sequence obtained on gorilla feces (G9) showed 98% identity with *A. baumannii* strain MS14413 (CP054302). Two human genotypes (Mbo033, Mbo064, Ibou02) and one gorilla isolate (G06B) were almost identical and closed together with *A. berezinae* strain YMC79 (JF302886) to which they highlighted 95%-99.5% of identity. Two other human isolates (Mbo047 and Mbo057) were closely similar and exhibited 92% and 93% similarity with *A. berezinae.* They constitute a potential new species. Three other sequences obtained on humans, potential new species, showed one of them (Mbo056) 88% similarity to *A. berezinae,* while the two others were 86% (Mbo058) and 93% (Mbo054) identical to *A. genomosp.* (EU477133 and KT997528). Sequence Mbo003 from a human constituted a separate branch (**Figure 4**) and showed 92% similarity with *A. tandoii* strain LUH 13385 (KU961639). In addition, two identical isolates (Mbo040, Mbo054) from human stools performed a separate branch and showed 91% identity to *A. bohemicus* strain ANC4315 (KJ124827). Another sequence (Mbo028) showed quasi-identity (98.5%) with *Acinetobacter sp*. strain WCHAc060041 (MH190065) found in waste water in China, it also showed 90% similarity with the official *A. defluvii* strain WCHA30 (KY435935). Mbo036 and Ibou001, almost similar two genotypes from humans, were close (93% of identity) to the species *A. venetianus*. Two others, one (Mbo062) showed 91% similarity to *A. nosocomialis* (KX444511), the other one (Mbo001) was 93.5% identical to *A. seifertii* (KJ956464). Three sequences from gorilla

feces and one other from macaque constituted a separate clade (G05, G05A, G06 and CC37). They were almost similar and were 92- 93% identical to *A. wuhouensis* strain WCHAc060049 (MK518338). Finally, two sequences from macaques (GC05 and CC10) closed with *Acinetobacter* sp. 'isolate 30Bi' (FJ157977) was detected in a dog **(Figure 4; Table S2)**.

We succeeded in obtaining at least 52 sequences of 760-870 bp of *Treponema* spp. 23S gene on NHP feces including 6 from chimpanzees (accession numbers: MT257111-MT257117), 13 sequences from gorillas (MT257084, MT257085, MT257088-MT257098), one from a green monkey (MT257101), 5 from Guinea baboons (MT257102-MT257106) and 4 from hamadryas (MT257107-MT257110) and 23 others from Barbary macaque samples (MT257228- MT257250) **(Figure 5)**. In addition, 4 good quality sequences were obtained from feces of humans from the Mbomo locality (MT257099, MT257100, MT257086, MT257087) **(Figure 6).** None of the identified *Treponema* species have been reported as clearly pathogenic. A specific DNA search for *T. pallidum* spp. gave negative results.

In human samples, we obtained two sequences (MT257099, MT257086) that were perfectly identical to *T. succinifaciens* DSM 2489 (CP002631). The two others seem to be new species, one of them (MT257100) was 83% identical to *T. succinifaciens* DSM 2489, while the second (MT257087) displayed 87% similarity with *T. pedis* str. T A4 (CP004120). Three gorilla genotypes, 4 genotypes from hamadryas, 3 others from baboons, one from green monkey and one from Barbary macaque were almost similar and closed to *T. succinifaciens* official strains to which they highlighted >99% of similarity. Three other similar gorilla genotypes constituted a separate cluster, they had 83.5% resemblance with *T. pectinovorum* strain Marseille-1-CSURP6641 (UOUI01000011) and 82.5% with *T. brennaborense* strain DD5/3 (NR076878). One other gorilla (MT257091) sequence split off separately, it was 81.5% identical to *T. brennaborense*. Three sequences from gorilla feces, 2 from baboons and 16 from macaques were almost similar and exhibited most similarity 97-99.5% to *T. berlinense* strain ATCC BAA-909 (FUXC01000026) recovered from Swine feces in Berlin. A distinct isolate from gorilla (MT257084) shared 86% of similitude with *T. berlinense.* Four chimpanzee sequences, grouped separately as one species, showed 93.5% identity with *T. berlinense* strain ATCC BAA-909. Two other chimpanzee sequences (MT257115, MT257116) were nearly similar to each other and showed 88-89% similarity to *T. berlinense* strain ATCC BAA-909, for which another gorilla isolate (MT257093) showed 84.5% identity and a more similar one with 95% similarity (MT257095).

Discussion

This study identified pathogenic bacteria in human feces and NHPs from Africa. The technic used, qPCR/ sequencing in feces, presented no ethical requirements. PCR was used in the diagnostic of gastrointestinal infections [16][17]. Using large specificity qPCR, we detected pathogenic bacteria, such as *Leptospira* spp. and nonpathogenic bacteria, such as *Treponema* spp., including several potential new species.

We found many *Treponema* spp. nonpathogenic despite the existence of the syphilis in NHPs. Most of the *Treponema* spp. detected (non-pathogenic) such as *T. succinifasciens* are part of the normal flora of primates, and all species in this study lose treponemes in their feces. The presence of Spirochaetes has been reported in the gut microbiota of NHPs [18]. High prevalence rates were observed in all primate species of *Treponema* spp. (not *pallidum*), *T. succinifasciens*, *T. berlinense* and at least six potential new species. *T. succinifasciens* and *T. berlinense* were highly prevalent species in the microbial genomes from NHPs gut metagenome [19]. It had been reported that traditional rural individuals were enriched with Spirochaetes, especially *Treponema. T. succinifasciens* and *T. berlinense* being the most prevalent species identified. This may be due to the rare use of antibiotics [20]. Special attention should be drawn to endemic *Treponema pallidum* infection with genital stigmata in NHPs from Guinea, Senegal and Tanzania. Many NHPs in Africa were found to suffer from treponematoses [21][22][23][24]. During fieldwork in Senegal, an epizootic of venereal disease was directly observed in green monkeys *(Chlorocebus sabaeus)* due to infection with *T. pallidum* subsp. *pertenue*, then an epizootic was observed in Senegal which spread to baboons one year later [25].

We agree with the conclusion drawn by Manna et al. 2019, the overlap (20% of microbial candidate species in NHPs also found in the human microbiome) occurs mainly between NHPs and non-Westernized human populations and NHPs living in captivity, suggesting that host lifestyle plays a significant role comparable to host speciation in shaping the primate intestinal microbiome. Several NHP-specific species are phylogenetically related to human-associated microbes, such as *Treponema*, and could be the consequence of host-dependent evolutionary trajectories. Gut *Treponema* have been found in NHPs and all traditional peoples studied to date, suggesting that they are lost symbionts in urban-industrialized societies [26].

Acinetobacter potential new species were detected in humans, gorillas and macaques. In humans, *Acinetobacter* is an organism of questionable pathogenicity for an infectious agent of importance to hospitals worldwide, it easily infects wounds [27]. The organism has the ability to accumulate diverse mechanisms of resistance, leading to the emergence of strains that are resistant to all commercially available antibiotics [28]. *Acinetobacter* is one of the main genera detected in primate gut microbiota. They might play a significant role in breaking down plant exudates [29].Very high diversity was observed in humans, gorillas and macaques.

Tuberculosis is rare in wild NHPs, but animals carrying *M. tuberculosis* could infect humans. Conversely, humans are the source of most NHP infections. *M. tuberculosis* and *M.*

bovis can be acquired from infected humans or ruminants [30]. In this study, *M. tuberculosis* has not been detected and great apes were found to be more carriers of *Mycobacterium* spp. than monkeys and humans. Natural infections with *M. leprae* was reported in chimpanzees and sooty mangabeys (*Cercocebusatys*) [31]. Recently, different strains of *M. leprae* have been isolated from NHPs, including chimpanzees, sooty mangabeys and cynomolgus macaques [32]. *Mycobacterium orygis* was isolated from captured rhesus monkeys [33]. It will be necessary to isolate the mycobacteria detected in this study to investigate their species diversity and identify their roles.

In addition, high prevalence for pathogenic *Leptospira* spp. has been observed in both humans and gorillas, which remains difficult to understand. All humans are apparently healthy and gorilla stools do not reflect any sign of diseases. Unfortunately, we were not able to genotype these *Leptospira* and the sequences obtained were unclear and suggested possible coinfection by more than one species. NHPs could be sensible to *Leptospira* infection, an outbreak of severe leptospirosis was reported in capuchin (*Cebus*) monkeys [34] . The presence of *Leptospira* in the feces of wild NHPs could be due to environmental contamination because the samples were collected from the soil, which is not the case in humans, leading to the weakness of this hypothesis. Inappropriate breeding of NHPs could create new reservoirs and transmission routes for *Leptospira*, threatening conservation efforts and public health. Furthermore, the extent of *Leptospira* transmission between humans and NHPs remains unknown.

Surprisingly, *Rickettsia* and *Wolbachia* had been detected in great apes and monkeys in the present study. For *Wolbachia*, we assume that it is *Wolbachia* eaten with their insect hosts by monkeys. For the *Rickettsia*, it is *R. africae*, very widespread on the continent, pathogenic agent, but very common in ticks. It is either the eaten ticks or the infection (very unlikely).

Enteric bacteria, such as *Salmonella* spp., including *S. typhi/paratyphi*, were detected in humans only, and pathogenic *H. pylori, T. whipplei* and *S. aureus* were detected in both humans and gorillas sharing the same living area, and humans were found to be more infected. *H. pylori* and *T. whipplei* are *a priori* anthroponotics and their origin remains unknown. These results suggest a transmission of pathogens from humans to gorillas. The threat is a direct function of the pathogens' mode of transmission and their ability to survive in aerosols, soil, water, food or feces [9]. In Uganda, it was observed that the number of gorillas carrying human gut *Salmonella* or *Campylobacter* had doubled in 4 years, and *Shigella* was isolated for the first time in this group of apes, probably because of ecotourism [35] .In addition, the fecal-oral transmission of *Shigella flexneri* and *S. sonnei*, enteropathogenic *Escherichia coli, Salmonella enteritidis, S. typhimurium, Campylobacter fetus, C. jejuni, Helicobacter pylori* and many others infections are common in NHPs [36]. For *T. whipplei*, two important but as yet unresolved issues are the natural habitat and the route of infection. Analysis of stool samples by PCR has detected *T. whipplei* DNA in patients with Whipple disease. Recovery of *T. whipplei* from culture of stool from a patient with Whipple disease has highlighted the presence of viable bacteria, suggesting that the disease could be linked to fecal-oral transmission [37] . Asymptomatic carriage in stool was found in humans (ranging

from a prevalence of 4% in the control group, to 12% among a subgroup of sewer workers), but not in monkeys and apes [37]. Thus, *T. whipplei* identified here in the gorilla most likely originates from humans.

Also, the other bacteria searched were negative, including the sexually transmitted pathogens in the current study (*Chlamydia* spp., *T. pallidum*, *N. gonorrhea*). The diversity of sample natures would also broaden the range of pathogens not found in stools and provide a clearer diagnostic vision in NHPs. It would therefore be interesting, in further studies, to use other types of excrement and biological fluids from these animals, especially for those in contact with humans, in order to assess the potential risks of interspecies transmission.

The prevalence of extra-intestinal infections might be underestimated in this study. Despite this, this method (search for pathogens in the feces) nevertheless made it possible to discover the origins of *Plasmodium* and HIV.

This work has contributed to the expansion of knowledge on NHP threatening bacteria in Africa by using a molecular stool technique. In addition, we have also shown that humans and gorillas in the same ecosystems share pathogens, indicating a real interspecies transmission. Constant monitoring is highly recommended to prevent any overflow of these pathogens. This effort entails a combination of epidemiology, molecular ecology, behavioral ecology, social and clinical survey, and spatially explicit modeling. The end outcomes are achievable plans to protect the health of humans and wildlife, while ensuring the sustainability of the ecosystems in which they live. In addition to better understanding the role of human-induced habitat change on pathogen dynamics, this work allows for the early detection of emerging pathogens that may pose a threat to global health and/or wildlife conservation.

Supplementary files

Table S1.

Table S2. Table summarizes blast results for *Acinetobacter* spp. detected on African humans, gorillas and macaques.

Disclosure statement

No potential conflict of interest was reported by the author (s).

Funding

This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program « Investissements d'avenir », reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI, Service de Coopération et d'Action Culturelle (SCAC) de l'Ambassade de la France en République du Congo, Laboratoire National de Santé Publique de Brazzaville.

ORCID

Medkour Hacène: https://orcid.org/0000-0002-5208-2576

Oleg Mediannikov: http://orcid.org/0000-0001-6039-2008

References

1. Kowalewski MM, Gillespie TR. Primatology , Biocultural Diversity and Sustainable Development in Tropical Forests. 2018;

2. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. Nature. 2008;451: 990–993. doi:10.1038/nature06536

3. Gillespie TR, Nunn CL, Leendertz FH. Integrative approaches to the study of primate infectious disease: Implications for biodiversity conservation and global health. Am J Phys Anthropol. 2008;137: 53–69. doi:10.1002/ajpa.20949

4. Calvignac-Spencer S, Leendertz SA, Gillespie TR, Leendertz FH. Wild great apes as sentinels and sources of infectious disease. Clin Microbiol Infect. 2012;18(6):521-527. doi:10.1111/j.1469-0691.2012.03816.x

5. Negrey JD, Reddy RB, Scully EJ, Phillips-garcia S, Owens LA. Simultaneous outbreaks of respiratory disease in wild chimpanzees caused by distinct viruses of human origin. 2019;8.

6. Estrada A, Garber PA, Rylands AB, Roos C, Fernandez-Duque E, Fiore A Di, et al. Impending extinction crisis of the world's primates: Why primates matter. Sci Adv. 2017;3. doi:10.1126/sciadv.1600946

7. Parsons MB, Travis D, Lonsdorf E V, Lipende I, Roellig DMA, Kamenya S, et al. Epidemiology and Molecular Characterization of Cryptosporidium spp . in Humans , Wild Primates , and Domesticated Animals in the Greater Gombe Ecosystem , Tanzania. 2015; 1–13. doi:10.1371/journal.pntd.0003529

8. Dunay E, Apakupakul K, Leard S, Palmer JL, Deem SL. Pathogen Transmission from Humans to Great Apes is a Growing Threat to Primate Conservation. Ecohealth. Springer US; 2018;2050. doi:10.1007/s10393-017-1306-1

9. Devaux CA, Mediannikov O, Medkour H, Raoult D. Infectious Disease Risk Across the Growing Human-Non Human Primate Interface: A Review of the Evidence. Front Public Heal. 2019;7: 1–22. doi:10.3389/fpubh.2019.00305

10. Gillespie TR, Chapman CA. Prediction of Parasite Infection Dynamics in Primate Metapopulations Based on Attributes of Forest Fragmentation. 2006;20: 441–448. doi:10.1111/j.1523-1739.2006.00290.x

11. Fuentes A, Kalchik S, Gettler L, Kwiatt A, Konecki M, Jones-Engel L. Characterizing human-macaque interactions in Singapore. Am J Primatol. 2008;70(9):879-883. doi:10.1002/ajp.20575

12. Faust CL, Dobson AP, Gottdenker N, et al. Null expectations for disease dynamics in shrinking habitat: dilution or amplification?. Philos Trans R Soc Lond B Biol Sci. 2017;372(1722):20160173. doi:10.1098/rstb.2016.0173

13. Letters E. Pathogen spillover during land conversion. 2018; doi:10.1111/ele.12904

14. Dridi B, Henry M, El Khéchine A, Raoult D, Drancourt M. High prevalence of Methanobrevibacter smithii and Methanosphaera stadtmanae detected in the human gut using an improved DNA detection protocol. PLoS One. 2009;4. doi:10.1371/journal.pone.0007063

15. Medkour H, Varloud M, Davoust B, Mediannikov O. New Molecular Approach for the Detection of Kinetoplastida Parasites of Medical and Veterinary Interest. Microorganisms. 2020;8: 356. doi:10.3390/microorganisms8030356

16. Binnicker MJ. Multiplex Molecular Panels for Diagnosis of Gastrointestinal Infection: Performance, Result Interpretation, and Cost-Effectiveness. J Clin Microbiol. 2015;53(12):3723-3728. doi:10.1128/JCM.02103-15

17. Platts-Mills JA, Liu J, Houpt ER. New concepts in diagnostics for infectious diarrhea. Mucosal Immunol. 2013;6(5):876-885. doi:10.1038/mi.2013.50

18. Angelakis E, Yasir M, Bachar D, Azhar EI, Lagier J, Bibi F, et al. Gut microbiome and dietary patterns in different Saudi populations and monkeys. Nat Publ Gr. Nature Publishing Group; 2016; 1–9. doi:10.1038/srep32191

19. Manara S, Asnicar F, Beghini F, Bazzani D, Cumbo F, Zolfo M, et al. Microbial genomes from non-human primate gut metagenomes expand the primate-associated bacterial tree of life with over 1000 novel species. Genome Biol 20, 299 (2019). https://doi.org/10.1186/s13059-019-1923-9.

