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Mr le Docteur Pascal DELAUNAY	Université de Nice	Rapporteur
Mr le Professeur Philippe PAROLA	Aix-Marseille Université	Directeur de thèse

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AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie 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 publiée dans un journal scientifique 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 où soumis associée 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.

Professeur Didier RAOULT

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RESUME

Les vecteurs sont des arthropodes hématophages assurant la transmission biologique active des nombreux pathogènes (bactéries, virus et parasites), d'un animal à un autre, dont l'homme. Les agents infectieux transmis sont responsables de millions de cas d'infections humaines et animales et de décès chaque année à travers le monde. Les pathogènes transmis par ces arthropodes causent parfois des maladies émergentes ou réémergentes peu connues ou sous estimées surtout dans les pays du sud. La lutte anti-vectorielle et la surveillance épidémiologique des vecteurs sont essentielles dans les stratégies de lutte et de contrôle de ces maladies vectorielles.

L'identification correcte des arthropodes, la détermination de l'origine du repas sanguin ainsi que la caractérisation du répertoire des pathogènes associés sont des étapes cruciales dans la lutte contre les vecteurs. Nos travaux ont ainsi porté sur une combinaison des méthodes (morphologie, moléculaire et MALDI-TOF MS) pour l'identification des arthropodes, la détermination de repas de sang et des pathogènes associés avec les hôtes et leurs arthropodes.

Dans la première partie de notre travail, nous avons utilisé l'outil MALDI-TOF MS pour identifier des tiques collectées au Mali et conservées dans de l'éthanol 70%. Les tiques étaient aussi testées par la biologie moléculaire pour rechercher les microorganismes associés. Nous avons également identifiés par MALDI-TOF MS des moustiques collectés au Tchad et conservés avec du silicagel. L'origine animale du repas sanguin de ces moustiques a également pu être étudiée par cet outil.

La seconde partie nous a permis de détecter à l'aide d'outils de biologie moléculaire des microorganimes dans des animaux (bœufs, moutons, chèvres, chevaux, ânes, rongeurs et insectivores) et leurs ectoparasites (tiques, puces et mites) en Algérie et au Mali.

Nos travaux nous ont donc permis de confirmer l'intérêt du MALDI-TOF MS en entomologie médicale, avec pour la première fois son application sur des moustiques collectés sur le terrain au sud et conservé avec du siligalel, et de compléter le répertoire des agents pathogènes associés aux arthropodes au Mali et en Algérie. Nos perspectives comprennent un travail de collaboration pour l'utilisation de ces outils innovants dans une étude des tiques de la République Démocratique du Congo.

ABSTRACT

Vectors are blood-sucking arthropods that ensure the active biological transmission of many pathogens (bacteria, viruses and parasites) from one animal to another, including humans. Transmitted infectious agents are responsible for millions of cases of human and animal infections and deaths each year around the world. Pathogens transmitted by these arthropods sometimes cause emerging or re-emerging diseases that are little known or underestimated, especially in southern countries. Vector control and epidemiological surveillance of vectors are essential in strategies for the control and monitoring of these vector-borne diseases.

The correct identification of arthropods, the determination of the origin of the blood meal and the characterization of the repertoire of associated pathogens are crucial steps in vector control. Our work thus focused on a combination of methods (morphology, molecular and MALDI-TOF MS) for identifying arthropods, determining blood meals and pathogens associated with hosts and their arthropods.

In the first part of our work, we used the MALDI-TOF MS tool to identify ticks collected in Mali and stored in 70% ethanol. Ticks were also tested by molecular biology to look for associated microorganisms. We also identified by MALDI-TOF MS mosquitoes collected in Chad and preserved with silicagel. The animal origin of the blood meal of these mosquitoes could also be studied by this tool.

The second part allowed us to detect microorganisms in animals (oxen, sheep, goats, horses, donkeys, rodents and insectivores) and their ectoparasites (ticks, fleas and mites) in Algeria and mali using molecular biology tools.

Our work therefore enabled us to confirm the interest of MALDI-TOF MS in medical entomology, with for the first time its application on mosquitoes collected in the field in the

south and stored in silica gel, and to complete the directory of agents associated with arthropods in Mali and Algeria. Our perspectives include collaborative work to use these innovative tools in a study of ticks in the Democratic Republic of Congo.

INTRODUCTION

L'entomologie médicale est l'étude des insectes et plus globalement des arthropodes qui ont un impact sur la santé humaine. Elle a des liens étroits avec l'entomologie vétérinaire et les sciences de l'environnement, dans le concept « one health ». Elle aborde plusieurs domaines tel que la taxonomie, la biologie, la physiologie, l'écologie, les interactions entre les pathogènes et leurs vecteurs, les stratégies de contrôle et de lutte contre les populations de vecteurs [1].

Les arthropodes sont le phylum le plus diversifié avec près de 85% des espèces animales existantes décrites [2]. Les arthropodes vecteurs sont des hématophages assurant la transmission biologique active d'un agent pathogène (bactéries, parasites, virus) d'un vertébré à un autre lors de leur repas sanguin [3]. Les moustiques et les tiques considérés respectivement comme premier et second vecteurs de maladies transmissibles chez les humains et animaux sont responsables de centaines de milliers de cas d'infections humaines et de décès à travers le monde chaque année [1]. Depuis plusieurs décennies, les moustiques et les tiques sont à l'origine de l'émergence ou réémergence de plusieurs maladies infectieuses partout dans le monde [4]. Cette émergence et réémergence résulte d'une part à la réponse évolutive des agents pathogènes eux même ou d'autre part à la modification de leur écosystème constituant ainsi une menace pour la santé humaine et animale [5,6].

La recherche et le suivi entomologique, essentiels dans la lutte contre les arthropodes vecteurs, nécessitent une identification correcte des arthropodes, la détermination du statut infectieux ainsi que de leur préférence trophique. Les méthodes classiques utilisées pour cela sont la morphologie et la biologie moléculaire. L'identification morphologique basée sur l'observation des caractères morphologiques nécessite une expertise et une documentation appropriées. Les experts en entomologie sont de plus en plus rares, et en général il s'agit

d'expert d'un groupe d'arthropodes. Même pour eux, il est difficile de distinguer les espèces d'un même complexe, ou des spécimens immatures ou endommagés. La biologie moléculaire a été utilisée pour l'identification des arthropodes depuis plus d'une vingtaine d'année. Cette technique est limitée par son coût relativement élevé, le temps assez long, l'absence d'une séquence génomique « idéale » à étudier, mais aussi par la présence de séquences de référence incomplètes ou erronées dans GenBank [7].

Depuis 15 ans, la spectrométrie de masse MALDI-TOF (Matrix-assisted Laser Desorption/Ionization Time-of-Flight) a révolutionné le domaine de la microbiologie clinique par l'identification rapide, fiable et moins couteuse des micro-organismes (bactéries, parasites et champignons) [8,9]. Cette approche est basée sur l'ionisation des molécules protéiques d'un organisme d'intérêt qui vont co-cristaliser avec une matrice. Les molécules seront propulsées dans un tube sous vide selon le ratio masse/charge et leur temps de vol sera capté par un détecteur placé à la fin du tube. Un pic sera associé à chacune des molécules détectées et un spectre global spécifique de l'organisme d'intérêt sera généré. Une base de données de spectres de référence sera construite à partir d'échantillons formellement identifiés morphologiquement et confirmés par biologie moléculaire. Cette base de données permettra d'identifier ou non les échantillons inconnus par comparaison de spectres à une base de donnée.

Ces dernières années la spectrométrie de masse a été utilisée avec succès pour l'identification de nombreux arthropodes élevés au laboratoire [10–13], collectés sur le terrain et congelés [14–21] ou conservés dans l'alcool [22,23]. Le MALDI-TOF MS a aussi été utilisé pour l'identification du repas sanguins des moustiques [20,24,25] et la détermination du statut infectieux de certains arthropodes au cours des modèles expérimentaux [26–32]. L'UMR VITROME a été pionnière dans ce domaine par la création et l'implémentation continue d'une base de données de spectre d'arthropodes [1]. Si le spectre obtenu est de

qualité et que la valeur du score identification est ≥ à 1.7 cela signifie qu'il est correctement identifié et lorsqu'il est faible (<1.7), cela signifie généralement que les spectres de cette espèce ne sont pas dans la base de données. L'arthropode est alors identifié par morphologie et biologie moléculaire puis son spectre introduit dans la base de données comme référence.

Au cours de cette thèse, mon souhait était d'étudier en premier lieu les tiques et les pathogènes associés au Mali dont je suis originaire. Avant d'entamer ce travail et faire un état des lieux, j'ai rédigé une revue intitulée "Ticks and tick-borne diseases to humans and animals in West Africa*"* pour faire le point sur les tiques et les maladies transmises par les tiques aux humains et aux animaux en Afrique de l'Ouest (Article 1).

Les objectifs généraux des travaux originaux de cette thèse étaient dans un premier temps d'appliquer et mettre au point l'outil MALDI-TOF Ms pour identification des arthropodes et de leur repas de sang collectés sur le terrain dans deux pays du sud (Mali, Tchad). Le premier travail de cette partie consistait à l'identification des tiques collectées sur les bovins et les chiens au Mali et conservées dans de l'alcool (Article 2). Le second portait sur l'évaluation de la capacité du MALDI-TOF MS à identifier les moustiques et leur repas de sang collectés au Tchad et conservés dans du silicagel (Article 3).

Dans un second temps, nous avons étudié et complété le répertoire de micro-organismes associés aux arthropodes et aux animaux en Algérie et Mali en utilisant les méthodes de biologie moléculaire. Nous avons détecté les bactéries dans les tiques collectées au Mali (Article 2). Nous avons recherché les protozoaires et les bactéries dans les animaux domestiques et leurs tiques dans le Nord-Est d'Algérie (Article 4). Enfin avons aussi recherché les microorganismes chez les petits mammifères (rongeurs et insectivores) et leurs ectoparasites (tiques, puces, mites) au Mali (Article 5).

ARTICLE 1: REVUE DE LA LITTERETURE

Ticks and tick-borne diseases to humans and animals in West Africa.

Adama Zan DIARRA, Carole ELDIN, Philippe PAROLA

(Article à soumettre à Parasites and vectors)

Préambule

Les tiques sont des acariens hématophages, assurant la transmission de nombreux agents pathogènes (bactéries, parasites, virus) responsables de maladies humaines et animales dans le monde. Actuellement considérées comme les seconds vecteurs de maladies humaines et animales, les tiques et les maladies transmises par les tiques restent mal connues ou sous-estimés en Afrique de l'Ouest. Plusieurs raisons peuvent l'expliquer comme le manque d'expert sur les tiques, de personnel qualifié pour le diagnostic clinique et biologique de ces maladies, ou du fait de la présence de maladies dont l'importance en santé publique est connue comme le paludisme, la tuberculose et le VIH SIDA. Malgré l'existence des données sur des tiques et des agents pathogènes associés aux animaux, peu revue de la littérature dans ce domaine n'est disponible en Afrique de l'Ouest.