20. Angelakis E, Bachar D, Yasir M, Musso D, Djossou F, Gaborit B, et al. Treponema species enrich the gut microbiota of traditional rural populations but are absent from urban individuals. New Microbes New Infect. Elsevier Ltd; 2019;27: 14–21. doi:10.1016/j.nmni.2018.10.009

21. Knauf S, Gogarten JF, Schuenemann VJ, Nys HM De, Düx A, Strouhal M, et al. Nonhuman primates across sub-Saharan Africa are infected with the yaws bacterium Treponema pallidum subsp . pertenue. 2018; doi:10.1038/s41426-018-0156-4

22. Strouhal M, Mikalova L, Darina C, Zobanı M, Fulton LL, Chen L, et al. Whole Genome Sequence of the Treponema Fribourg- Blanc : Unspecified Simian Isolate Is Highly Similar to the Yaws Subspecies. 2013;7. doi:10.1371/journal.pntd.0002172

23. Knauf S, Liu H, Harper KN. Treponemal infection in nonhuman primates as possible reservoir for human yaws. Emerg Infect Dis. 2013;19(12):2058-2060. doi:10.3201/eid1912.130863

24. Mubemba B, Chanove E, Mätz-Rensing K, et al. Yaws Disease Caused by *Treponema pallidum* subspecies *pertenue* in Wild Chimpanzee, Guinea, 2019. Emerg Infect Dis. 2020;26(6):1283-1286. doi:10.3201/eid2606.191713.

25. Mediannikov O, Fenollar F, Davoust B, Amanzougaghene N, Lepidi H, Arzouni J, et al. Epidemic of venereal treponematosis in wild monkeys : a paradigm for syphilis origin. New Microbes New Infect. Elsevier Ltd; 2020;35: 100670. doi:10.1016/j.nmni.2020.100670

26. Obregon-tito AJ, Tito RY, Metcalf J, Sankaranarayanan K, Clemente JC, Ursell LK, et

al. distinguish gut microbiomes. Nat Commun. Nature Publishing Group; 2015; 1–9. doi:10.1038/ncomms7505

27. Fournier PE, Richet H. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. Clin Infect Dis. 2006;42(5):692-699. doi:10.1086/500202.

28. Lolans K, Rice TW, Munoz-price LS, Quinn JP. Multicity Outbreak of Carbapenem-Resistant Acinetobacter baumannii Isolates Producing the Carbapenemase OXA-40. 2006;50: 2941–2945. doi:10.1128/AAC.00116-06

29. Clayton JB, Gomez A, Amato K, Knights D, Travis DA, Blekhman R, et al. The gut microbiome of nonhuman primates : Lessons in ecology and evolution. 2018; 1–27. doi:10.1002/ajp.22867

30. Fourie PB, Odendaal MW. *Mycobacterium tuberculosis* in a closed colony of baboons (*Papio ursinus*). Lab Anim. 1983;17(2):125-128. doi:10.1258/002367783780959376.

31. Gormus BJ, Wolf RH, Baskin GB, et al. A second sooty mangabey monkey with naturally acquired leprosy: first reported possible monkey-to-monkey transmission. Int J Lepr Other Mycobact Dis. 1988;56(1):61-65..

32. Honap TP, Pfister LA, Housman G, et al. *Mycobacterium leprae* genomes from naturally infected nonhuman primates. PLoS Negl Trop Dis. 2018;12(1):e0006190. Published 2018 Jan 30. doi:10.1371/journal.pntd.0006190.

33. Rahim Z, Thapa J, Fukushima Y, Zanden AGM Van Der, Gordon S V, Suzuki Y. Tuberculosis Caused by Mycobacterium orygis in Dairy Cattle and Captured Monkeys in Bangladesh : a New Scenario of Tuberculosis in South Asia. 2016; doi:10.1111/tbed.12596

34. Hemmige V, Tanowitz H, Sethi A. *Trypanosoma cruzi* infection: a review with emphasis on cutaneous manifestations. Int J Dermatol. 2012;51(5):501-508. doi:10.1111/j.1365-4632.2011.05380.x

35. Nizeyi JB, Innocent RB, Erume J, Kalema GR, Cranfield MR, Graczyk TK. Campylobacteriosis, salmonellosis, and shigellosis in free-ranging human-habituated mountain gorillas of Uganda. J Wildl Dis. 2001;37(2):239-244. doi:10.7589/0090-3558-37.2.239.

36. Raoult D, Fenollar F, Birg ML. Culture of *T. whipplei* from the stool of a patient with Whipple's disease. N Engl J Med. 2006;355(14):1503-1505. doi:10.1056/NEJMc061049.

37. Fenollar F, Trani M, Davoust B, et al. Prevalence of asymptomatic *Tropheryma whipplei* carriage among humans and nonhuman primates. J Infect Dis. 2008;197(6):880-887. doi:10.1086/528693

Figure legends

Figure 1. Map indicating the countries where fecal samples were collected. **In Senegal:** 48f ecal samples of western chimpanzees (*Pan troglodytes verus*) were collected from three sites located within the Dindefelo Community Natural Reserve in the Kédougou region. Site 1: 3samples (12°22′57.1404″ N, 12°17′16.7172″ W), Site 2: seven samples (12°22′53.1732″N, 12°17′26.7936″W) and Site 3: 38 samples (12°22′47.7084″ N, 12°17′48.588″ W). Four green monkeys (*Chlorocebus sabaeus*) and 7 Guinea baboons (*Papio papio*) fecal samples were collected near the Niokolo forestry guardhouse of the NKNP (13°04′28.6″ N 12°43′18.2″ W).**In the Republic of Congo:** 28 gorilla (*Gorilla gorilla*) feces were collected from the Lésio-Louna (LLR) and South-West Léfini gorilla reserves (2°58′33.1″S 15°28′33.4″E), 10 from Odzala-Kokoua National Park (OKNP) (1.3206°"N, 14.8455°"E), 35 human feces from (1.3206°"N, 14.8455°"E) village of Mbomo, located within the OKNP, and 3 from eco-guards in the LLR. **In Djibouti:** 6 hamadryas (*Papio hamadryas*) feces. These baboons lived outside the village of Oueah, 38 km from the city of Djibouti (11°29′56.1″N 42°51′14.8″E). **In Algeria:**69 Barbary macaques (*Macaca sylvanus*), including 30 samples collected from two sites, the Stream of Monkeys and the Gorges of la Chiffa in Blida Province, 50 km north of Algiers (36°23′42.9″N 2°45′53.6″E), and 39 samples from Cap Carbon (36°46′31.6″ N 5°06′11.2″ E) in the suburbs of Béjaïa, 250 km east of Algiers.

Figure 2. Infections detected in African NHPs and humans.

Figure 3. Phylogenetic tree for *Rickettsia* spp. in detected in African NHPs. The evolutionary history, based on 700 bp gltA partial gene, was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.10688518 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (1000 replicates is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were discarded. There were a total of 682 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Figure 4. Phylogenetic tree for *Acinetobacter* spp. detected in African human and NHP feces. The evolutionary history based on rpoB partial gene was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.17635033 is shown. The confidence probability (multiplied by 100) of the interior branch length is greater than 0, as estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 50 nucleotide sequences. All positions containing gaps and missing data were discarded. There were a total of 260 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Figure 5. Phylogenetic tree for *Treponema* spp. detected in NHP feces. The evolutionary history based on 23S rRNA gene was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2,23995197 is shown. The confidence probability (multiplied by 100) of the interior branch length is greater than 0, as estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 81 nucleotide sequences. All positions containing gaps and missing data were discarded. There were a total of 594 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Figure 6. Phylogenetic tree of *Treponema* spp. detected in gorilla and human feces living in the same area in Mbomo, Republic of Congo. The evolutionary history based on 23S rRNA partial gene was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2,51751187 is shown. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons. The analysis involved 36 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 361 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

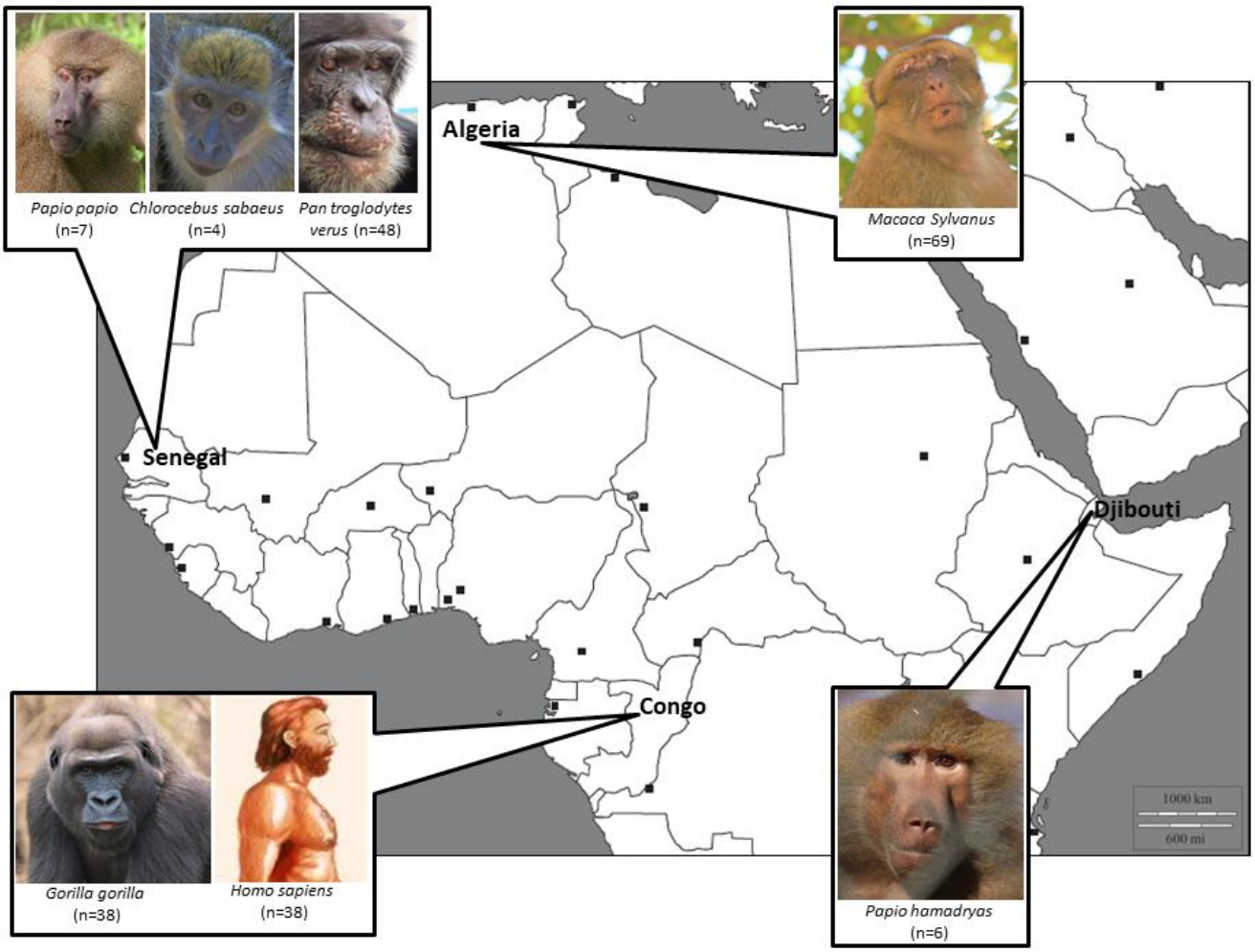
611 **Table 2.** Prevalence of bacterial infections detected on feces from different African NHP species as well as humans

Species	Origin	Number	*Leptospira* spp.		*Treponema* spp.		*Mycobacterium* spp.		*Acinetobacter* spp.		*Rickettsia* spp.		*Wolbachia* spp.*		*Mycoplasma* spp.		*S. aureus*		*H. pylori*		*Salmonella* spp.		*T. whipplei*	
			Pos	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**	**Pos**	**%**
Gorilla	Congo	38	25	65,8	38	100,0	20	52,6	13	34,2	5	13,2	3	7,9	3	7,9	3	7,9	1	2,6	0	0	1	2,6
Chimpanzee	Senegal	48	1	2,1	22	45,8	38	79,2	1	2,1	2	4,2	0	0,0	0	0,0	0	0,0	0	0,0	0	0	0	0,0
Guinea baboon		7	0	0,0	6	85,7	2	28,6	0	0,0	0	0,0	0	0,0	0	0,0	0	0,0	0	0,0	0	0	0	0,0
Hamadryas baboon	Djibouti	6	0	0,0	6	100,0	0	0,0	0	0,0	0	0,0	0	0,0	0	0,0	0	0,0	0	0,0	0	0	0	0,0
Green Monkey	Senegal	4	0	0,0	3	75,0	0	0,0	0	0,0	0	0,0	0	0,0	1	25,0	0	0,0	0	0,0	0	0	0	0,0
Macaque	Algeria	69	20	29,0	61	88,4	6	8,7	3	4,3	1	1,4	0	0,0	0	0,0	0	0,0	0	0,0	0	0	0	0,0
Great Apes	-	86	26	30,2	60	69,8	58	67,4	14	16,3	7	8,1	3	3,5	3	3,5	3	3,5	1	1,2	0	0	1	1,2
Monkeys	-	86	20	23,3	76	88,4	8	9,3	3	3,5	1	1,2	0	0,0	1	1,2	0	0,0	0	0,0	0	0	0	0,0
Total NHP	-	172	46	26,7	136	79,1	66	38,4	17	9,9	8	4,7	3	1,7	4	2,3	3	1,7	1	0,6	0	0	1	0,6
Human	Congo	38	31	81,6	30	78,9	12	31,6	28	73,7	0	0,0	0	0,0	0	0,0	4	10,5	9	23,7	**4**	**10,5**	4	10,5

In bolt: Number include 2 samples positive by *S. typhi/paratyphi* qPCR.