L'objectif cette revue était de faire un état des lieux des connaissances sur les tiques et les maladies humaines et animales associées au Benin, Burkina Faso, Cape Vert, Cote d'Ivoire, Gambie, Ghana, Guinée, Guinée Bissau, Liberia, Mali, Mauritanie, Niger, Nigeria, Sénégal, Sierra Leone et Togo dans le concept du « One Health ».

Nous avons trouvé que de nombreux travaux rapportent la détection des pathogènes dans les tiques vectrices et chez les animaux sauvages et domestiques. Cependant très peu d'études rapportent la présence des ces pathogènes chez les humains. Cela pourrait être due aux raisons que nous avons évoqué précédemment. La présence de vecteurs compétents et des pathogènes dans une même localité devraient attirer l'attention des cliniciens sur leur possible transmission chez humains et des causes probable de fièvres inconnues.

Ticks and tick-borne diseases in humans and animals in West Africa

Adama Zan DIARRA[1,2], Carole ELDIN[1,2], Philippe PAROLA[1,2*]

[1] IHU-Méditerranée Infection, Marseille, France.

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

*** Corresponding author**: Prof. Philippe Parola. VITROME, Institut Hospitalo-Universitaire Méditerranée Infection, 19-21 Boulevard Jean Moulin 13385 Marseille Cedex 05, France. Phone: + 33 (0) 4 13 73 24 01. Fax: + 33 (0) 4 13 73 24 02.

Email: philippe.parola@univ-amu.fr.

Abstract

Ticks are an important group of arthropod vectors, transmitting many pathogens responsible for human and animal diseases worldwide. Currently considered as the second biggest vector of human and animal diseases, the infections transmitted by ticks remain poorly known or underestimated in West Africa. This may be explained by the lack of qualified personnel and specialised laboratories. Despite the existence of numerous research projects in the field of ticks and associated pathogens, no review of current literature in this field is available in West Africa. In this review, we provide an update on pathogens (bacteria, parasites and viruses) reported in ticks, humans and animals in West Africa by molecular, serological and microscopic methods to alert the professionals working in the field of human and animal health about the presence of these pathogens.

Keywords: Ticks, Tick-borne diseases, Humans, Animal, West Africa,

Introduction

Ticks are invertebrate animals belonging to the phylum Arthropoda, class Arachnida and to the order Acari, and are obligate parasites that feed on blood [1]. Ticks are divided into two main families including Ixodidae (hard ticks) comprising over 700 species worldwide, and Argasidae (soft ticks) comprising roughly 200 species [2]. Currently, in Africa, domestic animals are most often infested with ten genera of ticks, seven of which are ixodidae and three are Argasidae, including approximately 48 species [1]. Ticks are parasites of most vertebrates in the world and can sometimes bite humans [3]. After mosquitos ticks are the second biggest vector and reservoir in the world of animal and human pathogens ranging from viruses, bacteria and protozoa [4]. More than 80% of the world's cattle population is at risk of contracting tick-borne diseases [5]. Today, ticks and tick-borne diseases (TBD) are a significant threat to the health, welfare and productivity of livestock in the whole of sub-Saharan Africa. The impact of ticks on animals is related to their blood-feeding, from which both their direct and indirect pathogenicity originates [6]. Despite the existence of numerous research projects into ticks and associated pathogens, no review of literature in this field is currently available in West Africa.

West Africa is a land-based region spanning the entire western part of sub-Saharan Africa. It roughly includes the coastal countries north of the Gulf of Guinea to the Senegal River, the countries covered by the Niger River Basin and the countries of the Sahel hinterland [7]. This zone represents one fifth of the area of the African continent with an area of 6,140,000 km^2 and covers 16 countries including Benin, Burkina Faso, Cape Verde, Côte d'Ivoire, Gambia, Ghana, Guinea, Guinea Bissau, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone, Togo [7].

In this review, we address human and animal tick-borne diseases in West Africa under the concept of "One Health". One Health is a concept that encourages the spirit of cooperation among animal, environmental and human health professionals and the development of integrated solutions for complex problems that impact on the health of animals, humans and the planet [8]. To carry out this review, we searched the Pubmed and Google scholar databases by entering the following keywords: Ticks, Tick-borne diseases, Humans, Animal, West Africa, *Rickettsia*, *Borrelia*, *Anaplasma*, *Ehrlichia*, *Bartonella*, *Coxiella*, *Theileria*, *Babesia*, *Hepatozoon* and Crimean-Congo haemorrhagic fever virus. Only articles in English

or with a summary in English were selected with no time limit and we included both molecular biology and serology studies.

A. Bacterial diseases

- Tick-borne spotted fever group Rickettsioses:

Bacteria of the genus Rickettsia are obligate intracellular organisms responsible for rickettsioses. The genus includes 31 species, of which about 17 are considered pathogens of animals and humans. These bacteria are divided into two main groups: the spotted fever group (SFG) and the typhus group (TG) [9]. The typhus group comprises two species, *Rickettsia typhi* and *Rickettsia prowazekii*, respectively transmitted by human body lice and fleas, and the SFG comprises around 30 species mainly transmitted by ticks, with the exception of *Rickettsia felis* which is transmitted by fleas and mosquitoes [10-12]. In humans, SFG rickettsioses manifest mainly themselves through fever, headache, muscle pain, regional lymphadenitis and an inconstant rash which may be maculopapular but also vesicular [9].

In West Africa, *R. africae*, the agent of African tick bite fever (ATBF), *R. conorii,* the agent of Mediterranean spotted fever (MSF), and *R. mongolitimonae* the agent of Lymphangitis-associated rickettsioses, and two other agents of emerging rickettsioses in humans (*R. aeschlimannii* and *R. massiliae)* have been reported [9] (Figure 1).

R. africae is transmitted by ticks of the genus *Amblyomma*, primarily *Am. variegatum* and *Am. hebraeum* (Figure 2). The infection rate of *R. africae* in these ticks is often very high, up to 100% in endemic areas [9]. ATBF is the second most common documented etiology of fever in ill travellers returning from sub-Saharan Africa, after malaria [13]. More than 350 travel-associated cases of African tick bite fever have been reported in travellers from EU member countries, North America, Australia, Argentina and Japan. Most of these travellers were infected in South Africa. However, in West Africa, cases of ATBF have only been reported among travellers who had stayed in The Gambia [13]. The reasons for this may be the lack of diagnostic laboratories, lack of knowledge of these diseases by clinicians, lack of specialists, by the fact that the disease is most often benign, and the fact that the eschar and discreet rash can pass unnoticed on black skin [9]. However, in humans, the prevalence of antibodies against *R. africae* was 19.6% among blood donors and patients from Mauritania [14], 1.1 to 25.4% in the Republic of Guinea [15], and 20.6% to 45.6% in

patients who had recently experienced a fever and had negative results for malaria in the villages of Dielmo and Ndiop in Senegal respectively [16].

In animals, a single animal study showed that the prevalence of antibodies against R. africae in farmed animals ranged from 0.6 to 18.8% in the Republic of Guinea [15].

In ticks, *R. africae* has been detected in *Am. variegatum* from Côte d'Ivoire, Mali, Niger, Liberia, Guinea, Nigeria, Senegal, Benin and Burkina Faso [16-25] and in *Am. compressum* from Liberia [21] (Table 1). This bacterium has been found in *Hyalomma marginatum rufipes* from Mali, Côte d'Ivoire, Guinea and Senegal [18,19,21,24], *Hy. truncatum* from Mali and Côte d'Ivoire [18,19], *Hy. impeltatum* from Nigeria [26], and *Hy. impressum* ticks from Côte d'Ivoire [19] (Table 1). It was also detected in *Haemaphysalis paraleachi* from Guinea [21]; in *Rhipicephalus Boophilus annulatus* from Senegal, Guinea and Nigeria [16,21,23]; in *Rh. evertsi* from Mali, Senegal and Nigeria [16,18,23]; in *Rh.*(*B*) *decoloratus* from Guinea and Nigeria and in *Rh.*(*B*) *geigyi*, *Rh. sanguineus* s.l. and *Rh.*(*B*) *microplus* from Liberia, Nigeria, and Côte d'Ivoire respectively [19,21,26] and recently in *Rh.* (*B*) *microplus* collected in cattle from Mali [18] (Table 1).

R. africae is transmitted by ticks of the genus *Amblyomma* [9]; its detection in other tick genera and species doesn't prove that they are all vectors for this pathogen. Further studies should be undertaken to test vector competence of these tick species for this pathogen.

R. conorii, an agent of MSF mainly transmitted by *Rh. sanguineus* s.l. (Figure 2) [9] is one of the oldest diseases transmitted by arthropods, and is endemic in the Mediterranean region, where the majority of cases are encountered in the warmest months [27]. MSF is characterised by high fever, flu-like symptoms, a black pressure ulcer at the site of the tick bite and a macula-papular rash, neurological disorders and the involvement of several organs can occur in severe forms [28].

In West Africa only one case of molecular detection of *R. conorii* has been identified in a patient with a recent fever and who was negative for malaria in Senegal [16]. However, the seroprevalence of antibodies against *R. conorii* was 38.2%, 31.5% and 27% using indirect immunofluorescent antibody (IFA) and 47.8%, 34.8% and 30% by western blot testing in apparently healthy adults (blood donors or volunteers) from Burkina Faso, Côte d'Ivoire and Mali respectively [29], and 13.5% in blood donors from Mauritania [14]. However, serological diagnostic methods are limited by cross-reactivity with other species of Rickettsia SFG. To date *R. conorii* has not been reported in animals in West Africa.

In ticks, DNA of *R. conorii* was detected in two *Rh. sanguineus* s.l. collected from rodents in Nigeria [30] and in one *Rh. e. evertsi* collected from a horse in Senegal [16] (Table 1).

R. aeschlimannii, a member of SFG, was first isolated from *Hy. m. marginatum* but several tick species are now considered as confirmed or potential vectors [9,28]. The pathogenicity of this bacterium was demonstrated in a French patient returning from a trip from Morocco [31]. In humans, a few cases of rickettsiosis caused by *R. aeschlimannii* have been reported in Europe [28].

In West Africa, *R. aeschlimannii* has not been detected in humans and domestic animals. However, the DNA of *R. aeschlimannii* has been detected in *Hy. truncatum*, *Hy. m.rufipes* and *Rh. sanguineus* s.l. from Mali, [18], in *Hy. m. rufipes* collected in Côte d'Ivoire, Senegal, Burkina Faso and Nigeria [16,19,24,25,32], in *Hy. truncatum* collected in Côte d'Ivoire, Senegal and Burkina Faso [16,19,25], in *Hy. impeltatum* collected in Senegal and Nigeria [24,32] and in *Rh. e. evertsi* collected in Senegal [16,24] (Table 1).

R. massiliae transmitted by *Rhipicephalus* spp. has been reported only in humans in South America and Europe and manifests itself in fever, a palpable purpuric rash and eschar [9]. In West Africa this bacterium has not been detected in human. In animals, one study has detected the DNA of *R. massiliae* in 3.5% of cattle from Nigeria [20]. In ticks, *R. massiliae* has been detected in *Rhipicephalus* sp. and *Rh. senegalensis* from Côte d'Ivoire [19,33], in *Rh. guilhoni* and *Rh. senegalensis* from Senegal and Liberia respectively [16,21] (Table 1).

Finally, *R. mongolitimonae,* mainly transmitted by ticks of the genus *Hyalomma,* has been reported in humans in France, Greece, Portugal and Spain but also in travellers returning from North Africa (Algeria and Egypt) [9]. The clinical symptoms are fever, maculopapular rash, one or more pressure ulcers, enlarged regional lymphadenitis and lymphangitis, which is characteristic of this disease, hence its name "rickettsiosis associated with lymphangitis" [28]. No case of *R. mongolitimonae* rickettsiosis has been reported in West Africa in humans or animals. However, the DNA of *R. mongolitimonae* was detected in *Hy. truncatum* ticks collected from cattle in Senegal and Mali [16,18] (Table 1).

The very few data on human rickettsioses in West Africa may be due to the lack of specialised laboratories capable of performing differential diagnoses in patients presenting

166 with fevers of unknown origin and the difficulty of identifying maculo-popular rashes and
167 eschars on black skin [9].

168 - **Tick Borne Relapsing Fevers**

169 TBRFs are acute febrile illnesses, characterised by multiple recurrences of fever, headache,
170 myalgia and arthralgia, caused by spirochetes bacteria of the genus *Borrelia* (*B. crocidurae*,
171 *B. duttonii* and *B. hispanica*) [34]. In subtropical regions around the world, TBRF are endemic
172 and transmitted by soft ticks of the genus *Ornithodoros.* It is a major cause of febrile illness in
173 several regions of Africa [35]. In West Africa TBRFs are mainly caused by *B. crocidurae* and
174 are transmitted by *O. sonrai*; in the savanna zones of eastern and southern Africa, *B. duttonii*
175 is responsible for TBRF transmitted by *O. moubata* s.l. and, finally, in North Africa, TBRF is
176 caused by *B. hispanica* with *O. erraticus* as vector [35].
177 In West Africa *B. crocidurae* and *B. duttonii* are responsible for TBFR, *B. theileri* responsible
178 for bovine borreliosis and other *Borrelia* species of unknown pathogenicity have been
179 detected in humans, animals and ticks [36] (Figure 3). TBRF has been studied more in
180 Senegal than in other countries in West Africa [37]. In humans from Senegal, the microscopic
181 incidence of *B. crocidurae* varies depending on the study and location. It was 0.9% between
182 1989–1990 in children under the age of 14, who were patients with acute fevers presenting at
183 the Keur Moussa dispensary [38]. The average incidence of TBRF was 11 per 100 person-
184 years between 1990 and 2003 [39]. The DNA of *B. crocidurae* was detected in 5.1% of
185 patients with a fever in Dielmo [40], in 0.42% patients suspected of having malaria who were
186 examined at the Institut Pasteur in Dakar between October 1999 and October 2003 [41], in
187 15% and 0.3% of patients who tested negative for malaria in Dielmo and Ndiop respectively
188 in 2011 [42], 9.5% in febrile patients from Dielmo and Ndiop between February 2011 and
189 January 2012 [43]. Tick-borne relapsing fever was reported in patients with a fever returning
190 from Senegal [44]. The DNA of *B. crocidurae* and B. *duttonii* was detected in 8.8% and 1.2%
191 respectively of febrile patients from Togo [45] and *B. crocidurea* was detected in 3.4% of
192 febrile patients who tested negative for Malaria in Mali [46]. However, in animals, *B.*
193 *crocudirae* was detected in asymptomatic small mammals in Senegal, Mali and Mauritania
194 [47]. In Senegal, 17.6% of 740 rodents and 7.3% of 55 musk shrews tested had positive blood
195 smears for *B. crocidurae* [48]. In Mali 11.3% (82/726) of animals (rodents and shrews) were
196 positive for antibodies against relapsing fever spirochetes, while 2.20% (16/724) were
197 revealed to have spirochetes in their blood by microscopic analysis [49]. In ticks, *B.*

198 *crocidurae* has been reported in *O. sonrai* from Mauritania, Senegal, Mali and The Gambia
199 [35,49,50] (Table 1).
200 *Borrelia theileri*, a spirochete responsible for bovine borreliosis, has been reported in Africa,
201 Australia, and North and South America in cows, goats and sheep. In cattle, infection usually
202 manifests itself through fever and anaemia [51]. In West Africa, the DNA of *B. theileri* has
203 been detected in *Rh.* (*B*) *geigyi* ticks collected from cattle in Mali [51] (Table1).
204 Other *Borrelia* species of unknown pathogenicity for humans and animals have been detected
205 in ticks and cattle in West Africa. *Borrelia* spp. were detected in domestic animals in Ghana
206 [52], in ticks collected from vegetation in Nigeria [23], and in *Am variegatum* and *Hy.*
207 *truncatum* ticks collected in Mali [18] (Table 1). Two potential new species of *Borrelia*
208 (*Candidatus* Borrelia africana and *Candidatus* Borrelia ivorensis) that are phylogenetically
209 distant from both the relapsing fever group and the Lyme disease group borreliae have been
210 detected in *Am. variegatum* from Côte d'Ivoire [19] (Table 1).
211 - **Anaplasmosis:**
212 Anaplasmosis is a vector-borne infection of humans and animals, caused by several
213 *Anaplasma* species, mainly transmitted by ticks in vertebrate hosts (Figure 4) [53-55]. In
214 humans, anaplasmosis is mainly caused by *A. phagocytophilum* [56] and *A. marginale*. *A.*
215 *centrale*, *A. ovis*, *A. phagocytophilum*, *A. platys* and *A. bovis* is responsible for animals
216 anaplasmosis, manifested by fever, jaundice, pale mucous membranes, loss of appetite,
217 weight loss, lethargy, jaundice, gastrointestinal signs, abortion, a reduction in milk production
218 and often death. The manifestations are more serious in old animals than young animals [56].
219 *Anaplasma phagocytophilum* is a mandatory intragranulocytic bacterium of major
220 importance for human and veterinary health, present worldwide and capable of infecting
221 many hosts, including humans and many animals. In humans, it is responsible for human
222 granulocytic anaplasmosis (HGA) which manifests itself in fever, malaise, headache, myalgia,
223 neck stiffness and nausea [56]. *Anaplasma phagocytophilum* is mainly transmitted by ticks of
224 the genus Ixodes but has also been detected in other tick species such as *D. reticulatus*, *D.*
225 *variabilis*, *D. occidentalis*, *Hae. concinna*, *Am. americanum* and *Am. ventalloi* whose
226 vectorial capacities in terms of transmission are not known [56]. In West Africa, the DNA of
227 *A. Phagocytophilum* was detected in one healthy dog from Nigeria [57] and in two febrile
228 sheep from Senegal [58]. In ticks, no detection of *A. Phagocytophilum* has been reported in
229 West Africa. To date, no human cases of anaplasmosis have been reported in West African
230 countries, this may be due to the lack of a laboratory specialising in the differential diagnosis

231 of this infection and the lack of clinicians capable of distinguishing between clinical signs of
232 the disease and other prevalent febrile diseases in these areas (malaria and typhoid fever).

233 *A. marginale* is an obligate intra-erythrocyte bacterium of ruminants responsible for
234 bovine anaplasmosis transmitted by tick species mainly belonging to the *Rhipicephalus* and
235 *Dermacentor* genera [56]. *A. marginale* has a wide distribution in tropical and subtropical
236 regions. It is probable that the transport of cattle from endemic areas to non-endemic areas
237 and global warming, which influences the movement of vectors, are the cause of its rapid
238 spread [56]. In animals from West Africa, *A. marginale* has been detected by microscopy in
239 cattle in various areas of Senegal [59-61] and the antigen against *A. marginale* was detected in
240 cattle from The Gambia [62]. The DNA of *A. marginale* has been reported in cattle from
241 Nigeria [20,63]. In ticks from West Africa, the DNA of *A. marginale* has been reported in *Rh.*
242 (*B*) *decoloratus* collected in Nigeria [26], in *Rh.*(*B*) *microplus* from Côte d'Ivoire, Mali and
243 Benin [18,19,64], and in *Am. variegatum* from Benin [65] (Table 1).

244 *Anaplasma centrale* is responsible for bovine anaplasmosis at a lower pathogenicity for
245 cattle and is principally transmitted by the African tick *Rhipicephalus simus* [56]. Because
246 *Anaplasma centrale* is less pathogenic, it is used as a live vaccine against *A. marginale*
247 infections in cattle in many countries [56]. In animals from West Africa, the DNA of *A.*
248 *centrale* has only been reported in cattle in Nigeria [20]. In ticks, the DNA of *A. centrale* was
249 found in *Am. variegatum* from Côte d'Ivoire and *Rh. (B) annulatus* and *Hy. impeltatum* from
250 Nigeria [19,23] (Table 1).

251 *Anaplasma ovis*, responsible for anaplasmosis in sheep, goats and wild ruminants in
252 tropical and subtropical regions, is an obligate intra-erythrocytic bacterium transmitted by
253 *Rhipicephalus* spp. [56]. *Anaplasma ovis* has been reported in Africa, Asia, Europe and the
254 United States [56]. In West Africa, *A. ovis* have been detected by microscopy in the blood of
255 small ruminants in Senegal [58-61] and DNA from this bacterium has been detected in dogs
256 from Nigeria [57]. Currently, *A. ovis* has not been reported in ticks collected in West Africa.

257 *Anaplasma bovis* is an obligate bacterium that mainly parasitizes cattle and buffalo
258 monocytes, but also occurs in many domestic and wild animals [56]. Several species of ticks
259 are suspected to be vectors of this bacterium, including *Hyalomma* sp., *Am. variegatum*, *Rh.*
260 *appendiculatus*, *Rh. sanguineus* s.l. and *Haemaphysalis* sp. and it is found in Africa, Asia
261 United States and Europe [56]. To date, *Anaplasma bovis* has not been reported in West
262 Africa.

263 *Anaplasma platys* is an obligate platelet bacterium responsible for infectious canine
264 cyclic thrombocytopenia (ICCT), mainly transmitted by *Rh. sanguineus* s.l. but detected in *D.*

auratus, *I. persulcatus*, *Ha. longicornis*, *R. turanicus*, despite the fact that these ticks are not involved in its transmission and it is a cosmopolitan bacterium [56]. Although *A. platys* is considered to be canine pathogen, it has also been reported in other animals such as cats, foxes, Bactrian camels, deer, sika deer, cattle and humans [56]. In West Africa, the DNA of *A. platys* has been detected in dogs, camels and cattle from Nigeria [20,66,67], in dogs from the Maio Island in the Cape Verde archipelago [68,69], in dogs from Ghana and Côte d'Ivoire [70,71], and in sheep from Senegal [58]. In ticks, the DNA of *A. platys* has been identified in *Rh. sanguineus* s.l. collected from dogs in Côte d'Ivoire [71] and in *Hy. truncatum* collected from cattle in Nigeria [20] (Table 1).

Other unidentified species of *Anaplasma* have been reported in ticks, including, *Candidatus Anaplasma ivorensis*, which is similar to *A. phagocytophilum* and which was identified in *Rh. (B) microplus* collected from cattle in Mali [19] and *Am. variegatum* in Côte d'Ivoire [19] (Table 1). Finally, *Anaplasma* spp. (Badiuoré Ziguinchor) has been found in sheep in Senegal and dogs and cattle in Nigeria [20,58,72].