*****: where also positive by *Anaplasmataceae* qPCR

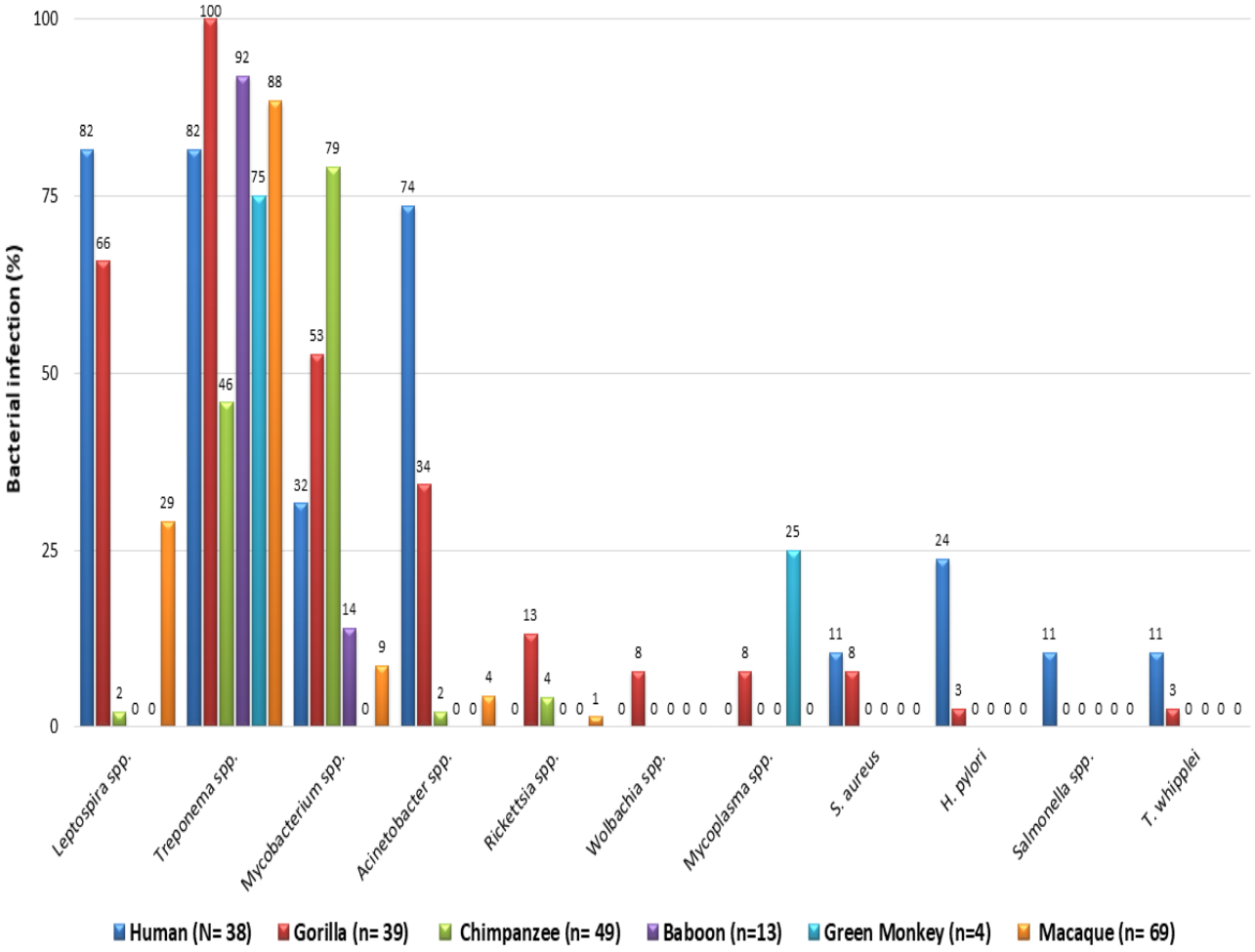
612 **Fig 1.**



613

614

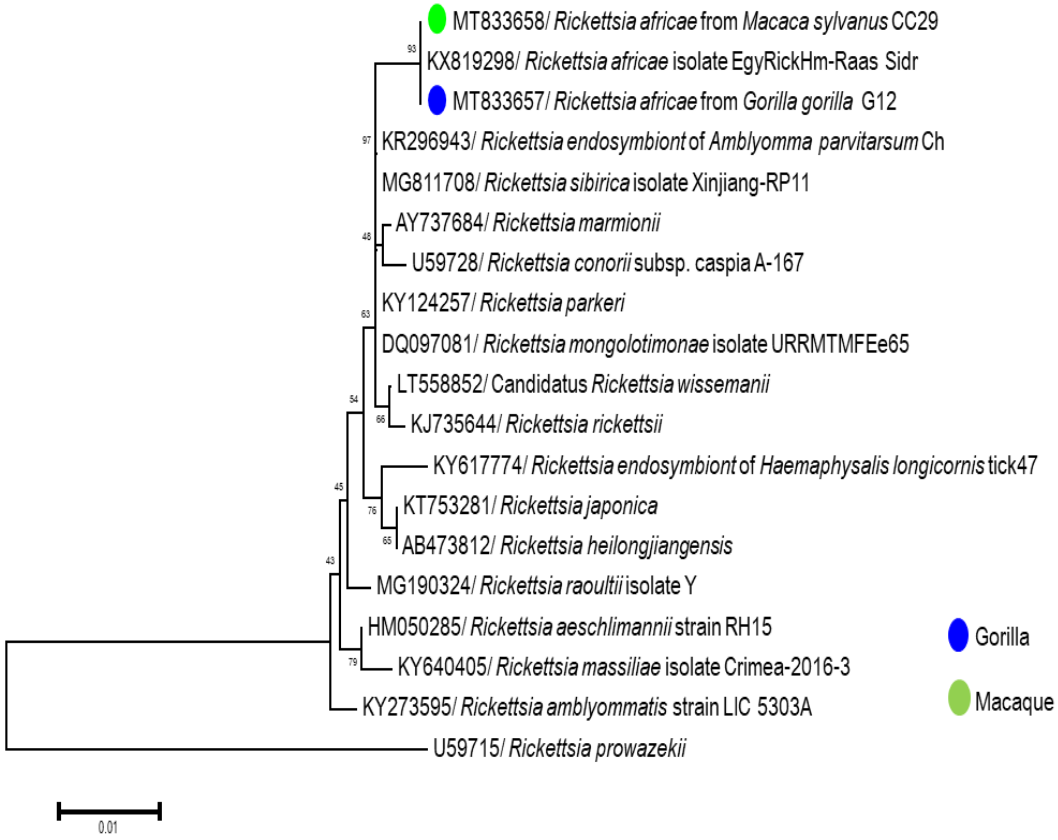
615 **Fig 2.**



616

617

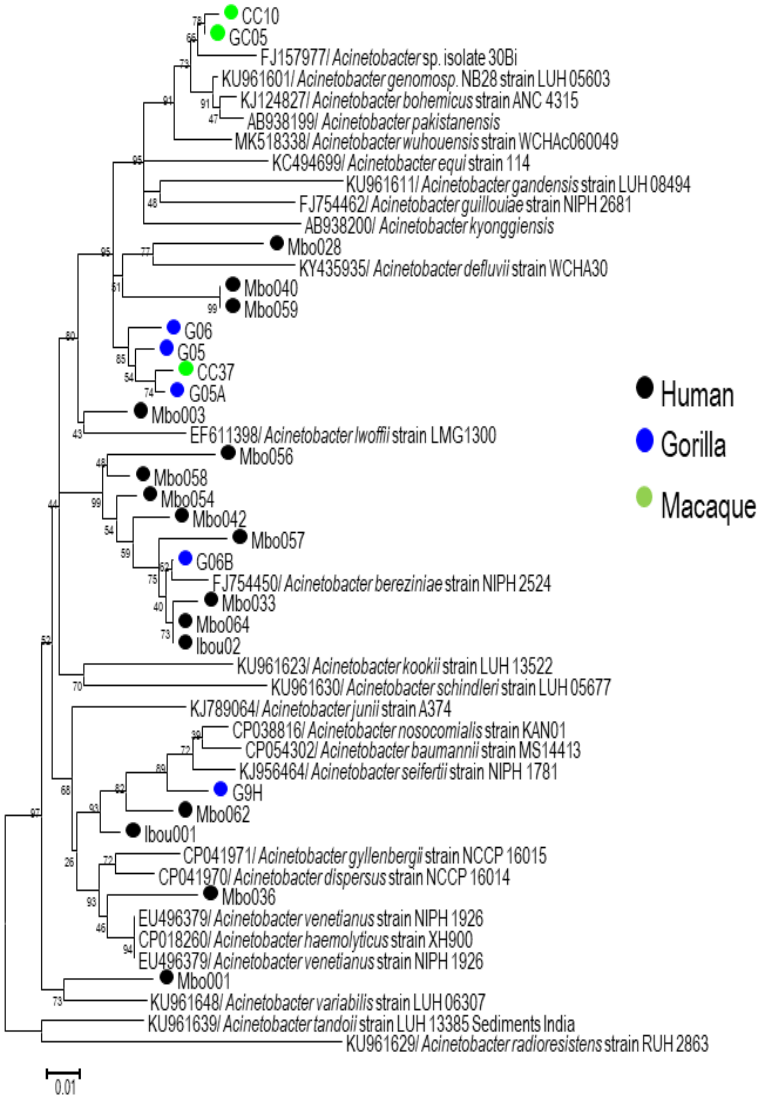
618 **Fig 3.**



619

620

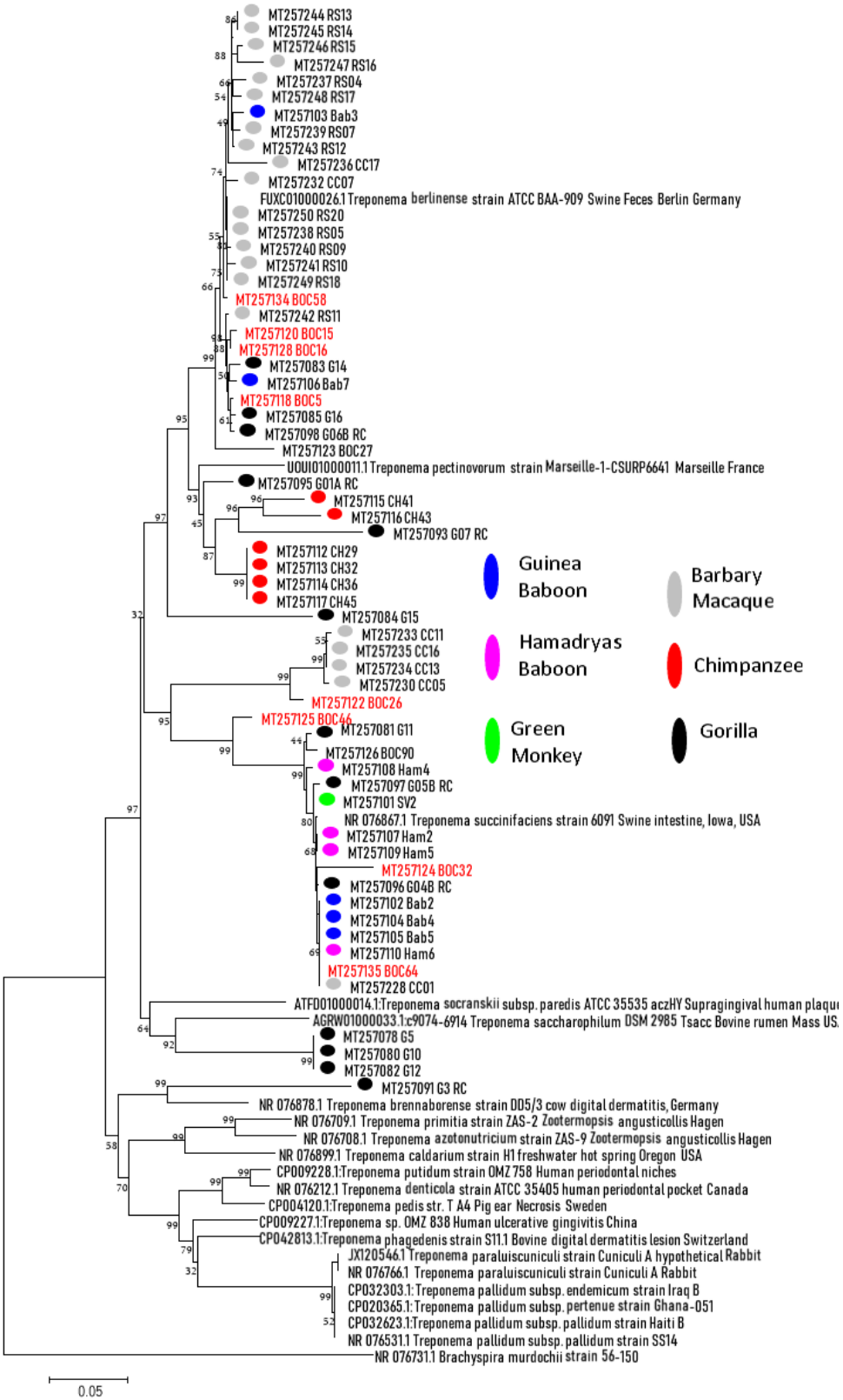
621 **Fig 4.**



622

623

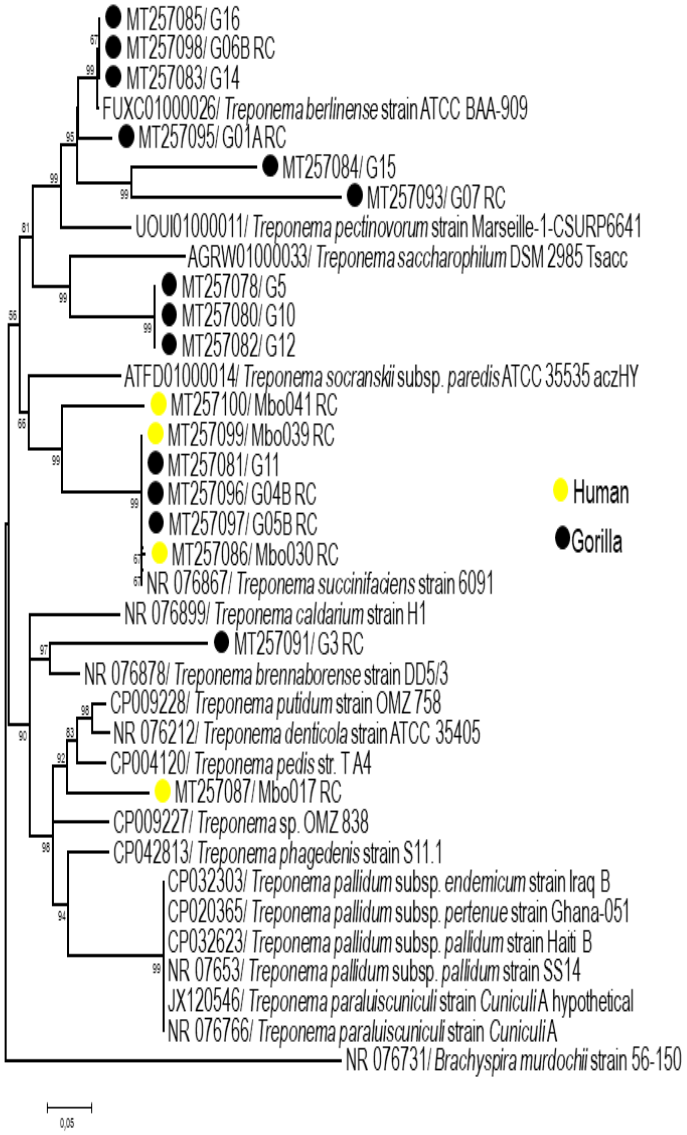
624 **Fig 5.**



625

626

627 **Fig 6.**



628

629

Annexe 2

Isolation of *Rodentibacter rarus* Strain in a Street Rat (*Rattus norvegicus*)

Hacène Medkour[1,2†], **Younes Laidoudi**[1,2†], Handi Dahmana[1,2], Hubert Lepidi[1,2,3], Oleg Mediannikov[1,2], and Bernard Davoust[1,2*]

[1] Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France
[2] IHU Méditerranée Infection, Marseille, France
[3] Laboratoire d'anatomo-pathologie, CHU La Timone, Assistance Publique – Hôpitaux de Marseille, France
† Equal contribution for the first and the second authors

RAPPORT DE CAS

Soumis à : Pathogens (en révision)

pathogens

MDPI

Article

Isolation of *Rodentibacter rarus* Strain in a Street Rat (*Rattus norvegicus*)

Hacène Medkour [1,2,†], Younes Laidoudi [1,2,†], Handi Dahmana [1,2], Bastien Salvi[2], Hubert Lepidi [1,2,3], Oleg Mediannikov [1,2] and Bernard Davoust [1,2,*]

[1] Aix Marseille Univ, IRD, AP-HM, MEPHI, IHU-Méditerranée Infection, Marseille, France; hacenevet1990@yahoo.fr (H. M.); younes.laidoudi@yahoo.com (Y.L.); handy92@hotmail.fr (H. D.); olegusss1@gmail.com (O. M.); bernard.davoust@gmail.com (B. D.)

[2] IHU Méditerranée Infection, Marseille, France; bastiensalvi36@gmail.com (B.S)

[3] Laboratoire d'anatomo-pathologie, CHU La Timone, Assistance Publique – Hôpitaux de Marseille, France; hubert.lepidi@ap-hm.fr (H. L.)

[†] Equal contribution for the first and the second authors

* Correspondence: bernard.davoust@gmail.com; Tel.: +33-0413732401

Abstract: Rodents are one of the most dangerous reservoirs of infectious diseases. Gradually, rats have become predominant in cities, increasing contact with humans and other animals and thus the risk of pathogen transmission. Here, we report an original case of bacterial pneumonia in a street rat (*Rattus norvegicus*). The rat was found dead in a street in downtown Marseille after a car accident. The necropsy revealed a generalized granulomatous pneumonia in almost all the pulmonary lobes. Pathological lesions are indicative of pneumonia due to multiple fibro-inflammatory scar foci located in the lung. We performed a bacterial culture from a lung sample. The colonies were identified by MALDI-TOF MS and universal PCR for all bacteria targeting the 16S rRNA gene. The following bacteria were identified: *Staphylococcus cohnii*, *Bordetella bronchiseptica*, *B. parapertussi*, *Corynebacterium glucuronolyticum*, *Pelistega suis* and *Rodentibacter rarus*. As all the other bacteria detected here are not known to be pathogenic to rats, the most likely etiology of the rat pneumonia studied is therefore *R. rarus*, a little-known bacterium that is closely related to R. *pneumotropicus.* This case is important to report because it concerns a pathology (due to *R. rarus*) affecting commensal rodent species living in close proximity to other animals (dogs, cats, birds) as well as humans. The microbial diversity associated with street rats, a worldwide population, needs to be better explored.