Ehrlichiosis

Ehrlichiosis is caused by tick-borne intracellular Gram-negative bacteria of the genus *Ehrlichia*, responsible for animal and human diseases. The genus *Ehrlichia* comprises five species: *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris* and *E. ruminantium* [73]. Transmission takes place through different species of ticks depending on the species of *Ehrlichia*, thus *E. canis* is mainly transmitted by *Rh. sanguineus* s.l., *E. ruminantium* by ticks of the genus *Amblyomma*, *E. chaffeensis* by *Am. americanum*, *E. ewingii* by *D. variabilis* and *E. muris* by *Haemaphysalis flava* and *Ixodes persulcatus* [73]. Several species of *Ehrlichia* have been reported in West Africa, including *E. ruminantium*, *E. canis*, *E. chaffeensis*, *E. ewingii* and other unidentified *Ehrlichia* species [36] (Figure 5). Human ehrlichiosis is characterised by fever, myalgia, headache, nausea, vomiting, acute renal failure, leukopenia, thrombocytopenia, and increased liver enzyme activity. In dogs, the symptoms of canine Ehrlichiosis are most often fever, thrombocytopenia and joint pain [74]. *E. ruminantium* and *E. canis,* which are responsible for animal ehrlichiosis can be benign or can be manifested by fever, loss of appetite, heavy breathing, hanging head, stiff gait, depression, lethargy, anorexia, lymph adenomegaly and splenomegaly [75].

Ehrlichia chaffeensis is an obligate intracellular Gram-negative bacterium responsible for human monocytotropic ehrlichiosis (HME), an important emerging zoonosis designated as a reportable disease by the Centers for Disease Control and Prevention, transmitted by *Am.*

americanum [76]. The majority of cases of human ehrlichiosis have been diagnosed in the United States, although cases have also been reported in Europe and Africa [77]. In humans in West Africa, a case of human ehrlichiosis has been reported in Mali [78] and antibodies against *E. chaffeensis* have been found in an asymptomatic blood donor in Burkina Faso [79]. In ticks, *E. chaffeensis* was reported in *Hy. impeltatum* and three genotypes of *Ehrlichia* which were phylogenetically close to *E. chaffeensis* in *Rh. e. evertsi* were collected in Nigeria [23] (Table 1).

Ehrlichia ewingii, an obligate intracellular bacterium that mainly infects granulocytes, is the etiologic agent of granulocytic ehrlichiosis in dogs and humans [80]. Recently *E. ewingii* DNA was found in peripheral blood leukocytes from patients with suspected ehrlichiosis in Missouri [80]. The main vector of the bacterium is *Am. americanum* in the USA and its reservoir is deer [80]. In West Africa, no human cases of granulocytic ehrlichiosis have been reported to date. In ticks, the DNA of *E. ewingii* was detected in *Rh. (B) annulatus* collected in Nigeria and two other genotypes that are close to *E. ewingii* in *Am. variegatum* and *Hy. impeltatum* [23] (Table 1).

E. ruminantium, the causative agent of heartwater, or cowdriosis, in wild and domestic ruminants, usually known as *Cowdria ruminantium*, is generally transmitted by ticks of the genus *Amblyomma* known as the main vector in Africa [75]. Heartwater is a notifiable disease according to the World Organisation for Animal Health (OIE) and a serious economic problem for pastoralists in sub-Saharan Africa. In Africa it affects most of sub-Saharan Africa (except the very dry south-west), the islands of Madagascar, Mauritius, Réunion, Grande Comore and São Tomé. In the new world, *E. ruminantium* is present on the islands of Guadeloupe, Antigua and Marie-Galante [75]. In animals from West Africa, antibodies against *E. ruminantium* have been detected in small ruminants in The Gambia [81] in cattle in Côte d'Ivoire [82] and in domestic ruminants in Ghana [83,84]. The DNA of *E. ruminantium* has been detected in lambs in The Gambia [85], in sheep in Senegal [58], in cattle from Burkina Faso [86] and in cattle and dogs in Nigeria [20,72]. In ticks, *E. ruminantium* was detected in *Am. variegatum* collected in The Gambia, Burkina Faso, Benin, Côte d'Ivoire and in Mali [18,19,86-88]. It has also been detected in other species of tick including *Rh. (B) microplus* collected on cattle in Mali, Côte d'Ivoire and Burkina Faso [18,19,89], *Hy. truncatum*, *Hy.m. rufipes* and *Rh. e. evertsi* from Mali [18] (Table 1).

Ehrlichia canis is a cosmopolitan dog bacterium which is responsible for canine monocytic ehrlichiosis transmitted by *Rh. sanguineus* s.l. In animals, the antibodies against *E. canis* have been only reported in West Africa in dogs from Côte d'Ivoire [90,91]. The DNA of

E. canis was detected in dogs from Nigeria [32,66,72,92,93], Senegal [94], Ghana [70] and Cape Verde [68,69]. In ticks, the DNA of *E. canis* was detected in *Rh. sanguineus* s.l. collected from watchdogs in Côte d'Ivoire [95] (Table 1).

The DNA of potential new species of *Erhlichia* named *Candidatus Ehrlichia rustica* and *Candidatus Ehrlichia urmitei* have been detected in *Am. variegatum*, *Hy. truncatum* and *Rh. (B) microplus* ticks collected in Côte d'Ivoire and Mali [18,19]. *Candidatus Neoehrlichia mikurensis* has been identified in *Rh. sanguineus* s.l. in Nigeria [66], *Erhlichia* spp. (Omatjenne), which is phylogenetically close to *Ehrlichia ruminantium*, has been detected *in Hy. truncatum* collected from cattle in Nigeria [20] and two genotypes of *Ehrlichia* sp. (ERm58 and EHt224) from the *E. canis* group in *Rh. muhsamae* and *Hy. truncatum* collected in Mali and Niger respectively [22] (Table 1).

- **Bartonellosis**

Bartonellosis are zoonoses which are often chronic or asymptomatic infections in reservoir hosts such as dogs, cats, bats and rodents [96]. They are caused by *Bartonella* species that are fastidious haemotropic Gram-negative organisms that have been recently identified in a wide range of domestic animals, wild mammals and humans. At least 20 species of *Bartonella* are known to be responsible for specific intra-erythrocyte infections in the host. *Bartonella bacilliformis*, *B. quintana* and *B. henselae* are the main causative agents of human diseases [96]. In West Africa, several species of *Bartonella* have been reported in humans, animals and arthropods (Figure 6).

Bartonella henselae more commonly infect domestic and wild cats and, accidentally, humans. It is the main causative agent of cat scratch disease and other life-threatening disorders in immunocompromised individuals. This bacterium is the second most common cause of endocarditis in the world and can also cause bacteraemia and bacillary angiomatosis [97]. In West Africa, no cases of *B. henselae* in humans have been reported. However, the antibodies against *B. henselae* have been reported in the serum of a cat from Ghana [98]. In ticks, one study found *B. henselae* DNA in *Rh. sanguineus* s.l. from dogs in Ghana [70].

Bartonella quintana, the agent responsible for louse-borne trench fevers transmitted by body lice, was detected by PCR in febrile patients in rural areas of Sine-Saloum in Senegal [43,99] and the antibodies against *B. quintana* were found in the sera of asymptomatic humans who had close contact with fruit bats [98].

Bartonella bovis, a small Gram-negative bacterium, is the etiological agent of cattle bartonellosis causing bacteraemia and endocarditis in cattle. Its transmission may take place through arthropods such as fleas, flies, lice, mites and ticks that are naturally found to be

infected [100]. Reported in Europe, USA, Asia and Africa, the prevalence of *B. bovis* in cattle is generally high but varies widely across studies and countries [100]. Cattle bartonellosis is manifested in symptoms such as anorexia, weight loss, wasting syndrome and abnormal cardiac auscultation [101]. In West Africa, *B. bovis* has only been reported in cattle from Côte d'Ivoire and Senegal [102,103].

Several isolate *Bartonella* spp. have also been reported in small mammals in West Africa. Among those of *Bartonella* spp. close *to B. elizabethae* were amplified in rodents from Benin [104], Nigeria [105] and recently in Mali (Adama et al revised in PlosNTD); *Bartonella* spp. close *to B. rochalimae* was reported in Benin [104]; *Bartonella* spp. close *to B. tribocorum* was reported in Benin and Nigeria [104,105]; *Bartonella* spp. close *to B.grahamii* was found in Nigeria [105] and unidentified *Bartonella* spp. were detected in rodents from Benin [104], in rodents, bats and their ectoparasites from Nigeria [105,106] and rodents from Mali (Adama *et al*. revised in PlosNTD). In addition, two potential new species of *Bartonella* including *Candidatus* Bartonella davoustii have been reported in cattle in Senegal [102] and *Candidatus* Bartonella mastomydis has been reported for the first time in rodents in Benin [104] and recently in rodents in Mali (Adama *et al.* revised in PlosNTD). Finally *B. senegalensis* and *B. massiliensis*, two newly recognised species, have been reported in *O. Sonrai* ticks collected in Senegal [107].

Q fever or coxiellosis

Coxiella burnetii is the causative agent of Q fever; it is a small Gram negative intracellular bacterium highly resistant to the environment and which is mainly transmitted to humans by aerosols generated by exposure to infected placentas and birth liquids contaminated by Q fever [108]. It is isolated in many tick species, suggesting that these arthropods play a role in the transmission of the bacterium [108]. Q fever has been reported almost everywhere it has been researched, with the exception of New Zealand. In humans, the infection can occur in two forms: the primary and persistent form or in the form of long-term complications. The primary infection is mainly asymptomatic, sometimes causing fever, malaise, headache, fatigue, and abdominal pain [108]. Serious obstetric complications and foetal malformations may occur in pregnant women. Persistent infections can cause endocarditis, vascular infections, osteoarticular infections and lymphadenitis [108].

In humans from West Africa, Q fever was reported in a man returning from Guinea Bissau with acute lobar pneumonia with fever, headache, haematuria and hepatitis [109] and also in tourists who had stayed in Gambia, Côte d'Ivoire, and Burkina Faso [13].

Antibodies reactive to *C. burnetti* have been reported in Nigerian patients hospitalised for various acute medical conditions [110], in apparently healthy adults from Mali, Burkina Faso and Côte d'Ivoire [29], in children aged between one month and five years in Niger [111], in children and adults from Ghana [112], in nomads in rural areas of northern Burkina Faso [113], in non-Fulanis and Fulanis in northern Togo [114], in blood donors and patients in Mauritania [14] and in adults and children in The Gambia [115,116]. *C. burnetii* DNA was detected in febrile patients who tested negative for malaria using Paracheck in Mali [46] and in human and various environmental samples in Senegal [43,117,118].

In animals, the antibodies against *C. burnetii* have been detected in sera and milk in cows in Nigeria [119], in dogs in Senegal and Côte d'Ivoire[120], in livestock In the Republic of Guinea [15], in cattle, sheep and goats in Togo and Côte d'Ivoire [114,121], in small ruminants (goats and sheep) in Gambia [115,122], and in rodents in Cape Verde [123]. The DNA of *C. burnetii* has been detected in milk for consumption in Senegal [124], in rodents in Nigeria [30] and recently in rodents in Mali (Adama *et al.* revised in PlosNTD).

DNA of *C. burnetii* has been detected in several species of ticks. The presence of *C. burnetii* DNA in these ticks is not synonymous with their vector roles in bacterial transmission as there is no specific vector involved in the epizootic cycle of *C. burnetii*. In Senegal *C. burnetii* was detected in *Am. variegatum*, *Rh.* (*B*) *annulatus*, *Rh.* (*B*) *decoloratus*, *Rh. e. evertsi*, *Rh. guilhoni*, *Hy.m. rufipes*, *Hy. truncatun* and *O. sonrai* [117] (Table 1). Similarly, it has also been identified in *Am. variegatum*, *Rh.* (*B*) *annulatus*, *Rh. e. evertsi* and *Hy. impeltatum* collected from cattle in Nigeria and *Am. variegatum* collected from cattle in Côte d'Ivoire [19,23] (Table 1). Recently *C. burnetii* has been identified in *Am. variegatum*, *Rh.* (*B*) *microplus*, *Rh. sanguineus* s.l., *Rh. e. evertsi*, *Hy.m. rufipes* and *Hy. truncatun* collected from cattle and head lice collected from patients in Mali [18,125] (Table 1).

B. Parasitic diseases

- Theileriosis

Theileriosis is a tropical haemoprotozoal disease caused by obligate intracellular parasites of the genus *Theileria* that infect both leukocytes and host erythrocytes and are transmitted by hard tick genera, mainly *Amblyomma*, *Haemaphysalis*, *Hyalomma*, and *Rhipicephalus* [126]. Wild and domestic ruminants are the main hosts in which the infection causes enlargement of the lymph nodes, fever, anaemia, jaundice, leukopenia, pulmonary oedema, lethargy, thrombocytopenia and death [127]. A large number of *Theileria* species have been reported in

domestic animals around the world including *Theileria parva*, *T. annulata*, *T. taurotragi*, *T. lestoquardi*, *T. orientalis*, *T. velifera*, *T. mutans*, *T. uilenbergi*, *T. lowenshuni*, *T. sinensis*, *T. ovis* and *T equi* [127]. Currently in West Africa the species of *Theileria* which have been reported are *T. annulata*, *T. mutans*, *T. velifera*, *T. taurotragi*, *T equi* and *Theileria* sp. (Figure 7).

T. annulata is responsible for the tropical theileriosis transmitted by several species of *Hyalomma* ticks, and is present around the Mediterranean basin, northeast Africa, the Middle East, India and South Asia. In West Africa, *T. annulata* has only been detected in *Hy. dromadarii* and *Hy. m. rufipes* collected from cattle in Mauritania [128] (Table 1).

Theileria mutans is a parasite that infects buffalo and cattle in Africa transmitted by Amblyomma sp. ticks. *T. mutans* has very rarely been associated with severe clinical illness. In West Africa *T. mutans* was detected by microscopy in asymptomatic cattle from Senegal [59-61], from Nigeria [20] and from Ghana [52]. DNA of *T. mutans* was detected in *Hy. impeltatum* and in *Rh.* (*B*) *annulatus* ticks collected from cattle in Nigeria [23], and in *Rh.* (*B*) *microplus* and *Am. variegatum* ticks collected from cattle in Benin [64,65] (Table 1).

Theileria velifera is a parasite that also infects buffalo and cattle in Africa and the infection is benign, transmitted by *Amblyomma* sp. ticks [127]. In West Africa *T. velifera* was detected in cattle from Nigeria and Ghana [20,52]. *T. velifera* has been detected in *Am. variegatum* ticks collected from cattle in the Republic of Guinea [129] (Table 1).

Theileria taurotragi is a parasite that exists in African eland and cattle and is transmitted by *Rhipicephalus* sp.; the infection is benign [127]. In West Africa, *T. taurotragi* has been reported in cattle from Nigeria [20] (Table 1).

Theileria equi infects horses and most often causes an asymptomatic infection but can cause epidemics and is a cosmopolitan parasite that is transmitted by *Hyalomma* sp., *Dermacentor* sp., *Rhipicephalus* sp. and *Amblyomma* sp [127]. In West Africa, *T equi* DNA was detected in asymptomatic dogs and horses from Nigeria [72,130], and in horses from Ghana [131]. In addition, *Theileria* sp. (sable) and *Theileria* sp. close to *T. ovis* were detected in Nigerian dogs [66,72] and *Theileria* sp. Was detected in sheep and goats from Ghana [52].

- **Babesiosis**

Babesiosis is a zoonotic disease caused by a parasite of the genus *Babesia* and usually transmitted by hard-bodied ticks. Worldwide, there are more than 100 species of *Babesia* that

infect a wide variety of domestic and wild animals, but only a few infect humans [132]. The human babesiosis caused by *B. microti* and *B. divergens* is characterised by unspecific fever that usually resolves spontaneously in immunocompetent patients. Sometimes haematuria and jaundice can also be observed depending on the degree of haemolysis [132]. Severe manifestations of the disease and fatal cases have been described in immunocompromised patients. In humans, only one probable case of human babesiosis has been reported in an infant in Côte d'Ivoire [133]. In animals, babesiosis is caused by *B. bigemina*, *B. bovis*, *B. gibsoni, B. canis a*nd *B. caballi* [134]. In West Africa, studies have reported the presence of several species of Babesia in animals and ticks (Figure 8).

Babesia microti, a zoonotic intra-erythrocyte parasite that occurs primarily in rodents is transmitted by *Ixodes* ticks in the Northern Hemisphere [134], and it is the main causative agent of human babesiosis in the United States [135]. In West Africa *B. microti* has only been found in house rats collected in Nigeria [136].

Babesia divergens is the major cause of human babesiosis in Europe, transmitted by the *Ixodes ricinus* tick which is the only known vector. [137]. In West Africa, *B. divergens* DNA was detected in *Am. variegatum* ticks in Nigeria [26] (Table 1).

Babesia bigemina is an intra-erythrocyte protozoan which parasitizes cattle, transmitted by *Rhipicephalus Boophilus*. *B. bigemina* has been reported on all continents [138]. In West Africa *B. bigemina* has been reported in cattle in Senegal [59-61], in Gambia [139], in Ghana [52], in Mali [140] and in Nigeria [20,141]. *B. bigemina* was detected in *Rh.* (*B*) *decoloratus* ticks and *Am. variegatum* in Nigeria [26,142] (Table 1).

Babesia bovis is an erythrocyte parasite transmitted by ticks to livestock in tropical and subtropical regions around the world. In animals, antibodies against *B. bovis* have been detected in cattle in Mali [140] and in Nigeria [141,143]. Studies have also reported the presence of *B. bovis* DNA in cattle in Nigeria [20] and in Ghana [144].

Babesia gibsoni is an intra-erythrocyte parasite recognised as being responsible for piroplasmosis in dogs. It is mainly transmitted to dogs by ticks. The parasite has been reported in Asia, North and East Africa, America and Europe. *Babesia gibsoni* has only been detected in dogs from Cape Verde in West Africa [145].

Babesia canis is an intracellular protozoan agent that infects canine red blood cells that are mainly transmitted by ticks. *B. canis* is widespread in Europe, corresponding with the

494 distribution area of *Dermacentor reticulatus* which is its known vector [134]. In West Africa,
495 *B. canis* has only been detected in dogs from Nigeria [146-148].

496 *Babesia caballi* is a haemoprotozoan parasite responsible for equine babesiosis which is an
497 infectious disease transmitted by ticks and which is present in most countries of the world
498 where the competent vectors are located [149]. In West Africa, *B. caballi* has been detected in
499 *Am. variegatum* collected from cattle in the Republic of Guinea [129].

500 Finally in West Africa, *Babesia perroncitoi* and *B. trautmanni* for swine babesiosis have
501 been reported in Nigeria and Ghana in pigs [150,151].

502 **- Hepatozoonosis**

503 Hepatozoonosis is an infection caused by hepatozoan species transmitted by definitive
504 haematophagous invertebrate hosts such as ticks, mites, mosquitoes, fleas, lice, sandflies,
505 tsetse flies and reduviid bugs [152]. Hundreds of *Hepatozoon* species infect birds, reptiles,
506 amphibians and mammals, and are found on every continent. It is not a human pathogen. Two
507 species, *Hepatozoon canis* and *H. americanum,* infect domestic dogs, the former has a global
508 distribution while the latter is limited to the United States [153].

509 *Hepatozoon canis* is widespread and has been reported on all continents, it is transmitted to
510 dogs by *Rh. sanguineus* s.l. and is often manifested by either a simple form with negligible
511 symptoms (asymptomatic) or a serious and fatal form with anaemia, fever, lethargy, cachexia,
512 weight loss in dogs, pale mucous membranes, eye discharges and hind limb weakness [153].
513 In West Africa *H. canis* has been reported in dogs from Nigeria [66,148], Cape Verde
514 [68,69,145] and Ghana [70].

515 **C. Viral diseases**

516 **- Crimean-Congo haemorrhagic fever (CCHF)**

517 CCHF is a zoonosis caused by a *Bunyaviridae* family virus causing haemorrhagic fever and
518 which has a significant mortality rate in humans. CCHF is a globally distributed disease and is
519 considered endemic in many countries in Asia, Europe and Africa. The disease is transmitted
520 to humans either by tick bites (*Hyalomma* spp., *Rh. rossicus* and *D. marginatus*) or by contact
521 with the blood or secretions of infected animals [154]. CCHF manifests in humans by fever,
522 chills, headache, dizziness, neck pain, nuchal rigidity, photophobia, retro-orbital pain,
523 myalgia, arthralgia, nausea, vomiting, diarrhoea, abdominal pain and haemorrhagic

manifestations such as petechial rashes, bruising, haematemesis and melena often associated with thrombocytopenia and leukopenia [154].

In West Africa, the first human case of CCHF virus was reported in 1983 in a febrile patient in southern Mauritania [155]. The CCHF virus was isolated from a dead patient and antibodies against the CCHF virus were detected in hospitalised patients with symptoms of haemorrhagic fever in southwestern Mauritania [156]. Subsequently, between February and August 2003, 38 people were diagnosed with the CCHF virus in Mauritania, 35 of whom resided in Nouakchott [157]. Anti-CCHF antibodies were reported in febrile patients from Nigeria [158,159], in people working in the slaughterhouse in Ghana [160], in Malian patients who had tested negative for *Plasmodium falciparum* and yellow fever but who had a history of fever, haemorrhagic, diarrheal or icteric syndromes [161]. One imported case has been reported in a woman in France after her return from Senegal [162]. CCHF virus RNA has been detected in humans in Nigeria and Mali [158,163].

In animals, antibodies against the CCHF virus have been detected in cattle from northern Nigeria [164], in cattle, rodents sheep and goats from Mauritania [156,157,165,166], in birds from Senegal [167] and cattle from Mali with variations from one region to another [168].