Keywords: Rodents; *Rattus norvegicus*; *Rodentibacter rarus*

1. Introduction

The brown rat or Norwegian rat (*Rattus norvegicus*) is actually native to Manchuria (northeast Asia). Arrived in Europe around 1750, it was transported worldwide by commercial vessels. It is a large rat that lives rather on the ground, especially in urbanized areas. Gradually, it became dominant in cities, supplanting the black rat (*Rattus rattus*), especially in the port cities (1). The brown rat mainly lives in the sewers. It also digs burrows. It swims well, unlike the black rat (1). In France, as in the rest of the world, its proliferation in the big cities (Paris, Marseille, Lyon, etc.) is a problem taken into account by the political authorities because of the negative effects on the image of their city. Public health is also evoked as a reminder of the devastation caused by past plague and typhus epidemics transmitted by rat fleas. In Marseille, which is the second largest commune in France with 863,310 inhabitants, we have already conducted studies to identify pathogens in 108 city rats (86 *R. norvegicus* and 22 *R. rattus*): *Hantavirus* (0%) *Bartonella* spp. (30%), *Leptospira*

interrogans (9%), *Streptobacillus moniliformis* (13%), *Calodium hepaticum* (44%), *Trichinella* spp. (0%), *Xenopsylla cheopis* (21%) (2,3,4,5). More recently, in an urban park near Paris, 86 brown rats were studied (6). Bacterial pathogens were identified: *Rickettsia* spp. (1.2%), *Bartonella* spp. (53%), *Francisella tularensis* (5%), *Leptospira* spp. (21%). This was apparently an asymptomatic carriage as no rats had any observable lesions at necropsy. Paradoxically, worldwide, although the wild brown rat is one of the most prevalent animals, investigations of its pathology are not numerous. The city where it has been the most investigated is Vancouver (631,486 inhabitants) in Canada, a port comparable to Marseille (7,8,9). In this city, researchers were able to necropsize 1383 brown rats, some of which had macroscopic and histologic lesions, particularly pulmonary, described in two of the only publications on the pathology of the wild brown rat (10,11). Within the lungs, peribronchiolar and/or perivascular lymphoplasmacytic cuffs were present and were also significantly associated with cilia-associated respiratory bacillus (CARB) and *Mycoplasma pulmonis* (10). In Baltimore, a US port (Maryland) of 622 104 inhabitants, antibodies against Seoul virus (57.7%), hepatitis E virus (HEV, 73.5%), *Leptospira interrogans* (65.3%), *Bartonella elizabethae* (34.1%) and *Rickettsia typhi* (7.0%) were detected in Norway rats (N= 201) (12). In Germany and neighboring countries (Denmark and Switzerland), it was mainly *L. interrogans* (17%) which was detected in 426 brown rats by PCR on kidneys (13). A recent review of the infectious agents [*Leptospira* spp., *Rickettsia* spp., orthopoxviruses (positive serology) and novel rat polyomavirus (unknown pathogenesis)] carried by rats in Europe shows that there is a need to monitor their development (14). In this context, to extend our knowledge on city rats, we present in this article an original case of exceptional bacterial pneumonia observed in a wild brown rat in Marseille (France).

2. Results

2.1. Pathological Investigations

Examination of the rat cadaver revealed that it died of head trauma as a result of impact with a vehicle. Necropsy revealed lesions of generalized granulomatous pneumonia. Granulomas, hard and whitish, 2 mm in diameter, occupied all the lung lobes (**Figure 1**). On the liver, we observed lesions of whitish fibrosis suggestive of *Calodium hepaticum* parasite. On the kidneys, we found a few foci of necrosis.

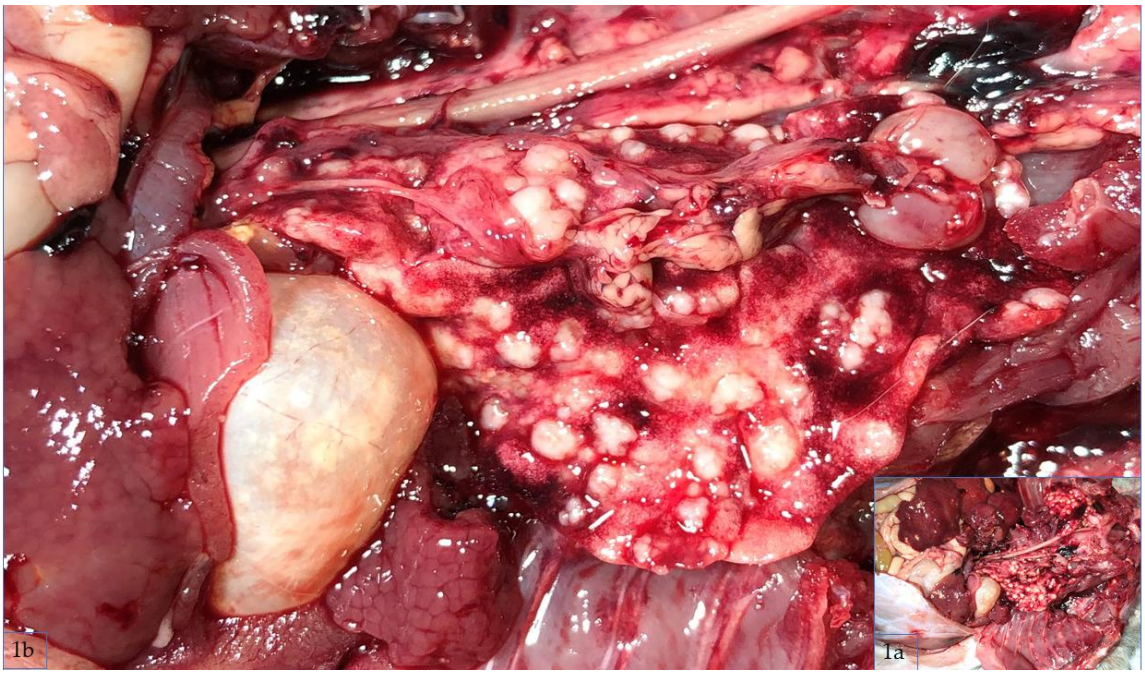
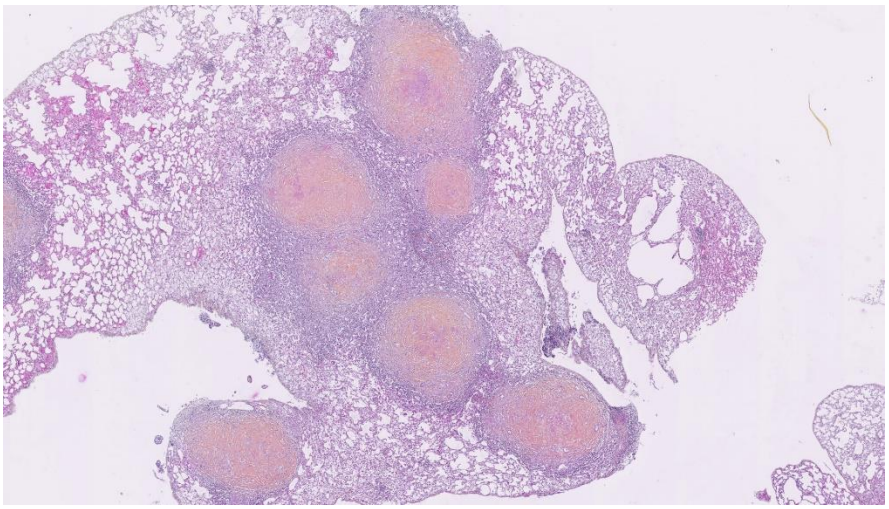
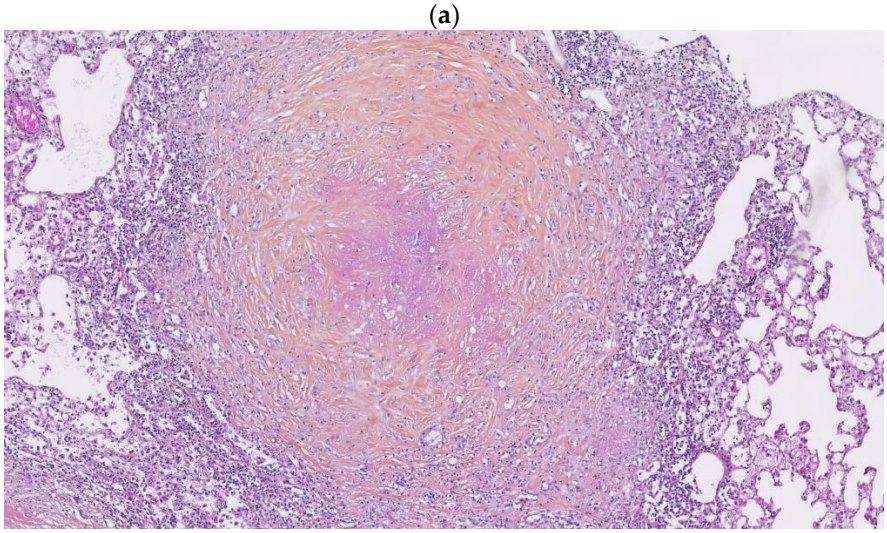


Figure 1: 1a. Thoracic and abdominal organs of the autopsied rat. **1b.** thoracic lesions

Histologic analysis revealed numerous several fibro-inflammatory scarring areas in the lungs measuring about 2 mm in diameter **(Figure 2a).** These foci were mainly composed of a hyaline fibrous scar containing inflammatory cells, mainly epithelioid macrophages with a rim of lymphocytes **(Figure 2b)**. Rare multinucleated giant cells were also observed in these lesions, but not central necrosis nor bacteria. In more recent lesions, inflammatory foci with a patchy distribution and filing of the alveoli, mainly composed of lymphocytes and alveolar macrophages, were also seen in lung tissues **(Figure 3).** These inflammatory exudates were often centered by an arterial vessel **(Figure 4).**



(**a**)



(**b**)

Figure 2. Multiple several fibro-inflammatory scarring lesions in lung sections with mononuclear inflammatory cells and hyaline center (hematoxylin-eosin-saffron, original magnification X20 and X80, 2A and 2B respectively).

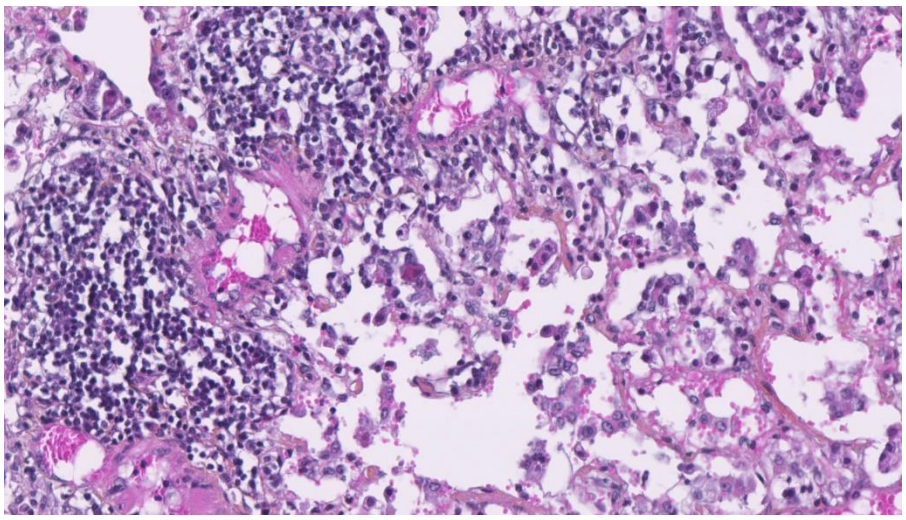


Figure 3. Dense inflammatory infiltrates composed of lymphocytes and alveolar macrophages with filing of alveoli (hematoxylin-eosin-saffron, original magnification X300).

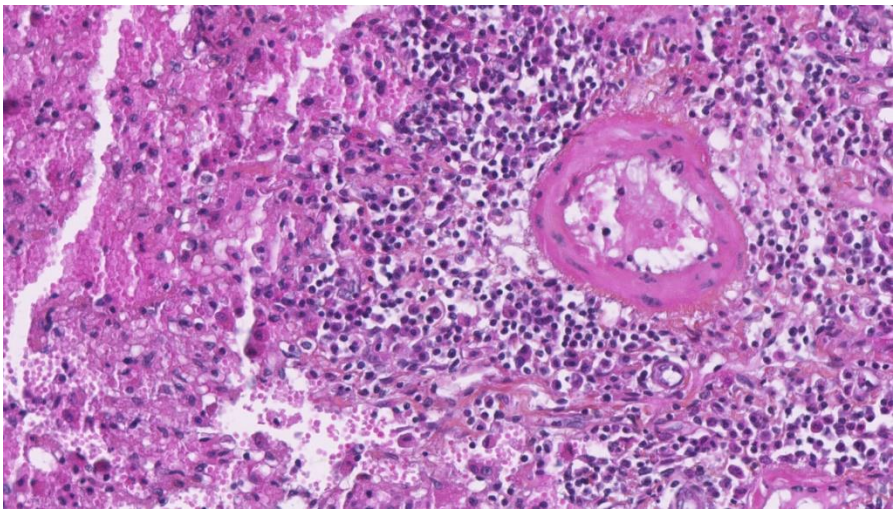


Figure 4. Alveolar inflammatory infiltrates surrounding an arterial vessel (hematoxylin-eosin-saffron, original magnification X300).

Histologic analysis confirmed the presence of the parasite *Calodium hepaticum* in the liver.

2.2. Microscopy, Culture, Molecular Analysis, MALDI-TOF MS and Antimicrobial Susceptibility Testing

The lung was bacteriologically cultured on agar medium at 37°C. Ten bacterial strains were isolated.

The MALDI-TOF MS analysis for the cultured-colonies in the three portions of lungs allowed these bacteria to be detected: *Staphylococcus cohnii*, *Bordetella bronschiseptica*, *Bordetella holnesii*, *B. bronchiseptica Corynebacterium glucuronolyticum*, and five colonies remained unidentified.

All colonies were subjected to PCR/sequencing of the 16S rRNA gene which confirmed the MALDI-TOF MS results and allowed the identification of the unidentified colonies. One sequence shared 99.9% identify with *S. cohnii* strains from GenBank database. *Bordetella bronschiseptica,* colonies identified by MALDI-TOF, were confirmed by 16S sequencing and with two others undefined colonies which resulted in almost identical sequences. These sequences were perfectly

similar (100%) to *B. parapertussis* and *B. bronchiseptica* strains from GenBank database. Two undefined colonies resulted in two almost identical sequences; they were 99.16% identical to *Pelistega suis* strain 3340-03 (NR_145928). Finally, another undefined colony by MALDI-TOF could be identified by 16S sequencing as *Rodentibacter rarus* (*rarus*, rare, referring to the very rare isolation of this species). The GenBank access number for the strain of *R. rarus* is MT 860347. This strain was deposited at the CSUR collection of strains (CSUR, IHU Méditérranée Infection, Marseille, France) under the number: Q5438. It exhibited 99% identity with *R. pneumotropicus* strain D2-1X-88 (MK287673) and 97.59% identity with *R. ratti* strain F75 (NR_156998) (**Figure 5**).