In ticks, three different genotypes of CCHF virus have been isolated from *Hy. m. rufipes*, *Am. variegatum*, *Rh. guilhoni* and *Rh. e. evertsi* collected from cattle and goats in Senegal [169] (Table 1). The virus was also isolated in Senegal in immature stages of *Rh. m. rufipes* collected from the hornbill [167]. The virus has also been isolated from *Hy. m. rufipes* ticks collected from camels and cattle in Mauritania [165], from *Hyalomma* ticks collected from cattle in Mali [170], and has recently been detected in *Hy. excavatum* and *Am. variegatum* collected from cattle in Ghana [160] (Table 1). *Hyalomma* spp. ticks are considered to be the only known vectors of the CCHF virus, so detecting virus RNA in *Amblyomma* ssp. and *Rhipicephalus* spp. does not make them potential vectors.

Conclusion

Tick-borne diseases are generally neglected or underestimated diseases in West Africa. In febrile autochthonous patients or travellers returning from West Africa, the most commonly reported diseases are African tick bite fever, tick borne relapsing fever, Q fever and Crimean-Congo haemorrhagic fever. However, in healthy blood donors, antibodies against some tick-borne pathogens have also been detected. Many species of pathogens have been detected in

556 wild and domestic animals as well as in ticks collected from animals. The detection of
557 pathogens in vector tick species such as *R. africae*, *E. ruminantium*, *T. mutans* and *T. velifera*
558 in *Amblyomma* spp.; *T. annulata*, *T. mutans*, *R. aesclimannii* and the CCHF virus in
559 *Hyalomma* spp.; *B. crocidurae* in *O. sonrai*; *A. marginale*, *B. bigemina*, *R. conorii* and *E.*
560 *canis* in *Rhipicephalus* spp. are indirect evidence of the presence of pathogens which have not
561 yet been described in animals or humans in this region. Ticks are, therefore, of major
562 epidemiological interest for surveillance in areas where there are no specialised laboratories to
563 detect these pathologies in humans and animals.

564 **List of abbreviations**

ATBF	African tick bite fever
CCHF	Crimean-Congo haemorrhagic fever
DNA	Deoxyribonucleic acid
EU	European Union
HGA	Human granulocytic anaplasmosis
HME	Human monocytotropic ehrlichiosis
ICCT	Infectious canine cyclic thrombocytopenia
MSF	Mediterranean spotted fever
OIE	World Organisation for Animal Health
PCR	Polymerase Chain Reaction
SFG	Spotted fever group
TBD	Tick-borne diseases
TBRF	Tick Borne Relapsing Fevers
TG	Typhus group
USA	United States of America
VITROME	Vecteurs Infections Tropicale et Méditerranéennes

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Authors' contributions

DZA wrote the initial draft of the manuscript, PP and EC added their contributions and comments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Contributor Information

Diarra Adama Zan, Email: adamazandiarra@gmail.com.

Eldin Carole, Email : carole.eldin@gmail.com.

Parola Philippe, Email: philippe.parola@univ-amu.fr.

592 **Table 1: Tick species tested positive for tick-associated pathogens in different West**
593 **African countries**

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Ticks species	Pathogens detected and vectorised*	Countries	References
Am. variegatum	*R. africae**	Côte d'Ivoire, Niger, Mali, Liberia, Guinea, Senegal, Benin, Burkina Faso	[16-25]
	Candidatus Borrelia africana	Côte d'Ivoire	[19]
	Candidatus Borrelia ivorensis	Côte d'Ivoire	[19]
	Borrelia spp.	Mali	[18]
	A. marginale	Benin	[65]
	Candidatus Anaplasma ivorensis	Côte d'Ivoire	[19]
	*E. ruminantium**	Mali, Côte d'Ivoire, Burkina Faso, Gambia, Benin,	[18,19,86-88]
	Ehrlichia ewingii	Nigeria	[23]
	Candidatus Ehrlichia rustica	Côte d'Ivoire	[19]
	Candidatus Ehrlichia urmitei	Côte d'Ivoire	[19]
	C. burnetii	Mali, Côte d'Ivoire, Senegal, Nigeria	[18,19,23,117]

	*T. mutans**	Benin	[65]
	*T. velifera**	Guinea	[129]
	B. bigemina	Nigeria	[26]
	B. divergens	Nigeria	[26]
	B. caballi	Guinea	[129]
	CCHF virus	Ghana, Senegal	[160,169]
Am. compressum	*R. africae**	Guinea	[21]
Hae. paraleachi	*R. africae*	Guinea	[21]
Hy. dromedarii	*T. annulata**	Mauritania	[128]
Hy. excavatum	CCHF virus	Ghana	[160]
	R. africae	Nigeria	[26]
	R. aeschlimannii	Senegal, Nigeria	[24,32]
Hy. impeltatum	*A. centrale*	Nigeria	[23]
	E. chaffeensis	Nigeria	[23]
	E. ewingii	Nigeria	[23]
	C. burnetii	Nigeria	[23]
	*T. mutans**	Nigeria	[23]
Hy. impressum	*R. africae*	Côte d'Ivoire	[19]
	R. africae	Mali, Côte d'Ivoire, Guinea, Senegal	[18,19,21,24]
Hy.m. rufipes	*R. aesclimannii**	Mali, Senegal, Burkina Faso, Nigeria	[16,18,24,25,32]
	E. ruminantium	Mali	[18]
	C. burnetii	Mali	[18]

	T. annulata	Mauritania	[128]
	CCHF virus*	Mauritania, Senegal	[165,167,169]
	R. africae	Mali, Côte d'Ivoire	[18,19]
	R. aeschlimannii	Mali, Côte d'Ivoire, Senegal, Burkina Faso	[16,18,19]
Hy. truncatum	*R. mongolitimonae*	Mali, Senegal	[16,18]
	A. platys	Nigeria	[20]
	E. ruminantium	Mali	[18]
	Ehrlichia sp.	Niger	[22]
	Candidatus Ehrlichia rustica	Mali, Côte d'Ivoire	[18,19]
	Candidatus Ehrlichia urmitei	Côte d'Ivoire	[19]
	C. burnetii	Mali, Senegal	[18,117]
	*B. crocidurae**	Mali, Senegal, Mauritania, Gambia	[35,49,50,133]
O. sonrai	*B. senegalensis*	Senegal	[107]
	B. massiliensis	Senegal	[107]
	C. burnetii	Senegal	[117]
	R. africae	Senegal, Nigeria, Guinea	[16,21,23]
Rh. (*B*) *annulatus*	*A. centrale*	Nigeria	[23]
	E. ewingii	Nigeria	[23]
	C. burnetii	Nigeria, Senegal	[23,117]
	T. mutans	Nigeria	[23]
	R. africae	Guinea, Nigeria	[21,26]

Rh. (*B*) *decoloratus*	*A. marginale**	Nigeria	[26]
	C. burnetii	Senegal	[117]
	*B. bigemina**	Nigeria	[26]
Rh. (*B*) *geigyi*	*R. africae*	Liberia	[21]
	B. theileri	Mali	[51]
	R. africae	Mali, Côte d'Ivoire	[18,19]
	*A. marginale**	Mali, Côte d'Ivoire, Benin	[18,19,64]
Rh. (*B*) *microplus*	*Candidatus Anaplasma ivorensis*	Mali	[18]
	E. ruminantium	Mali, Côte d'Ivoire, Burkina Faso	[18,19,89]
	Candidatus Ehrlichia urmitei	Mali, Côte d'Ivoire	[18,19]
	Candidatus Ehrlichia rustica	Côte d'Ivoire	[19]
	C. burnetii	Mali	[18]
	T. mutans	Benin	[64]
	R. africae	Mali, Senegal, Nigeria	[16,18,23]
	R. aeschlimannii	Senegal	[16,24]
Rh. e. evertsi	*R. conorii*	Senegal	[16]
	E. ruminantium	Mali	[18]
	E. chaffeensis	Nigeria	[23]
	C. burnetii	Mali, Nigeria, Senegal	[18,23,117]
	CCHF virus	Senegal	[167]

Rh. guilhoni	*R. massiliae*	Senegal	[16]
	C. burnetii	Senegal	[117]
	CCHF virus	Senegal	[167]
Rh. muhsamae	*Ehrlichia* sp.	Mali	[22]
	R. africae	Nigeria	[26]
	R. aeschlimannii	Mali	[18]
Rh. sanguineus s.l.	*R. conorii**	Nigeria	[30]
	A. platys	Côte d'Ivoire	[71]
	*E. canis**	Côte d'Ivoire	[95]
	Candidatus Neoehrlichia mikurensis	Nigeria	[66]
	B. henselae	Ghana	[70]
	C. burnetii	Mali	[18]
Rh. senegalensis	*R. massiliae*	Côte d'Ivoire, Liberia	[19,21]

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Figure 1: Tick-borne rickettsiae detected by PCR in humans, animals and ticks in West African countries.

Figure 2: Vector ticks of the group of spotted fever rickettsioses in West Africa. Above *Am. variegatum*, vector of *R. africae*, agent of ATBF, below *Rh. sanguineus*, the main vector of *R. conorii* subsp. *conorii*, agent of Mediterranean fever. A and C = females and B and D = males

Figure 3: Different species of *Borrelia* detected by PCR or microscopy methods in humans, animals and ticks in West Africa

Figure 4: Different species of *Anaplasma* detected by PCR in animals and ticks in West Africa

Figure 5: Different species of *Ehrlichia* detected by PCR in humans, animals and ticks in West Africa

Figure 6: Different species of *Bartonella* detected by PCR in humans, animals and ectoparasites in West Africa

Figure 7: Different species of *Theileria* detected by PCR in humans, animals and ticks in West Africa

Figure 8: Different species of *Babasia* detected by PCR or microscopy in animals and ticks in West Africa