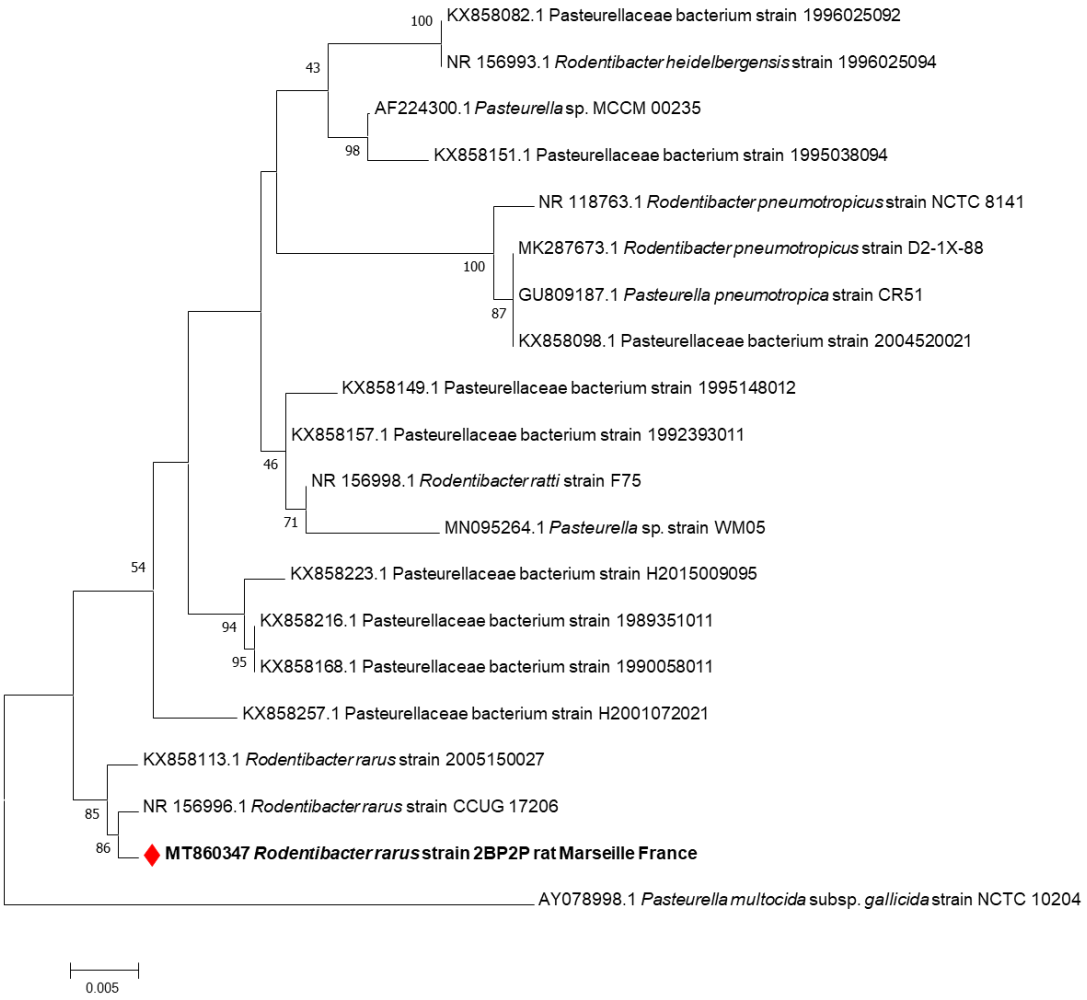


Figure 5. Molecular phylogenetic analysis by maximum likelihood method for *Rodentibacter rarus* strain 2BP2P.
The evolutionary history, based on 16S rRNA gene, was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3030.53) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1329 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

The antimicrobial susceptibility test of the strain *Rodentibacter rarus*, revealed that it is sensitive to all antibiotics tested, with the exception of ciprofloxacin, to which it is resistant (**Table 1**).

130 The qPCR assays for *Mycobacterium* spp., *Mycoplasma pneumoniae*, *M. hominis* and *Pneumocystis*
131 *jirovecii* (*P. carinii*) were all negative.
132

Table 1. *Rodentibacter rarus* strain 2BP2P detailed results of the antimicrobial susceptibility testing.

Antibiotic abbreviation	Antibioc	Ø (mm)	S/I/R
TIC	Ticarcillin	40.5	S
TCC	Ticarcillin/ Clavulanic acid	40.4	S
TPZ	Piperacillin/Tazobactam	38.5	S
ATM	Aztreonam	39.8	S
CAZ	Ceftazidime	33.4	S
FEP	Cefepime	43.2	S
MER	Meropenem	34.5	S
IPM	Imipenem	36.3	S
FF	Fosfomycin	41.3	S
RA	Rifampicin	27.8	S
SXT	Trimethoprim/Sulfamethoxazole	39.4	S
AK	Amikacin	20.2	S
CIP	Ciprofloxacin	22	R
DO	Doxycycline	29	S
CT	Colistin	25.2	S
CN	Gentamicin	29	S

S: Sensitive - **I**: Intermediate - **R**: Resistant.

3. Discussion

As soon as the cadaver was collected, at the time of the death, our objective was to highlight, using the diagnosis tools at our disposal, the causative agents (especially zoonotic ones) of the lesions observed in the rodent. During the necropsy that closely followed the death, we were surprised by the extent of the lung lesions observed. We had never encountered such a case in our experience in necropsy of several hundred wild rats in France and Africa (2,3,4,5).

No search for viruses has been undertaken; we favored other microbes because, according to the literature, no virus is known to induce such lesions in rats (10,15). The granulomatous features of the lesions that extended to almost all the pulmonary lobes allowed us to eliminate a parasitic diagnosis. *Pneumocystis carinii* infection was, however, investigated. Since the PCR was negative and pathological analysis did not reveal any cystic forms in the pulmonary alveoli, this etiology was discarded. *P. carinii* has been consistently identified in pneumonia of wild brown rats co-infected with *Mycoplasma pulmonis* (10). *M. pulmonis* is the most common cause of bronchopneumonia in rats (15). The extensive lesions observed in some rat strains after exposure to *M. pulmonis* may be attributable to an exaggerated and mis-directed cellular immune response. It is responsible for a purulent disease, not granulomatous. Our macroscopic and histological observations were not in favor of this diagnosis, and it was further excluded by the mycoplasma-negative bacterial culture and PCR. In this study, neither *P. carinii* nor *M. pneumoniae* or *M. hominis* were detected.

Similarly, mycobacteriosis (*Mycobacterium* spp.) was excluded after analysis (PCR, Ziehl-Neelsen staining). In the lungs, a characteristic granulomatous inflammatory reaction, forming both caseating and noncaseating granuloma was observed. Because of the importance of the fibrosis inside the granulomas, these histological features were in favor of an old bacterial infection with tissue areas at different stages of infection. Thanks to the bacterial culture carried out on three lung samples, followed by analyses in mass spectrometry and molecular biology, the etiology became clearer. Four bacterial strains were identified with a homology higher than 99%, compared to strains referenced in GenBank: *Staphylococcus cohnii, Pelistega suis, Bordetella parapertussis* and *Rodentibacter rarus.*

Staphylococcus cohnii is a bacterium that colonizes human skin. It may have been the cause of few cases of nosocomial infections (16). In rats, staphylococci cause abscesses but never pneumonia

(15). Exceptionally, this bacterium was found to be transmitted by rat bite in one of 40 rats studied (17).

Pelistega suis cannot cause pneumopathy. It is a bacterium isolated from pigs and wild boar, but there is no data available on rats, the pathogenicity of which is unknown (18).

Bordetella parapertussis is the agent of a mild form of whooping cough (20% of cases), a highly contagious disease of the upper respiratory tract in humans, severe in infants. There is no reason to suspect that this bacterium, of which humans are the only reservoir, is the cause of the lesions observed in rats (19). In contrast, *B. bronchiseptica* causes respiratory infections in many different mammals, including mice, rats, rabbits, cats, dogs, ferrets, foxes, pigs, hedgehogs, sheep, horses, and, occasionally, humans (20). *B. bronchiseptica* causes in rats a multifocal bronchopneumonia with polymorphonuclear cell and lymphocytic infiltration, and peribronchial lymphoid hyperplasia can be observed microscopically (15). In this study, *B. bronchiseptica* identified with MALDI-TOF was not confirmed by sequencing of 16S rRNA gene, the results showed same identity for both *B. parapertussis* and *B. bronchiseptica* and we therefore discarded this hypothesis as being the cause of the pneumonia.

Rodentibacter rarus is the most probable etiological agent of rat pneumopathies. The bacterium was first described by Adhikary et al., within the genus *Rodentibacter* in 2017 (21). The genus *Rodentibacter* belongs to the Pasteurellaceae family and comprised a total of eight type species. This includes, *Rodentibacter pneumotropicus,* which was reclassified in *Rodentibacter* instead of *Pasteurella* genus (formerly *Pasteurella pneumotropica* Jawetz 1950) and seven newly types species, including *R. rarus*. The strain *R. rarus*, DSM number: 103980, was first isolated in a biological sample from a rat in Denmark in 1979 by H. Christensen (University of Copenhage) and was used in genome-based description of the type species. Another genome of *R. rarus* is now available in GenBank database (NCBI ID 1908260). The strain was isolated from mouse in 1984 in USA known as Bisgaard taxon17 (21, 22).

Interestingly, we genetically demonstrated that *R. rarus* is very close to the well-known pathogenic species of *R. pneumotropicus* on the basis of the 16S gene (99% of identity). So far, data on the pathogenicity of *R. rarus* in rodents are lacking and have never been studied in other hosts.

Former *Pasteurella pneumotropica* is a Gram-negative, non-motile, short rod-shaped bacterium (21). In rodents, that are the main hosts of this bacterium, it causes pneumopathies in immunocompromised animals. Usually, immunocompetent rodents are asymptomatic carriers. Experimental infections of SCID mice cause the appearance of pneumonia lesions (23). Another study showed that a highly virulent strain of *R. pneumotropicus* causes severe pneumonia and septicaemia after intranasal infection of C57BL/6 and BALB/c mice (24). Alveoli and bronchioles of this mouse were infiltrated with a high number of neutrophils. *R. pneumotropicus* is best known in laboratory rodents (rat and mouse) animal houses as an opportunistic microbe that can seriously affect the health of rodents and thus disrupt experiments (25). Current protocols, implemented in rodent suppliers and pet shops, include routine screening for *R. pneumotropicus*, at best by PCR (feces and oral swab) and culture (26). Serological tests are also used (27). In case of infection, it is necessary to eliminate carrier animals and decontaminate the facilities (28). Furthermore, in humans, this bacterium can very rarely cause septicemia (29). Finally, this bacterium was transmitted three times out of 40 rat bites studied (17). According to our literature search, a virulent strain of *R. pneumotropicus* has never been isolated from a wild brown rat before. The reference strains or sequences deposited in GenBank all come from laboratory rodents, especially mice (30).

Here, we report the first isolation of a pathogenic strain of *R. rarus* from a street rat. The bacterium was genetically close to *R. pneumotropicus* and was involved in a severe pneumonia in a brown rat.

4. Materials and Methods

4.1. Case History

In December 2019, a rat was struck by a car in the middle of a small street (43°17'06.2 "N 5°23'46.7 "E) in the centre of Marseille (France). A few minutes after the accident, the rat, that died instantly, was picked up and brought to the *Institut hospitalo-universitaire* (IHU) Méditérrannée Infection - Veterinary Research Center. Initial morphological examinations confirmed that it was indeed a street rat (*Rattus norvegicus*), male, weighing 475 g, with a head + body length of 25 cm and a tail length of 22 cm. The animal was a carrier of few ectoparasites (*Ornithonyssus* spp.; family Macronyssidae; Acari).

222 *4.2. Pathological Investigations*

223 The necropsy and biological sampling were carried out as previously described (31). Lungs
224 and liver were removed from the rat and subsequently fixed with buffered formalin 4% to 10% and
225 embedded in paraffin. Serial sections (3 μm) of these specimens were obtained for routine stains
226 hematoxylin-eosin-saffron (HES) and Giemsa, and special stains including Grocott-Gomori
227 methanamine silver for fungi, periodic-acid Schiff to detect polysaccharides, mucosubstances and
228 mucin in tissus and Ziehl-Neelsen for mycobacteria.
229 The slide could be scanned and visualized on a computer. The microscopic diagnosis was
230 performed at magnifications of X 20, X 80 and X 300. Slides were examined with a Leica DM 2000
231 microscope (Germany). Microphotographs were taken with the image viewing software
232 NDP.view2 (Hamamatsu, Japan).

233

234 *4.3. Direct examination, Culture, MALDI-TOF identification*

235 The lung sample was divided in three parts inoculated into Columbia agar supplemented with
236 5% sheep blood (bioMérieux, Marcy l'Etoile, France) (COS) and incubated at 37°C for 24 hours. The
237 isolated bacterial colonies were identified by MALDI-TOF MS.
238 MALDI-TOF MS was performed on a Microflex LT spectrometer (Bruker Daltonics, Bremen,
239 Germany) as previously described (32). The obtained spectra were imported into MALDI Biotyper
240 3.0 software (Bruker) and compared to the main spectra of bacteria included in two databases
241 [Bruker as well as Microbes Evolution Phylogeny and Infections (MEPHI) lab, which is constantly
242 updated].
243 *4.4. Molecular Analysis*
244 PCR using the universal primers fd1 and rp2 targeting 16S rRNA gene, followed by
245 sequencing was performed for all colonies (33). For 16S gene amplifications, after DNA extraction
246 using EZ1®DNA tissue kit on BIOROBOT EZ1 (Qiagen, Hiden, Germany), PCR reactions were
247 performed in a total volume of 50 μL, consisting of 25 μL of AmpliTaq Gold master mix, 18 μL of
248 ultra-purified water DNAse-RNAse free, 1 μL of each primer and 5μL of DNA template. The
249 thermal cycling conditions were as follows: incubation step at 95°C for 15 minutes, 40 cycles of one
250 minute at 95°C, 30s at 50°C, 1.5 min of elongation time at 72°C followed by a final extension for five
251 minutes at 72°C. PCR amplification was performed in a thermocycler (Applied Biosystem, Paris,
252 France). The results of amplification were visualized by electrophoresis on 2% agarose gel. PCR
253 products were purified using NucleoFast 96 PCR plates (Macherey Nagel EURL, Hoerdt, France)
254 according to the manufacturer's instructions. The amplicons were sequenced using the Big Dye
255 Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) with an
256 ABI automated sequencer (Applied Biosystems).The electropherograms obtained were assembled
257 and edited using ChromasPro software (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin,
258 Australia) and compared with those available in the GenBank database by NCBI BLAST
259 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). For *Rodentibacter rarus*, the sequence obtained was
260 aligned with those available in the GenBank database. A maximum-likelihood methods was used to
261 infer the phylogenetic analyses and tree reconstruction was performed using MEGA software
262 version 7 (https://www.megasoftware.net/). Bootstrap analyses were conducted using 1000
263 replicates (**Figure 5**).
264 In addition, a specific real-time PCR, targeting the Internal Transcribed Space (ITS) for
265 *Mycobacterium* spp. (34), was performed directly on the DNA extracted from the lung. In addition,
266 we tested the lung sample for the presence of *Pneumocystis jirovecii/carinii* using primers
267 PCP_msg_F_CAAAAATAACATTGACATCAACGAGG,
268 PCP_msg_R_AAATCATGAACGAAATAACCATTGC and probe PCP_msg_P_6FAM-
269 TGCAAACCAACCAAGTGTACGACAGG as previously described (35), *Mycoplasma pneumoniae*
270 using a specific qPCR: F_CCGTTTTACTCGTGCCGCG, R_GGGAGCGCTAACCCCCG and P_6FAM-
271 AGCGTGGTGTACTATGA described previously (36) and *M. hominis* using the following primers:

Mhom_16S_F_GCTGTTATAAGGGAAGAACATTTGC, Mhom_16S_R_GGCACATAGTTAGCCATCGC and probe Mhom_16S_P_6FAM-AAATGATTGCAGACTGACGGTACCTTGTCAG.

4.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) was determined using disk diffusion test (bioMérieux, Marcy l'Etoile, France), in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

5. Conclusions

History and previous epidemics demonstrate the extent to which animal infections can affect human health. Our case study demonstrates the importance of rodent survey in cities. In New York, a metagenomics study on brown rats showed that, in addition to bacteria, numerous and various viral infections (37). This required implementation of effective control measures. Inhabitants do not throw food waste within the reach of rodents. The proliferation of rats, especially in cities, is a worldwide growing phenomenon. The World Health Organization is working to bring together experts in the control of rodent commensal reservoirs and potential vectors of zoonoses and to publish recommendations (38). Many researchers are looking for rat genetic resistances to rodenticides (6). Manufacturers are proposing new painless control methods for animals (electronic traps). Our initial work on the detection of pathogenic microorganisms in brown rats must be part of our ongoing efforts to inform health authorities about the risks these animals may present. Moreover, this case of pneumonia and the isolation of *Rodentibacter rarus* strain is of great importance as it concerns a species of commensal rodent living in the vicinity of other animals (dogs, cats, birds) as well as humans. Nevertheless, the microbial diversity of street rat populations around the world needs to be further explored.

Author Contributions: Conceptualization, B.D.; formal analysis and investigation, H.M., B.S., Y.L., H.L., H.D.; writing – original draft preparation, B.D., H.M., Y.L., H.D.; writing-review; B.D.; supervision, O.M. and B.D. All authors have read and agreed with the published version of the manuscript.

Funding: This study was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, the National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Acknowledgments: The authors thank Bastien Salvi and Lola Castinel for their technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Schweinfurth, M.K. The social life of Norway rats (*Rattus norvegicus*). *eLife* **2020**, *9*, e54020.
2. Davoust, B.; Boni, M.; Branquet, D.; Ducos de Lahitte, J.; Martet, G. [Recherche de trois infestations parasitaires chez des rats capturés à Marseille: évaluation du risque zoonosique]. *Bull. Acad. Natle Med*. **1997**, *181*, 887-897.
3. Boni, M.; Davoust, B.; Drancourt, M.; Louis, F.J.; André-Fontaine, G.; Jouan, A.; et al. [Rats et chats errants: enquête épidémiologique en milieu urbain]. *Bull.Soc.Vet.Prat.de France.* **1997**, *81*, 441–457.
4. Gundi, V.K.B.; Davoust, B.; Khamis, A.; Raoult, D.; La Scola, B. *Bartonella phoceensis* sp . nov . from European *Rattus norvegicus*. *J Clin Microbiol.* **2004**, *42(8)*, 3816–3818.
5. Socolovschi, C.; Angelakis, E.; Renvoisé, A.; Fournier, P.E.; Marié, J.L.; Davoust, B.; et al. Strikes, flooding, rats, and leptospirosis in Marseille, France. *Int. J. Infect. Dis.* **2011**, *15(10),* e710-5.
6. Desvars-Larrive, A.; Pascal, M.; Gasqui, P.; Cosson, J.F.; Benoît, E.; Lattard, V.; et al. Population genetics, community of parasites, and resistance to rodenticides in an urban brown rat (*Rattus norvegicus*) population. *PLoS One* **2017**, *12(9)*, 1–25.
7. Himsworth, C.G.; Jardine, C.M.; Parsons, K.L.; Feng, A.Y.T.; Patrick, D.M. The characteristics of wild rat (*Rattus* spp.) populations from an inner-city neighborhood with a focus on factors critical to the understanding of rat-associated zoonoses. *PLoS One* **2014**, *9(3),* e91654.
8. Himsworth, C.G.; Parsons, K.L.; Feng, A.Y.T.; Kerr, T.; Jardine, C.M.; Patrick, D.M. A mixed methods

approach to exploring the relationship between Norway rat (*Rattus norvegicus*) abundance and features of the urban environment in an inner-city neighborhood of Vancouver, Canada. *PLoS One* **2014**, *9(5),* e97776.
9. Himsworth, C.G.; Parsons, K.L.; Jardine, C.; Patrick, D.M. Rats, cities, people, and pathogens: A systematic review and narrative synthesis of literature regarding the ecology of rat-associated zoonoses in urban centers. *Vector-Borne Zoonotic Dis.* **2013**,*13(6),* 349–359.
10. Rothenburger, J.L.; Himsworth, C.G.; Clifford, C.B.; Ellis, J.; Treuting, P.M.; Leighton, F.A. Respiratory pathology and pathogens in wild urban rats (*Rattus norvegicus* and *Rattus rattus*). *Vet. Pathol.* **2015**, *52(6),*1210–1219.
11. Rothenburger, J.L.; Himsworth, C.G.; La Perle, K.M.D.; Leighton, F.A.; Nemeth, N.M.; Treuting, P.M.; et al. Pathology of wild Norway rats in Vancouver, Canada. *J. Vet. Diagnostic Investig.* **2019**, *31(2),*184–199.
12. Easterbrook, J.D.; Kaplan, J.B.; Vanasco, N.B.; Reeves, W.K.; Purcell, R.H.; Kosoy, M.Y.; et al. A survey of zoonotic pathogens carried by Norway rats in Baltimore, Maryland, USA. *Epidemiol. Infect.* **2007**, *135(7),*1192–1199.
13. Heuser, E.; Fischer, S.; Ryll, R.; Mayer-Scholl, A.; Hoffmann, D.; Spahr, C.; et al. Survey for zoonotic pathogens in Norway rat populations from Europe. *Pest Manag. Sci.* **2017**, *73(2),* 341–348.
14. Strand, T.M.; Lundkvist, Å. Rat-borne diseases at the horizon. A systematic review on infectious agents carried by rats in Europe 1995–2016. *Infect. Ecol. Epidemiol.* **2019**, *9(1),*1553461.
15. Kling, M.A. A review of respiratory system anatomy, physiology, and disease in the mouse, rat, hamster, and gerbil. Vet. Clin. North Am. - Exot. Anim. Pract. **2011**, *14*, 287–337.
16. Hu, X.J.; Li, A.; Lv, L.X.; Yuan, C.; Guo, L.; Jiang, X.; et al. High quality draft genome sequence of *Staphylococcus cohnii* subsp. *Cohnii* strain hu-01. *Stand Genomic Sci.* **2015**, *9(3),* 755–762.
17. Himsworth, C.G.; Zabek, E.; Tang, P.; Parsons, K.L.; Koehn, M.; Jardine, C.M.; et al. Bacteria isolated from conspecific bite wounds in Norway and black rats: Implications for rat bite-associated infections in people. *Vector-Borne Zoonotic Dis.* **2014,** *14(2),* 94–100.
18. Vela, A.I.; Sancho, M.P.; Domínguez, L.; Busse, H.J.; Fernández-Garayzábal, J.F. *Pelistega suis* sp. Nov., isolated from domestic and wild animals. *Int. J. Syst. Evol. Microbiol.* **2015,** *65(12),* 4909–4914.
19. Guiso, N. *Bordetella pertussis.* Mol. Med. Microbiol. **2014**, *3(3),* 1507–1527.
20. Mattoo, S.; Cherry, J.D. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin. Microbiol. Rev.* **2005,** *18(2),* 326–382.
21. Adhikary, S.; Nicklas, W.; Bisgaard, M.; Boot, R.; Kuhnert, P.; Waberschek, T.; et al. *Rodentibacter* gen. Nov. including *Rodentibacter pneumotropicus* comb. nov., *Rodentibacter heylii* sp. nov., *Rodentibacter myodis* sp. nov., *Rodentibacter ratti* sp. nov., *Rodentibacter heidelbergensis* sp. nov., *Rodentibacter trehalosifermentans* sp. nov., *Rodentibacter rarus* sp. nov., *Rodentibacter mrazii* and two genomospecies. *Int. J. Syst. Evol. Microbiol.* **2017**, *67(6),*1793–1806.
22. Bisgaard, M. Ecology and significance of *Pasteurellaceae* in animals. *Zentralbl. Bakteriol.* **1993**, *279,*7–26.
23. Sasaki, H.; Ueshiba, H.; Kanai, T.; Kawamoto, E. Experimental infection of immunodeficient and immunocompetent mice with *Rodentibacter pneumotropicus*. *Top 10 Contributions on Microbiology* Chapter 10, **2018**, pp 22. www.avidscience.com
24. Fornefett, J.; Krause, J.; Klose, K.; Fingas, F.; Hassert, R.; Benga, L.; et al. Comparative analysis of humoral immune responses and pathologies of BALB/c and C57BL/6 wildtype mice experimentally infected with a highly virulent *Rodentibacter pneumotropicus* (*Pasteurella pneumotropica*) strain. *BMC Microbiol.* **2018**, *18(1),* 1–11.
25. Benga, L.; Sager, M.; Christensen, H. From the [*Pasteurella*] *pneumotropica* complex to *Rodentibacter* spp.: an update on [*Pasteurella*] *pneumotropica*. *Vet. Microbiol.* **2018**, *217*, 121–134.
26. Otto, G.M.; Myles, M.H. Medical management and diagnostic approaches. *The Laboratory Rat.* **2020**, 421–450.
27. Fingas, F., Volke, D., Hassert, R., Fornefett, J., Funk, S., Baums, C.G.; et al. Sensitive and immunogen-specific serological detection of *Rodentibacter pneumotropicus* infections in mice. *BMC Microbiol.* **2019**, *19(1),*1–13.
28. Ragland, N.H.; Miedel, E.L.; Engelman, R.W. PCR prevalence of murine opportunistic microbes and their mitigation by using vaporized hydrogen peroxide. *J. Am. Assoc. Lab. Anim. Sci.* **2019**, *58(2),* 208–215.
29. Frebourg, N.B.; Berthelot, G.; Hocq, R.; Chibani, A.; Lemeland, J.F. Septicemia due to *Pasteurella pneumotropica*: 16S rRNA sequencing for diagnosis confirmation. *J. Clin. Microbiol.* **2002**, *40(2),* 687–689.

30. Benga, L.; Knorr, I.J.; Engelhardt, E.; Gougoula, C.; Benten, P.M.; Christensen, H.; et al. Current distribution of *Rodentibacter* species among the mice and rats of an experimental facility. *J. Am. Assoc. Lab. Anim. Sci.* **2019,** *58(4),* 475–478.
31. Herbreteau, V.; Jittapalapong, S.; Rerkamnuaychoke, W.; Chaval, Y.; Morand, S. Protocols for field and laboratory rodent studies. Retrieved from CERoPath project. Kasetsart University Press **2011,** pp 46.
32. Seng, P.; Drancourt, M.; Gouriet, F.; La Scola, B.; Fournier, P.; Rolain, J.M.; et al. Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis.* **2009**, *49(4),* 543–551.
33. Weisburg, W.G.; Barns, S.M.; Pelletier, D.A.; Lane, D.J.; 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **1991**,*173(2),* 697–703
34. Bruijnesteijn van Coppenraet, ES.; Lindeboom, J.A.; Prins, J.M.; Peeters, M.F.; Claas, E.C.J.; Kuijper, E.J. Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children. *J. Clin. Microbiol.* **2004**, *42(6),* 2644–2650.
35. Linssen, C., J. Jacobs, P. Beckers, K. Templeton, J. Bakkers, E. Kuijper, W. Melchers, M. Drent and C. Vink. Inter-laboratory comparison of three different real-time PCR assays for the detection of *Pneumocystis jiroveci* in bronchoalveolar lavage fluid samples. *J. Med. Microbiol.* **2006,** *55(9),* 1229-1235.
36. Cohen-Bacrie, S.; Ninove, L.; Nougairède, A.; Charrel, R.; Richet, H.; Minodier, P. ; et al. Revolutionizing clinical microbiology laboratory organization in hospitals with *in situ* Point-of-Care. *PLoS ONE* **2011**, *6(7),* e22403.
37. Firth, C.; Bhat, M.; Firth, M.A.; Williams, S.H.; Frye, M.J.; Simmonds, P.; et al. Detection of zoonotic pathogens and characterization of novel viruses carried by commensal *Rattus norvegicus* in New York city. *mBio* **2014**, *5(5),* 1–16.
38. Colombe, S.; Jancloes, M.; Riviere, A.; Bertherat, E. A new approach to rodent control to better protect human health: first international meeting of experts under the auspices of WHO and the Pan American Health Organization. *Weekly Epidemiological Record* **2019**, *17*, 197–203.

Annexe 3

Autochthonous human and animal leptospirosis, Marseille, France

Pablo Sanchez Fernandez[a], Angeli Kodjo[b], Hacène Medkour[a, c], **Younes Laidoudi**[a, c], Grégory Dubourg[a, c], Carole Eldin[c,d], Philippe Parola[c,d], Bernard Davoust[a,c*], Jean-Christophe Lagier[a,c]

[a]Aix Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France
[b]Leptospirosis Laboratory, VetAgro Sup, Veterinary Campus of Lyon, Marcy l'Etoile, France
[c]IHU-Méditerranée Infection, Marseille, France
[d]Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France

RAPPORT DE CAS

Publié dans : IDCases, 2020, 21, e00899

(doi.10.1016/j.idcr.2020.e00899)

IDCases 21 (2020) e00899

Contents lists available at ScienceDirect

IDCases

journal homepage: www.elsevier.com/locate/idcr

Case report

Autochthonous human and animal leptospirosis, Marseille, France

Pablo Sanchez Fernandez[a], Angeli Kodjo[b], Hacène Medkour[a,c], Younes Laidoudi[a,c], Grègory Dubourg[a,c], Carole Eldin[c,d], Philippe Parola[c,d], Bernard Davoust[a,c,*], Jean-Christophe Lagier[a,c]

[a] Aix Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France
[b] Leptospirosis Laboratory, VetAgro Sup, Veterinary Campus of Lyon, Marcy l'Etoile, France
[c] IHU Me'diterrane'e Infection, Marseille, France
[d] Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France

ARTICLE INFO

Article history:
Received 5 May 2020
Received in revised form 29 June 2020
Accepted 29 June 2020

Keywords:
France
Leptospirosis
Leptospira interrogans
Rattus norvegicus
Zoonosis

ABSTRACT

Autochtonous leptospirosis is an emerging zoonotic disease in Europe, particularly in France. We report a case of leptospirosis in a 36 year-old man, who is a recently arrived migrant from Tunisia and lives in a squat. He suffered from pulmonary and neurological involvement as well as hepatitis. Seven rats (*Rattus norvegicus*) were trapped in the squat where the patient lived. *Leptospira* spp. DNA was detected in the kidney of one rat, highlighting the most likely source of contamination. In addition to the classic recreational or professional exposure to fresh water and practice of outdoor sports as a source of leptospirosis contamination, unhealthy living conditions (homeless or squatting) and therefore frequent exposure to rats, are another risk factor for leptospirosis in Europe.

Introduction

Leptospirosis is an endemic zoonosis worldwide with a million cases per year, including 500,000 severe cases, and a mortality of around 60,000 cases per year [1]. This infectious disease is mainly encountered in South America, the Caribbean, South Asia and Oceania, favored by the tropical climate, stagnant waters and floods, as well as precariousness in urban areas [1]. Severe forms of human leptospirosis are mainly associated with the serovar Icterohaemorrhagiae present in *Rattus norvegicus* [2,3]. In France, the incidence of all forms of leptospirosis is one of the highest in Europe with an increase in the number of cases since 2014, i.e. 600 cases per year with an incidence of 1 case per 100,000 inhabitants [4]. Nevertheless, most of the cases (85 %) are imported [4]. Here, we report the clinical case of a patient suffering from autochthonous leptospirosis in Marseille, France, as well as the epidemiological survey of this patient living in a squat.

Case report

In 2018, during the winter season in December, a 36-year-old man was admitted in infectious diseases ward in Marseille (France) with fever, cough with hemoptysis, headache, stiff neck and photophobia, arthralgia, myalgia and jaundice. The symptoms were present for five days ago. This man was born in Tunisia and arrived in France three months ago. He left with his two children and wife in unsanitary conditions in a squat with presence of rat's infestation. Upon arrival to the emergency department, the patient had fever at 38.5 °C and dyspnea (respiratory rate 28/min) with cough and hemoptysis. He also presented meningismus syndrome with headache, neck stiffness, photophobia, phonophobia and scleral icterus. His main complaint was diffuse body pain with severe myalgia, especially his involving calves. Laboratory revelated an inflammatory syndrome with white blood cells at 18 giga/L, hepatic dysfunction (AST: 70 UI/L, ALT: 105 IU/L), jaundice (Total bilirubin: 54 μmol/L, conjugated bilirubin: 44 μmol/L,) and acute kidney injury (creatinin116 μmol/L). A chest and CT scan showed bilateral interstitial infiltrates (Fig. 1). A lumbar puncture was performed and revealed liquid that looks like rock water with hypercellularity (lymphocyte count at: 100 cells/mm^3, including 80 % mononuclear cells); glucose was 2.10 mmol/L and proteins were 0.67 g/L. Cerebrospinal fluid (CSF) cultures were sterile. Treatment with ceftriaxone 100 mg/kg every 12 h daily and acyclovir 15 mg/kg every 8 h was empirically initiated. After

* Corresponding author.
E-mail addresses: sanchezpfernandez@gmail.com (P. Sanchez Fernandez), angeli.kodjo@vetagro-sup.fr (A. Kodjo), hacenevet1990@yahoo.fr (H. Medkour), younes.laidoudi@yahoo.com (Y. Laidoudi), gregory.dubourg@univ-amu.fr (G. Dubourg), carole.eldin@univ-amu.fr (C. Eldin), philippe.parola@univ-amu.fr (P. Parola), bernard.davoust@gmail.com (B. Davoust), jean-christophe.lagier@univ-amu.fr (J.-C. Lagier).