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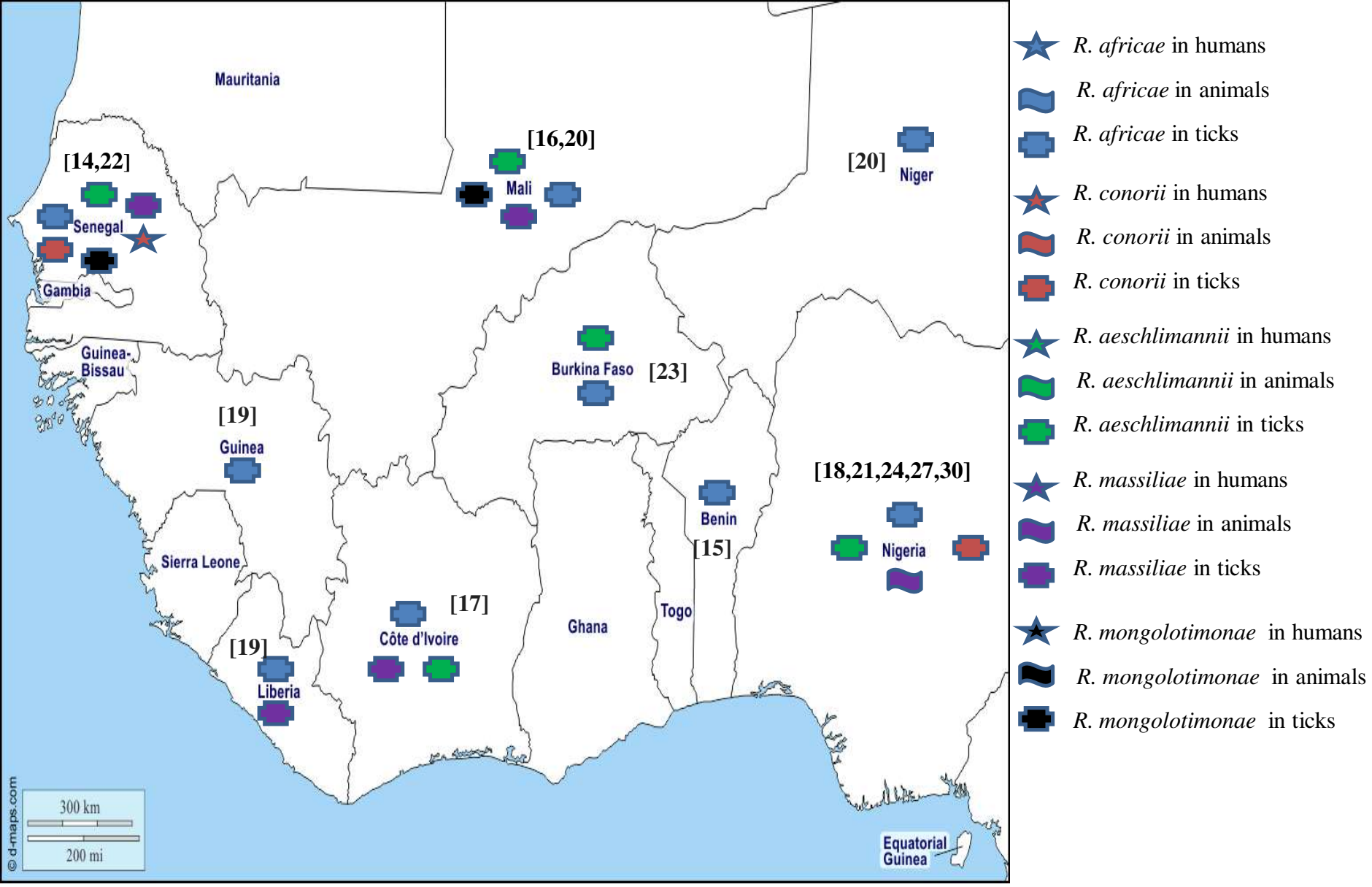
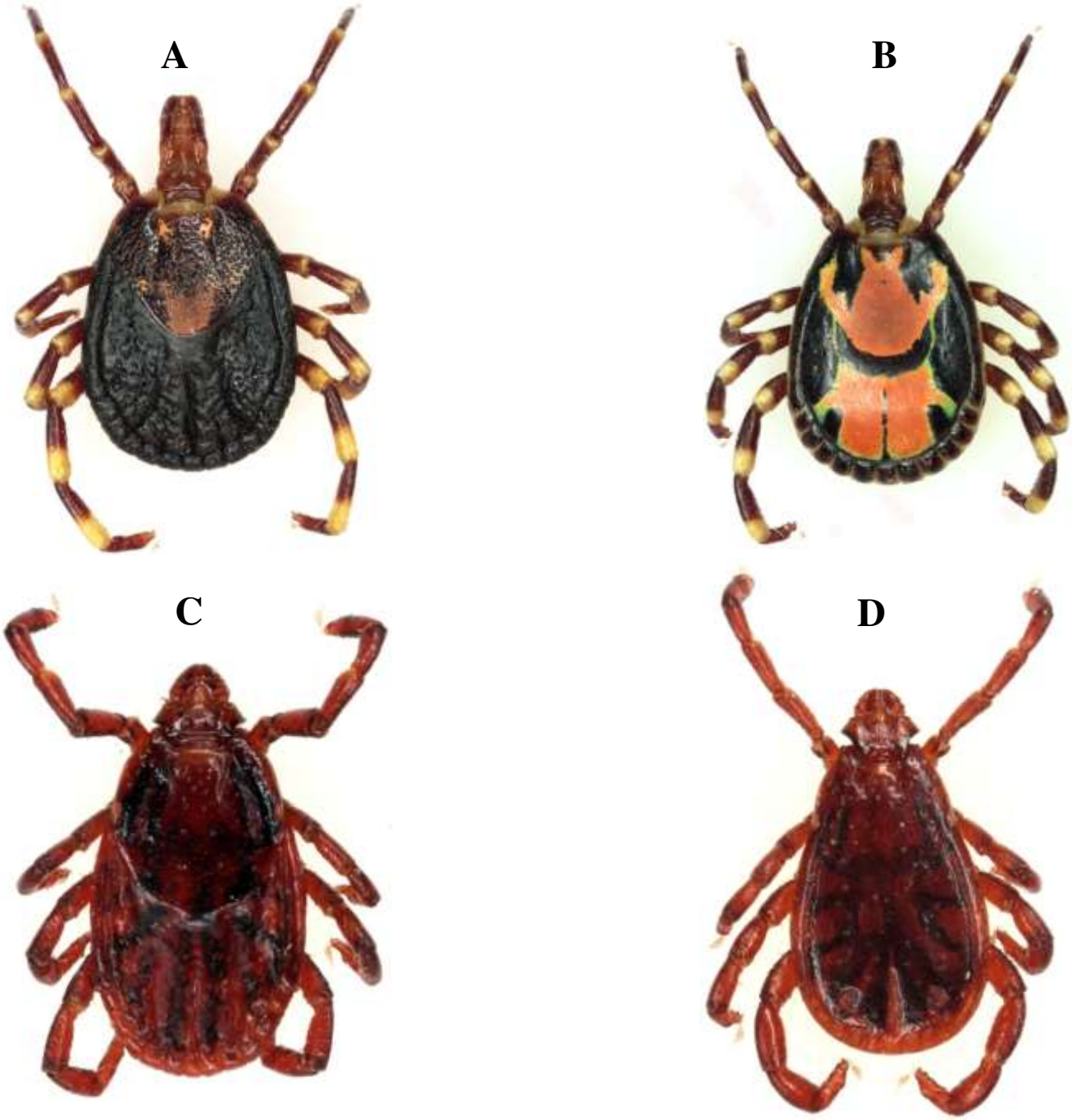


Fig 1:Tick-borne rickettsiae detected by PCR in humans, animals and ticks in West Africa countries.


A
B
C
D

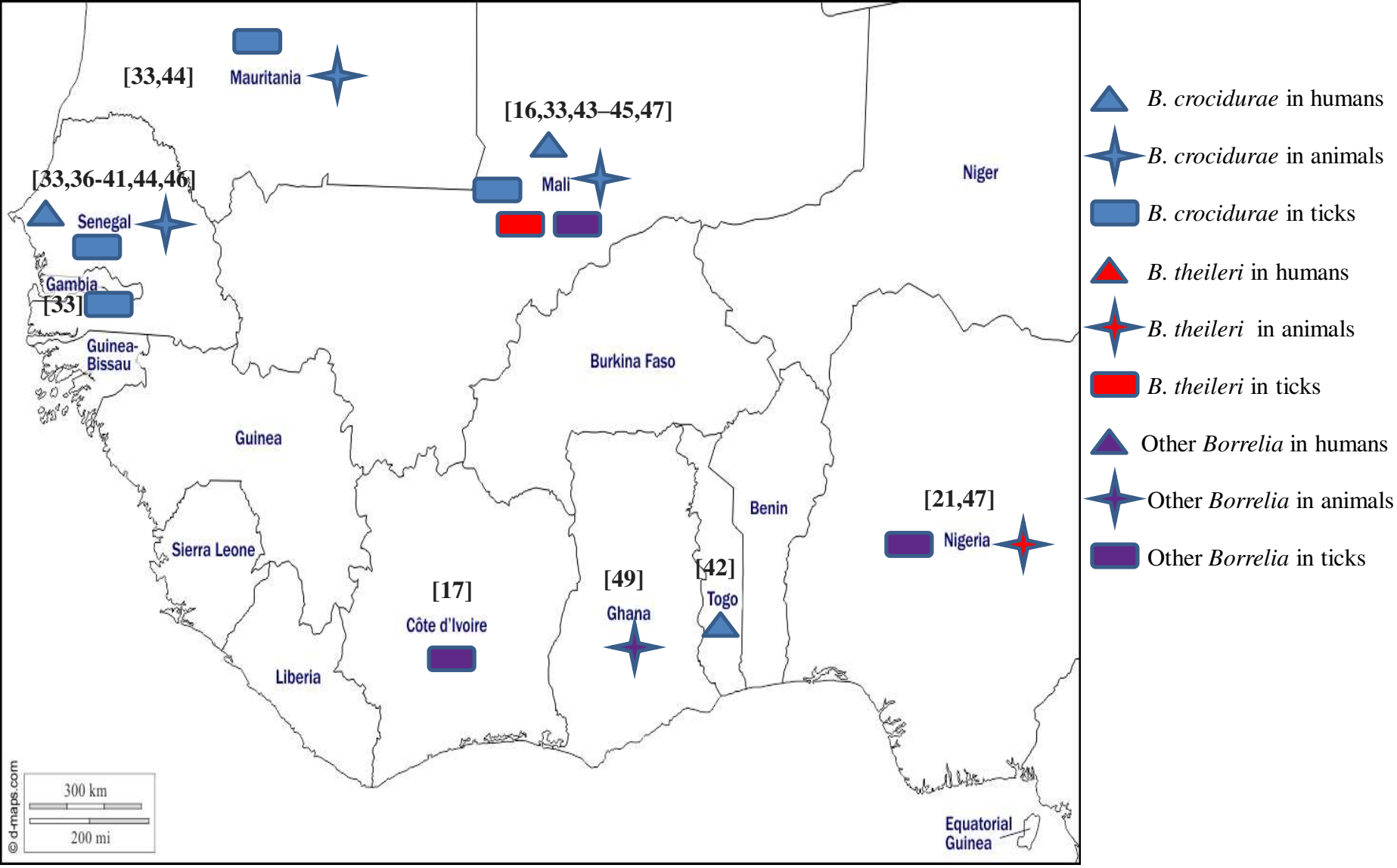
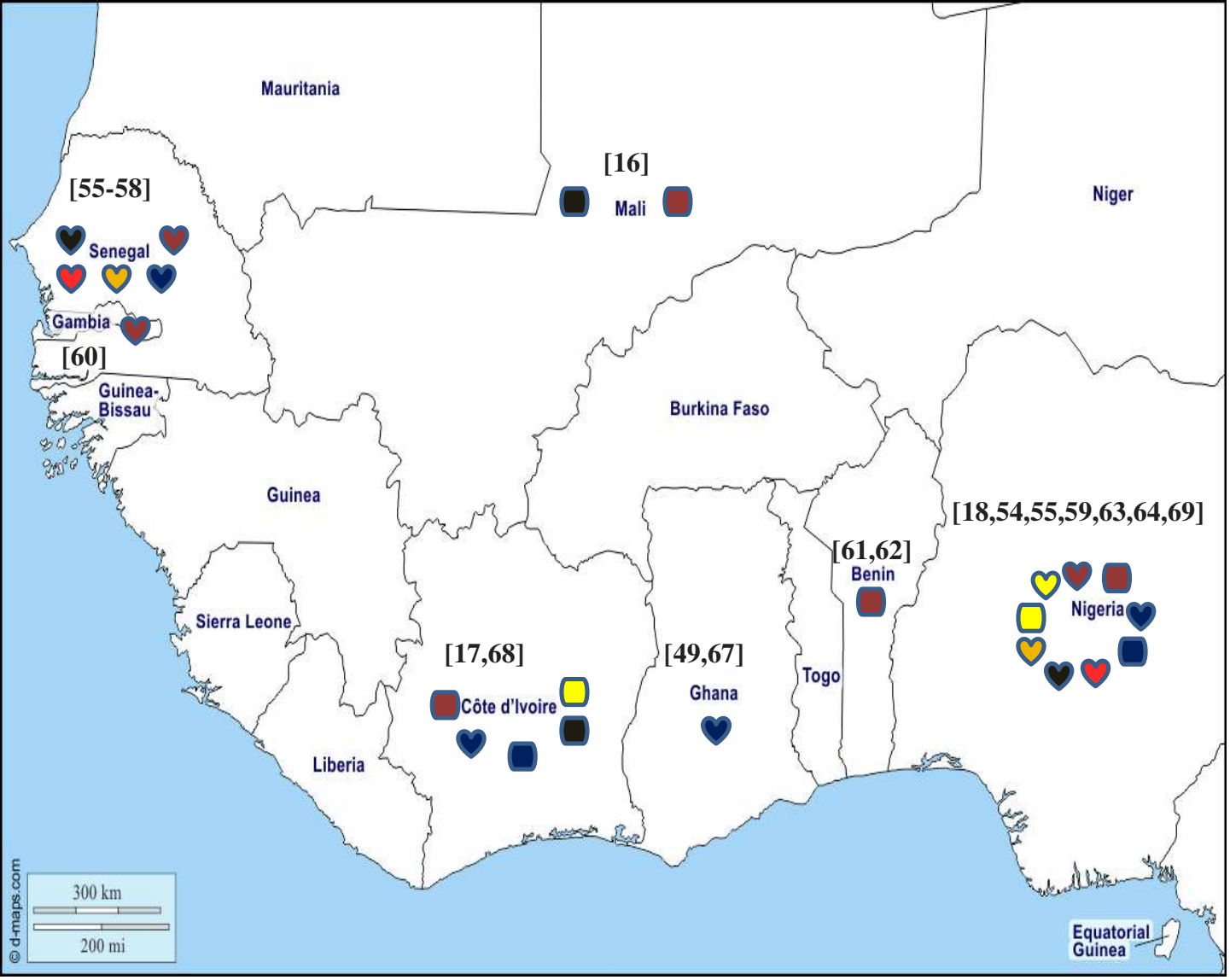