https://doi.org/10.1016/j.idcr.2020.e00899

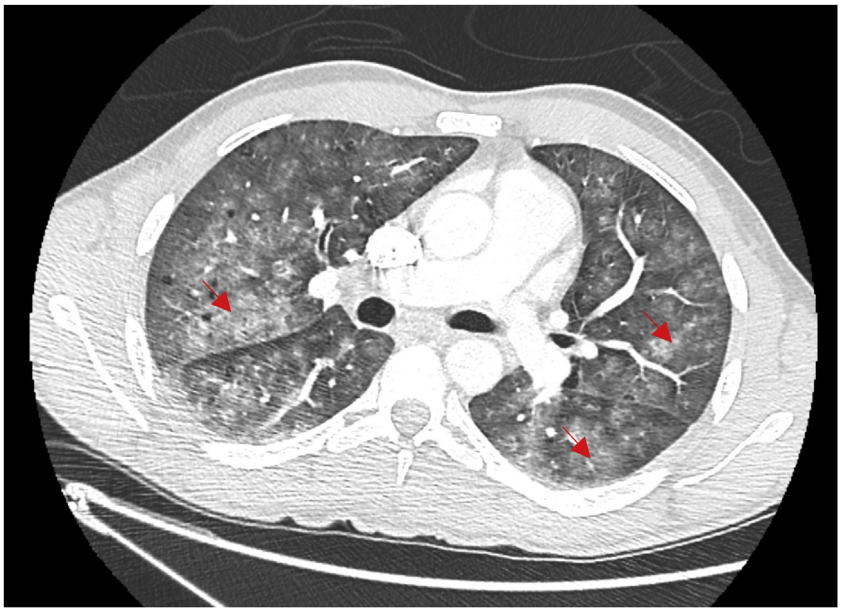


Fig. 1. Chest Ct-Scan of the patient showing interstitial bilateral pneumonia.

negative Herpes virus specific PCR, acyclovir was stopped and ceftriaxone was continued alone. Leptospirosis specific PCR targeting the 16S rRNA gene [6] was performed as from blood and urine and was found to be positive while detection was negative from CSF. In addition, the serology performed with a commercially available ELISA kit for both IgG and IgM (Institut Virion Serion GmbH, Warburg, Germany) showed positive IgM. The microscopic agglutination test (MAT) performed on serum was positive antibody titers for *Leptospira interrogans* Icterohaemorrhagiae Copenhageni (1/640), Bratislava (1/10) and Serjoe (1/160). Two and a half months later, the patient's serum was positive for Copenhageni (1/640), Icterohaemorrhagiae (1/160) and Serjoe (1/80). The patient received seven day of ceftriaxone resulting clinical improvement and discharge from hospital. He had complete clinical recovery one month post hospitalization. Interrogation of the patient showed that he lived with his family on the ground floor of a building. He had observed rat infestation outside his front door. Five weeks post hospitalization, an epidemiological veterinary investigation was conducted at the patient's home. Trapping was performed for four consecutive nights along the vicinity of a river and seven rats (*Rattus norvegicus*) were captured. After general anesthesia (ketamine) and euthanasia, the rats were autopsied. Serological analyses of these rats were performed using a MAT with a dilution threshold of 1:10 of 23 leptospire serovars. All the rats were serologically negative. The detection of *L. interrogans* DNA was performed according to the amplification protocol of the 16S locus of ribosomal DNA to which duplex PCR, amplification of the secY locus was added [5,6]. PCR was positive on the kidneys of one rat. Sequencing was not possible. All liver, lungs and urine samples were negative.

Discussion

We report a case of autochtonous human leptospirosis in Marseille (France) assessed by a veterinary survey exploring the source of contamination. We detected Leptospiral DNA in the kidney of one rat surrounding the housing of the patient, suggesting that it could be a possible source of contamination. We used a validated PCR technique with validated positive and negative controls, allowing prompt diagnosis in the patient, and DNA detection in the rat. The rat with positive kidney PCR had negative serology (at 1:10), which can be explained by the fact that animal reservoirs generally have immune tolerance to host strains [5]. It is also possible that the infection in the trapped rat had occurred recently before it seroconverted. The genus *Leptospira* includes 24 species, including 10 pathogens and more than 300 serovars. Some mammals are likely to harbor leptospires. Nevertheless, rodents are asymptomatic carriers, while dogs and livestock are susceptible animals [1,7]. Classical risks factors for Leptospirosis in Europe are recreational or professional exposure to fresh water and practice of outdoor sports, especially in the rivers after heavy rain [8,9]. However, urban or peri-urban leptospirosis has been reemerging in Europe in recent years, with reports from Italy, Greece and south of France (Marseille) [3,7,9,10]. France reports one of the highest endemicity levels in Europe, but this is mainly due to cases from the French overseas territories, with an incidence in these regions up to 10–100 times higher than in mainland France [4]. In a clinically compatible context, the association of fever, hepatitis with jaundice, acute kidney injury, conjontcivitis and neurological symptoms sould evoke the diagnosis. In most leptospirosis human cases diagnosed in metropolitan France, the origin of leptospires is unknown. They are often summer cases related to the aquatic activities [4,11]. No case of infection in the city among precarious people (immigrants without papers living in squats) has been described in France and only few case in the USA, Japan and Portugal, in a homeless person [12–15]. The proliferation of rats has already been implicated as a possible reservoir and source of transmission of leptospirosis in Marseille France, associated with the accumulation of garbage and rainfall [7]. Our case is original and the associated veterinary investigations, rarely highlighted in the literature, illustrate the persistence of this reservoir in Marseille. This case also highlights that recent immigration and unstable housing (homeless or squatting) with subsequent rat exposure is another risk factor for leptospirosis that should be taken into account by the clinicians. Our case is in addition to those already described recently. Leptospirosis of the homeless in cities is a topical disease [16].

Funding source

This work has benefited from the French State support, managed by 'Agence Nationale pour la Recherche' including the 'Programme d'Investissements d'avenir' under the reference

Méditerranée Infection 10-IAHU-03. This work was supported by the Région Provence-Alpes-Côte d'Azur and European funding FEDER PRIMI.

Contribution

Study design: JCL, BD; Management of the patient: PS, CE, GD, JCL; Data collection: PS, AK, HM, YL, GD, PP; Data analysis: PS, CE, GD, PP, BD, JCL; Writing: PS, PP, BD, JCL

Ethical approval

Not required.

Declaration of Competing Interest

None.

Acknowledgments

We thank Audrey Chapron and Sergei Castañeda for their kind cooperation.

References

[1] Levett PN. Leptospirosis. Clin Microbiol Rev 2001;14(2):296–326.
[2] Dupouey J, Faucher B, Edouard S, Richet H, Kodjo A, Drancourt M, et al. Human leptospirosis: an emerging risk in Europe? Comp Immunol Microbiol Infect Dis 2014;37(2):77–83.
[3] Dupouey J, Faucher B, Edouard S, Richet H, de Broucker C-A, Marié J-L, et al. Epidemiological investigation of a human leptospirosis case reported in a suburban area near Marseille. New Microbes New Infect 2014;2(3):82–3.
[4] Bourhy P, Septfons A, Picardeau M. Diagnostic, surveillance et épidémiologie de la leptospirose en France. Bull Epidémiol Hebd 2017;(8-9):131–7. http://invs.santepubliquefrance.fr/beh/2017/8-9/2017_8-9_1.html.
[5] Morel AS, Dubourg G, Prudent E, Edouard S, Gouriet F, Casalta JP, et al. Complementarity between targeted real-time specific PCR and conventional broad-range 16S rDNA PCR in the syndrome-driven diagnosis of infectious diseases. Eur J Clin Microbiol Infect Dis 2015;34(3):561–70.
[6] Mérien F, Amouriaux P, Perolat P, Baranton G, Saint Girons I. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. J Clin Microbiol 1992;30(9):2219–24.
[7] Socolovschi C, Angelakis E, Renvoisé A, Fournier P-E, Marié JL, Davoust B, et al. Strikes, flooding, rats, and leptospirosis in Marseille, France. Int J Infect Dis 2011;15(10):e710–715.
[8] Matsushita N, Ng CFS, Kim Y, Suzuki M, Saito N, Ariyoshi K, et al. The non-linear and lagged short-term relationship between rainfall and leptospirosis and the intermediate role of floods in the Philippines. PLoS Negl Trop Dis 2018;12(4) e0006331.
[9] Vitale M, Agnello S, Chetta M, Amato B, Vitale G, Bella CD, et al. Human leptospirosis cases in Palermo Italy. The role of rodents and climate. J Infect Public Health 2018;11(2):209–14.
[10] Panagopoulos P, Ganitis A, Papanas N, Iosifidou G, Maltsan T, Kioutsouk S, et al. Leptospirosis: a report on a series of five autochthonous cases in a Greek region. J Chemother 2016;28(5):428–31.
[11] Brockmann S, Piechotowski I, Bock-Hensley O, Winter C, Oehme R, Zimmermann S, et al. Outbreak of leptospirosis among triathlon participants in Germany, 2006. BMC Infect Dis 2010;10:91.
[12] Jones S, Kim T. Fulminant leptospirosis in a patient with human immunodeficiency virus infection: case report and review of the literature. Clin Infect Dis 2001;33(5):31–3.
[13] Bini Viotti J, Chan JC, Rivera C, Tuda C. Sporadic leptospirosis case in Florida presenting as Weil`s disease. IDCases 2019;19:e00686.
[14] Kang Ym, Hagiwara A, Uemura T. Leptospirosis infection in a homeless patient in December in Tokyo: a case report. J Med Case Rep 2015;9:198.
[15] Moreira Marques T, Nascimento PO, Almeida A, Tosatto V. Weil's disease in a young homeless man living in Lisbon. BMJ Case Rep 2020;13(6):e233543.
[16] Hagan JE, Moraga P, Costa F, Capian N, Ribeiro GS, Wunder Jr EA. Prospective study of leptospirosis transmission in an urban slum community: role of poor environment in repeated exposures to the *Leptospira* agent. PLoS Negl Trop Dis 2016;10(1) e0004275.

Références bibliographiques

Abramides GC, Roiz D, Guitart R, et al. Effectiveness of a multiple intervention strategy for the control of the tiger mosquito (*Aedes albopictus*) in Spain. *Trans R Soc Trop Med Hyg*. 2011;105(5):281-288.

Anderson RC. Nematode parasites of vertebrates: Their development and transmission. Oxfordshire, UK: CABI Publishing. 2000.

Atawodi JC, Dzikwi AA, Odoba MB, et al. Animals as reservoir of some human diseases. *Electron J Biol.* 2013;9(2):24–28.

Backer LC, Grindem CB, Corbett WT, et al. Pet dogs as sentinels for environmental contamination. In *Science of the Total Environment*. 2001;274.

Bentley SD, Maiwald M, Murphy LD, et al. Sequencing and analysis of the genome of the Whipple's disease bacterium *Tropheryma whipplei*. *Lancet*. 2003;361(9358):637-644.

Beugnet F, Marié JL. Emerging arthropod-borne diseases of companion animals in Europe. *Vet Parasitol*. 2009;163(4):298-305.

Bian G, Joshi D, Dong Y, et al. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. *Science*. 2013;340(6133):748-751.

Bourguinat C, Lee AC, Lizundia R, et al. Macrocyclic lactone resistance in *Dirofilaria immitis*: Failure of heartworm preventives and investigation of genetic markers for resistance. *Vet Parasitol*. 2015;210(3-4):167-178.

Bourtzis K, Dobson SL, Xi Z, et al. Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. *Acta Trop*. 2014;132(1),S150-S163.

Bouwman H, van den Berg H, Kylin H. DDT and malaria prevention: addressing the paradox. *Environ Health Perspect*. 2011;119(6):744-747.

Bowman DD, Liu Y, McMahan CS, et al. Forecasting United States heartworm *Dirofilaria immitis* prevalence in dogs. *Parasit Vectors.* 2016;9:540.

Bustinduy AL, Sutherland LJ, Chang-Cojulun A, et al. Age-stratified profiles of serum IL-6, IL-10, and TNF-α cytokines among Kenyan children with *Schistosoma haematobium*, *Plasmodium falciparum*, and other chronic parasitic co-infections. *Am J Trop Med Hyg*. 2015;92(5):945-951.

Calic SB, Galvão MA, Bacellar F, et al. Human ehrlichioses in Brazil: first suspect cases. *Braz J Infect Dis*. 2004;8(3):259-262.

Canning D, Priority setting and the ‘neglected’ tropical diseases. *Trans R Soc Trop Med Hyg*. 2006;100(6):499–504.

Carrion MN, Hamer DH, Evans D. Parasitic diseases, an overview. In *International Encyclopedia of Public Health*. Academic Press, USA. 2016.

Cleaveland S, Meslin F, Breiman R. Dogs can play useful role as sentinel hosts for disease. *Nature.* 2006;440:605.

Colwell DD, Dantas-Torres F, Otranto D. Vector-borne parasitic zoonoses: emerging scenarios and new perspectives. *Vet Parasitol*. 2011;182(1):14-21.

Commission Européenne. European convention for the protection of vertebrate animals used for experimental and other scientific purposes. Strasbourg. 18/03/1986. https://www.coe.int/en/web/conventions/full-list/-/conventions/treaty/123

Corbin J & Strauss A. Basics of qualitative research: Techniques and procedures for developing grounded theory (3rd ed.). Thousand Oaks, CA: Sage. 2008.

da Costa PS, Brigatte ME, Greco DB. Antibodies to *Rickettsia rickettsii, Rickettsia typhi, Coxiella burnetii, Bartonella henselae, Bartonella quintana,* and *Ehrlichia chaffeensis* among healthy population in Minas Gerais, Brazil. *Mem Inst Oswaldo Cruz*. 2005;100(8):853-859.

Dahmana H, Mediannikov O. Mosquito-borne diseases emergence/resurgence and how to effectively control it biologically. *Pathogens*. 2020;9(4):310.

Dahmani M, Davoust B, Tahir D. et al. Molecular investigation and phylogeny of Anaplasmataceae species infecting domestic animals and ticks in Corsica, France. *Parasit Vectors.* 2017;10:302.

Davoust B, Roqueplo C, Parzy D. et al. A twenty-year follow-up of canine leishmaniosis in three military kennels in southeastern France. *Parasit Vectors.* 2013;6:323.

Duncan AW, Correa MT, Levine JF, et al. The dog as a sentinel for human infection: prevalence of Borrelia burgdorferi C6 antibodies in dogs from southeastern and mid-Atlantic states. *Vector Borne Zoonotic Dis*. 2004;4(3):221-229.

Egerton FN. History of ecological sciences, Part 52: Symbiosis studies. *Bull Ecol Soc Am*. 2015;96(1):80-139.

Fain A, Vandepitte J. Description des Physaloptères (*Abbreviata caucasica* Linstow, 1902) récoltés chez l'homme au Congo. *Bull Acad R Méd Bel*.1964;4:663-682.

Favia G, Ricci I, Marzorati M, et al. Bacteria of the genus *Asaia*: A potential paratransgenic weapon against malaria. In: Aksoy S. (eds) *Transgenesis and the management of vector-borne disease. Advances in experimental medicine and biology*. Springer, New York. 2008;627.

Fenga C, Gangemi S, de Luca A, et al. Seroprevalence and occupational risk survey for *Coxiella burnetii* among exposed workers in Sicily, Southern Italy. *Int J Occup Med Environ Health*. 2015;28(5):901-907.

Ferri E, Bain O, Barbuto M, et al. New insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. *PLOS One*.

2011;6(6):e20843.

Genchi C, Kramer LH, Rivasi F. Dirofilarial infections in Europe. *Vector Borne Zoonotic Dis.* 2011;11:1307–1317.

Ghigo E, Capo C, Aurouze M, et al. Survival of *Tropheryma whipplei*, the agent of Whipple's disease, requires phagosome acidification. *Infect Immun*. 2002;70(3):1501-1506.

Gibson G. and Colwell D. Editorial. *Med Vet Entomol.* 2007;21:1-1.

Espino CI, Gómez T, González G, et al. Detection of *Wolbachia* bacteria in multiple organs and feces of the triatomine insect *Rhodnius pallescens* (Hemiptera, Reduviidae). *Appl Environ Microbiol*. 2009;75(2):547-550.

Griffiths EC, Pedersen AB, Fenton A, et al. Analysis of a summary network of co-infection in humans reveals that parasites interact most via shared resources. *Proc Biol Sci*. 2014;281(1782):20132286.

Haines A, Kovats RS, Campbell-Lendrum D, et al. Climate change and human health: impacts, vulnerability and public health. *Public Health*. 2006;120(7):585-596.

Hertig M, Wolbach SB. Studies on *Rickettsia*-like micro-organisms in insects. *J Med Res*. 1924;44(3):329-374.

Horta MC, Labruna MB, Sangioni LA, et al. Prevalence of antibodies to spotted fever group rickettsiae in humans and domestic animals in a Brazilian spotted fever-endemic area in the state of São Paulo, Brazil: serologic evidence for infection by *Rickettsia rickettsii* and another spotted fever group Rickettsia. *Am J Trop Med Hyg*. 2004;71(1):93-97.

Hosokawa T, Koga R, Kikuchi Y, et al. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc Natl Acad Sci U S A*. 2010;107(2):769-774.

Hunt P. The human right to the highest attainable standard of health: New opportunities and challenges. *Trans R Soc. Trop. Med. Hyg.* 2006;100(7):603–607.

Islamuddin M, Chouhan G, Farooque A, et al. Th1-biased immunomodulation and therapeutic potential of *Artemisia annua* in murine visceral leishmaniasis. *PLoS Negl Trop Dis*. 2015;9(1):e3321.

Jeffries CL, Walker T. The potential use of *Wolbachia*-based mosquito biocontrol strategies for Japanese encephalitis. *PLOS Negl Trop Dis.* 2015;9(6):e0003576.

Kambris Z, Cook PE, Phuc HK, et al. Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science.* 2009;326(5949):134-136.

Kersh GJ, Oliver LD, Self JS, et al. Virulence of pathogenic *Coxiella burnetii* strains after growth in the absence of host cells. *Vector Borne Zoonotic Dis*. 2011;11(11):1433-1438.

Kramer LH, Passeri B, Corona S, et al. Immunohistochemical/immunogold detection and distribution of the endosymbiont *Wolbachia* of *Dirofilaria immitis* and *Brugia pahangi* using a polyclonal antiserum raised against WSP (*Wolbachia* surface protein). *Parasitol Res*. 2003;89(5):381-386.

Kutschera U, Niklas KJ. Endosymbiosis, cell evolution, and speciation. *Theory Biosci.* 2005;124:1–24.

Landmann F, Foster JM, Slatko B, et al. Asymmetric *Wolbachia* segregation during early *Brugia malayi* embryogenesis determines its distribution in adult host tissues. *PLOS Negl Trop Dis*. 2010;4(7):e758.

Latrofa MS, Annoscia G, Colella V, et al. A real-time PCR tool for the surveillance of zoonotic *Onchocerca lupi* in dogs, cats and potential vectors. *PLOS Negl Trop Dis*. 2018;12(4):e0006402.

Lefoulon E, Bain O, Bourret J, et al. Shaking the tree: Multi-locus sequence typing usurps current onchocercid (filarial nematode) phylogeny. *PLOS Negl Trop Dis*. 2015;9(11):e0004233.

Lefoulon E, Clark T, Borveto F, et al. Pseudoscorpion *Wolbachia* symbionts: diversity and evidence for a new supergroup S. *BMC Microbiol.* 2020;20:188.

Le Page D, Bordenstein SR. *Wolbachia*: Can we save lives with a great pandemic?. *Trends Parasitol*. 2013;29(8):385-393.

Lindenmayer JM, Marshall D, Onderdonk AB. Dogs as sentinels for Lyme disease in Massachusetts. *Am J Public Health*. 1991;81(11):1448-1455.

Lindsey ARI, Bordenstein SR, Newton ILG, et al. *Wolbachia pipientis* should not be split into multiple species: A response to Ramírez-Puebla et al., "Species in *Wolbachia*? Proposal for the designation of 'Candidatus *Wolbachia bourtzisii*', 'Candidatus *Wolbachia onchocercicola*', 'Candidatus *Wolbachia blaxteri*', 'Candidatus *Wolbachia brugii*', 'Candidatus *Wolbachia taylori*', 'Candidatus *Wolbachia collembolicola*' and 'Candidatus *Wolbachia multihospitum*' for the different species within *Wolbachia* supergroups". *Syst Appl Microbiol*. 2016;39(3):220-222.

Livio M, Mobilia A, Abbate S, et al. Punture di zecche in operai forestali [Ticks bite in foresters]. *G Ital Med Lav Ergon*. 2007;29(3 Suppl):811-812.

Luce-Fedrow A, Macaluso KR, Richards AL. Growth of *Rickettsia felis* in *Drosophila melanogaster* S2 cells. *Vector Borne Zoonotic Dis*. 2014;14(2):101-110.

Maina AN, Luce-Fedrow A, Omulo S, et al. Isolation and characterization of a novel *Rickettsia* species (*Rickettsia asembonensis* sp. nov.) obtained from cat fleas (*Ctenocephalides felis*). *Int J Syst Evol Microbiol*. 2016;66(11):4512-4517.

Makepeace BL, Tanya VN. 25 Years of the *Onchocerca ochengi* model. *Trends Parasitol*. 2016;32(12):966-978.

Mancini F, Ciccozzi M, Lo Presti A, et al. Characterization of spotted fever group Rickettsiae in ticks from a city park of Rome, Italy. *Ann Ist Super Sanita*. 2015;51(4):284-290.

Martin, By William F, and Marek Mentel. 2010. "The Origin of Mitochondria Mitochondria : A Ubiquitous and Diverse Family of Organelles The Endosymbiotic Origin of Mitochondria." *Nature*, 1–5. https://doi.org/10.1038/nature03343.

Masselot F, Boulos A, Maurin M, et al. Molecular evaluation of antibiotic susceptibility: *Tropheryma whipplei* paradigm. *Antimicrob Agents Chemother*. 2003;47(5):1658-1664.

Mateo M, Maynard L, Vischer C, et al. Comparative study on the short-term efficacy and adverse effects of miltefosine and meglumine antimoniate in dogs with natural leishmaniosis. *Parasitol Res*. 2009;105(1):155-162.

Miró G, Petersen C, Cardoso L, et al. Novel areas for prevention and control of canine leishmaniosis. *Trends Parasitol*. 2018;34(5):445.

Moran NA. Symbiosis. *Curr Biol*. 2006;16(20):R866-R871.

Morchón R, Carretón E, González-Miguel J, et al. Heartworm disease (*Dirofilaria immitis*) and their vectors in Europe - New Distribution Trends. *Front Physiol*. 2012;3:196.

Nikoh N, Hosokawa T, Moriyama M, Oshima K, Hattori M, Fukatsu T. Evolutionary origin of insect-*Wolbachia* nutritional mutualism. *Proc Natl Acad Sci U S A*. 2014;111(28):10257-10262.

O'Connor L, Plichart C, Sang AC, et al. Open release of male mosquitoes infected with a wolbachia biopesticide: field performance and infection containment. *PLOS Negl Trop Dis*. 2012;6(11):e1797.

OIE. World organisation for animal health. Terrestrial animal health code. Paris. 28th Edition 2019. Chapter 7.8. Use of animals in research and education. https://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_aw_research_education.htm

Omsland A, Cockrell DC, Howe D, et al. Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. *Proc Natl Acad Sci U S A*. 2009;106(11):4430-4434.

Otranto D, Capelli G, Genchi C. Changing distribution patterns of canine vector borne diseases in Italy: leishmaniosis vs. dirofilariosis. *Parasit Vectors*. 2009;2 Suppl 1(Suppl 1):S2.

Otranto D, Dantas-Torres F, Breitschwerdt EB. Managing canine vector-borne diseases of zoonotic concern: part one. *Trends Parasitol*. 2009;25(4):157-163.

Otranto D, Eberhard ML. Zoonotic helminths affecting the human eye. *Parasit Vectors*. 2011;4:41.

Otranto D, Dantas-Torres F, Brianti E, et al. Vector-borne helminths of dogs and humans in Europe. *Parasit Vectors*. 2013;6:16.

Pennisi E. The birth of the nucleus. *Science* 2004;766-768.

Plevraki K, Koutinas AF, Kaldrymidou H, et al. Effects of allopurinol treatment on the progression of chronic nephritis in Canine leishmaniosis (*Leishmania infantum*). *J Vet Intern Med*. 2006;20(2):228-233.

Pluemer M, Dubay S, Drake D, et al. 2019. Red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*) in an urban landscape: Prevalence and risk factors for disease. *J Urban Ecol*.

2019;5(1).

Ponte-Sucre A, Gamarro F, Dujardin JC, et al. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. *PLOS Negl Trop Dis*. 2017;11(12):e0006052.

Rabinowitz P, Gordon Z, Chudnov D, et al. Animals as sentinels of bioterrorism agents. *Emerg Infect Dis*. 2006;12(4):647-652.

Ramírez-Puebla ST, Servín-Garcidueñas LE, Ormeño-Orrillo E, et al. Species in *Wolbachia*? Proposal for the designation of 'Candidatus *Wolbachia bourtzisii*', 'Candidatus *Wolbachia onchocercicola*', 'Candidatus *Wolbachia blaxteri*', 'Candidatus *Wolbachia brugii*', 'Candidatus *Wolbachia taylori*', 'Candidatus *Wolbachia collembolicola*' and 'Candidatus Wolbachia multihospitum' for the different species within *Wolbachia* supergroups. *Syst Appl Microbiol*. 2015;38(6):390-399.

Raoult D, Abat C. Developing new insecticides to prevent chaos: the real future threat. *Lancet Infect Dis*. 2017;17(8):804-805.

Ravindran R, Varghese S, Nair SN, et al. Canine filarial infections in a human *Brugia malayi* endemic area of India. *Biomed Res Int*. 2014;2014:630160.

Reiter P, Sprenger D. The used tire trade: a mechanism for the worldwide dispersal of container breeding mosquitoes. *J Am Mosq Control Assoc*. 1987;3(3):494-501.

Roger AJ, Muñoz-Gómez SA, Kamikawa R. The origin and diversification of mitochondria. *Curr Biol*. 2017;27(21):R1177-R1192.

Shane JL, Grogan CL, Cwalina C, et al. Blood meal-induced inhibition of vector-borne disease by transgenic microbiota. *Nat Commun*. 2018;9:4127.

Shaw SE, Day MJ, Birtles RJ, et al. Tick-borne infectious diseases of dogs. *Trends Parasitol*. 2001;17(2):74-80.

Shen SS, Qu XY, Zhang WZ, et al. Infection against infection: parasite antagonism against parasites, viruses and bacteria. *Infect Dis Poverty*. 2019;8(1):49.

Strait K, Else JG, Eberhard ML. Parasitic Diseases of Nonhuman Primates. Nonhuman Primates In *Biomedical Research*. Second Edi. Elsevier Inc. 2012.

Tahir D, Bittar F, Barré-Cardi H, et al. Molecular survey of *Dirofilaria immitis* and *Dirofilaria repens* by new real-time TaqMan® PCR assay in dogs and mosquitoes (Diptera: Culicidae) in Corsica (France). *Vet Parasitol*. 2017;235:1-7.

Tahir D, Davoust B, Parola P. Vector-borne nematode diseases in pets and humans in the Mediterranean Basin: An update. *Vet World*. 2019;12(10):1630-1643.

Taylor LH, Latham SM, Woolhouse ME. Risk factors for human disease emergence. *Philos Trans R Soc Lond B Biol Sci*. 2001;356(1411):983-989.

Walker T, Moreira LA. Can *Wolbachia* be used to control malaria?. *Mem Inst Oswaldo Cruz*. 2011;106 Suppl 1:212-217.

Want MY, Islamuddin M, Chouhan G, et al. Therapeutic efficacy of artemisinin-loaded nanoparticles in experimental visceral leishmaniasis. *Colloids Surf B Biointerfaces*. 2015;130:215-221.

Want MY, Islammudin M, Chouhan G, et al. Nanoliposomal artemisinin for the treatment of murine visceral leishmaniasis. *Int J Nanomedicine*. 2017;12:2189-2204.

Wernegreen JJ. Endosymbiosis. *Curr. Biol.* 2012;22:R555–R561.

World Health Organisation.WHO Guidelines on hand hygiene in health care: First global patient safety challenge clean care is safer care. *World Health*. 2009.

World Health Organisation. A global brief on vector-borne diseases. (No. WHO/DCO/WHD/2014.1). 2014.

World Health Organization. Lymphatic Filariasis. Accessed May 12, 2020. https://www.who.int/news-room/fact-sheets/detail/lymphatic-filariasis.

Worobey J, Fonseca DM, Espinosa C, et al. Child outdoor physical activity is reduced by prevalence of the Asian tiger mosquito, Aedes albopictus. *J Am Mosq Control Assoc*. 2013;29(1):78-80.

Wysmołek, Magdalena E, Maciej Klockiewicz, and Małgorzata Sobczak-filipiak. 2020. "Case Studies of Severe Microfilaremia in Four Dogs Naturally Infected With Case Studies of Severe Microfilaremia in Four Dogs Naturally Infected With *Dirofilaria Repens* as the Primary Disease or a Disease Complicating Factor," no. September. https://doi.org/10.3389/fvets.2020.577466.

Zaim M, Guillet P. Alternative insecticides: an urgent need. *Trends Parasitol*. 2002;18(4):161-163.

Zhang D, Zheng X, Xi Z, et al. Combining the sterile insect technique with the incompatible insect technique: I-impact of wolbachia infection on the fitness of triple-and double-infected strains of *Aedes albopictus*. *PLOS One*. 2015;10(4):e0121126.

Helminthoses vectorielles : Caractérisation, diagnostic et moyens de contrôle

Par Younes Laidoudi

La présente thèse étudie de façon inductive, d'une part, le paradigme de lutte contre les maladies vectorielles (principalement des helminthoses) des animaux et d'autre part le rôle d'animaux réservoirs dans la transmission des VBD. Nous avons étudié les bactéries du genre *Wolbachia*, endosymbiotique des vecteurs et des filaires, souvent utilisées pour la lutte contre les VBD. Nous avons isolé et caractérisé le génome de *Wolbachia massiliensis* sp. nov. (*wChem*), souche type d'un nouveau supergroupe T, *Wolbachia* de *Cimex hemipterius* collectée au Sénégal. L'étude taxo-génomique souligne la nette distinction de cette nouvelle espèce de toutes les autres *Wolbachia*, et la nécessité de revoir la taxonomie de ce genre bactérien. L'analyse du génome de *W. massiliensis* montre un profil proche de celui de la *Wolbachia* mutualistique de la filariose humaine *Brugia malayi*, faisant d'elle une nouvelle piste pour approfondir les connaissances de cette relation symbiotique. D'un autre côté, l'implication des *Wolbachia* en tant que cible moléculaire pour le diagnostic des filarioses a permis d'améliorer la qualité de leur diagnostic. Ainsi, nous avons proposé de nouvelles approches moléculaires de génotypage et d'exploration des filaires et nématodes, et des outils de diagnostic combinant la détection multiple par qPCR, des filaires ainsi que de leurs *Wolbachia.* Les principales espèces ciblées sont celles sévissant dans le Bassin méditerranéen. Dans un second volet, on a caractérisé des nématodes de primates non-humains (PNHs) du Nouveau et de l'Ancien Monde. Nos résultats montrent qu'au moins onze espèces de nématodes gastro-intestinaux, ont été caractérisées à partir de fèces des PNHs d'Afrique, particulièrement *Abbreviata caucasica*. Nous avons également fourni des données préliminaires sur des filaires, non identifiées auparavant, chez des singes hurleurs de l'Amazonie, infectés avec au moins trois génotypes, dont un du genre *Brugia*, potentiellement zoonotique. En termes de protection contre les CVBD, nous avons prouvé d'abord, au laboratoire, l'effet protecteur d'un insecticide (Vectra® 3D) à la fois sur les hôtes traités et non traités présents à proche distance de ces derniers. Ensuite, via un essai terrain de supériorité, nous avons prouvé l'efficacité de la stratégie prophylactique mensuelle multimodale, basée sur l'utilisation de deux produits (Vectra® 3D associé au Milbactor®) contre les dirofilarioses, la Leishmaniose canine (LCan) et l'ehrlichiose, en Corse. En outre, on a démontré, via un essai de non-infériorité, l'efficacité de l'artésunate dans le traitement de la LCan. Dans le cadre de la lutte biologique contre les vecteurs, on a partiellement caractérisé une amine produite par la souche *Serratia marcescens* P400, dotée d'un effet insecticide supérieur à celui de l'ivermectine contre les larves d'*Aedes albopictus*. On a également détecté l'infection naturelle des tiques par des guêpes parasitoïdes. Ces données peuvent offrir une excellente alternative de lutte biologique contre ces vecteurs. Finalement, on a démontré le rôle joué par les animaux réservoirs et sentinelles de la transmission et de la propagation des VBD. Ainsi, l'hôte canin, particulièrement le chien, en Guyane, en France métropolitaine, en Algérie et en Côte d'Ivoire, a un rôle sentinelle relatif au maintien et à la propagation des VBD (dirofilarioses, leishmanioses et trypanosomose). En Italie, on a mis en évidence le rôle de sentinelle des rickettsioses joué par les reptiles, alors que les gorilles, en République du Congo, ont un rôle sentinelle pour des nematodoses gastro-intestinaaux. Finalement, ma thèse montre l'utilité de développer et d'adapter de nouvelles stratégies de contrôle et de surveillance des maladies vectorielles. Elle vise, modestement, à suppléer au manque de mise en œuvre de techniques performantes et donc de connaissances dans la perspective d'un paradigme complet d'étude des phénomènes infectieux dans leur écosystème.

Mots-clés : *Wolbachia*, filaires, nématodes, vecteurs, sentinelle, réservoir, diagnostic, prophylaxie, traitement.