Fig 3: Different species of *Borrelia* detected by PCR or microscopy methods in humans, animals and ticks in West Africa



A. marginale in humans
A. marginale in animals
A. marginale in ticks
A. centrale in humans
A. centrale in animals
A. centrale in ticks
A. bovis in humans
A. bovis in animals
A. bovis in ticks
A. ovis in humans
A. ovis in animals
A. ovis in ticks
A. platys in humans
A. platys in animals
A. platys in ticks
A. pagacytophilum in humans
A. pagacytophilum in animals
A. pagacytophilum in ticks
Other *Anaplasma* in humans
Other *Anaplasma* in animals
Other *Anaplasma* in ticks

Fig 4: Different species of *Anaplasma* detected by PCR in animals and ticks in West Africa

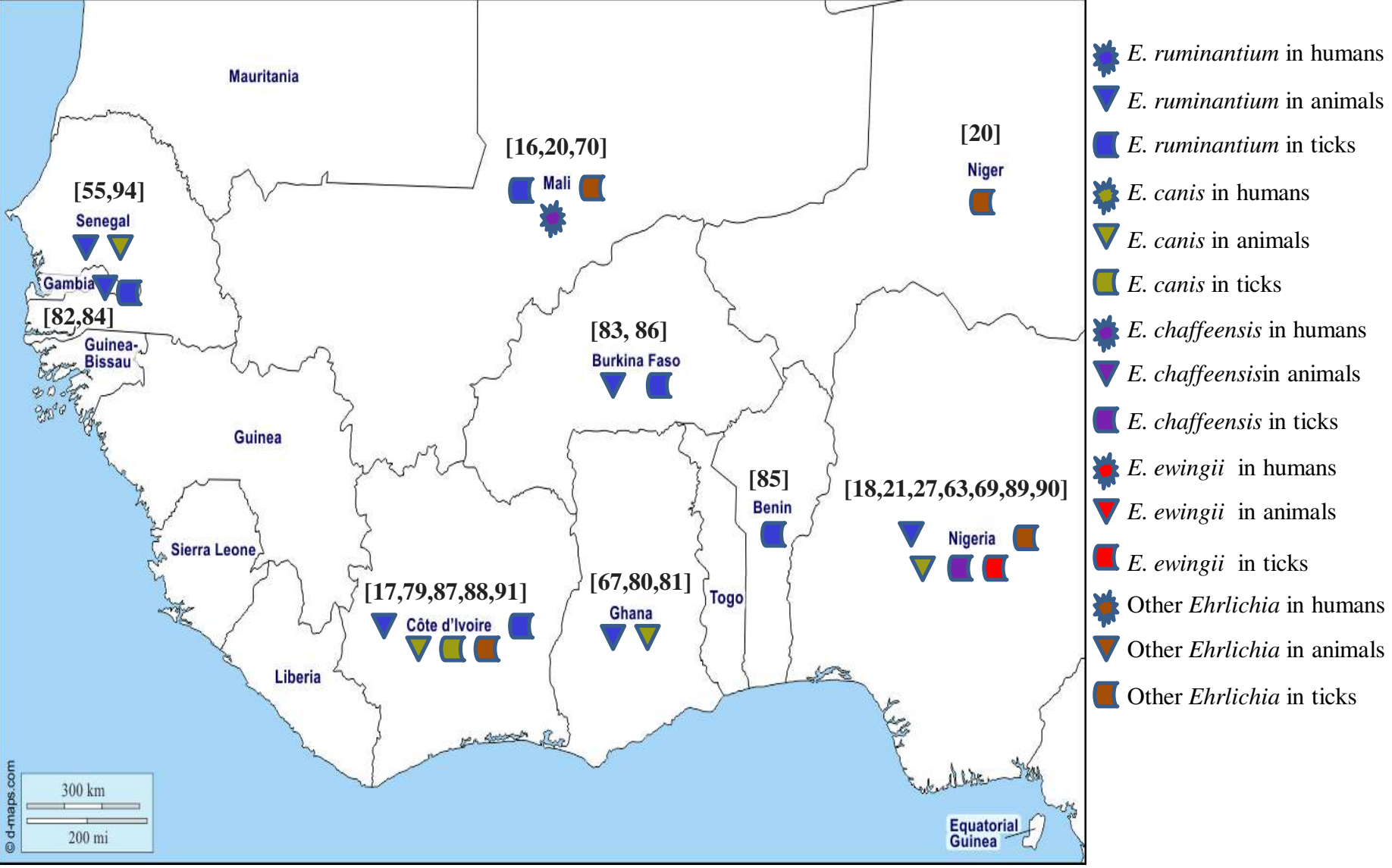


Fig 5: Different species of *Ehrlichia* detected by PCR in humans, animals and ticks in West Africa

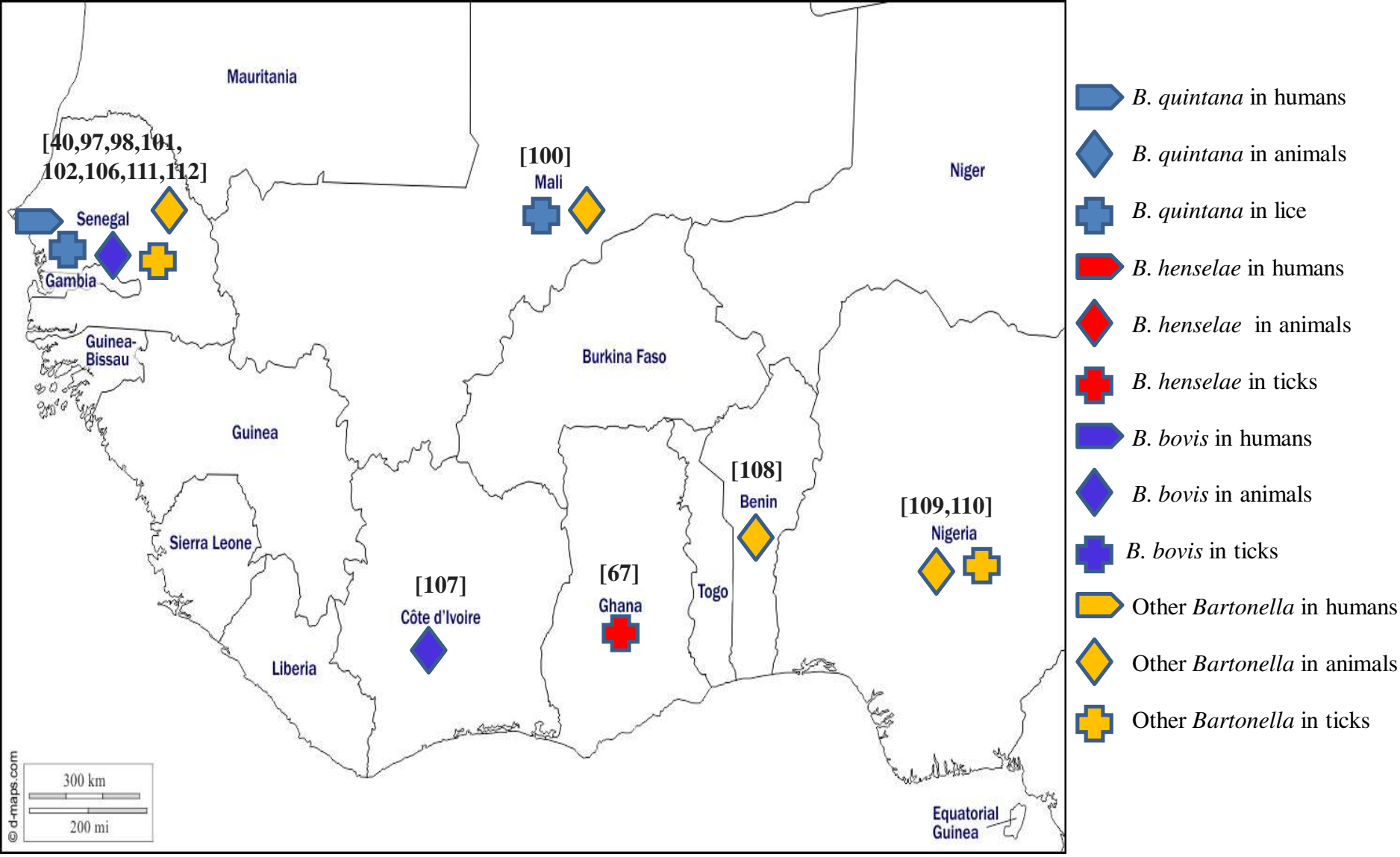


Fig 6: Different species of *Bartonella* detected by PCR in humans, animals and ectoparasites in West Africa

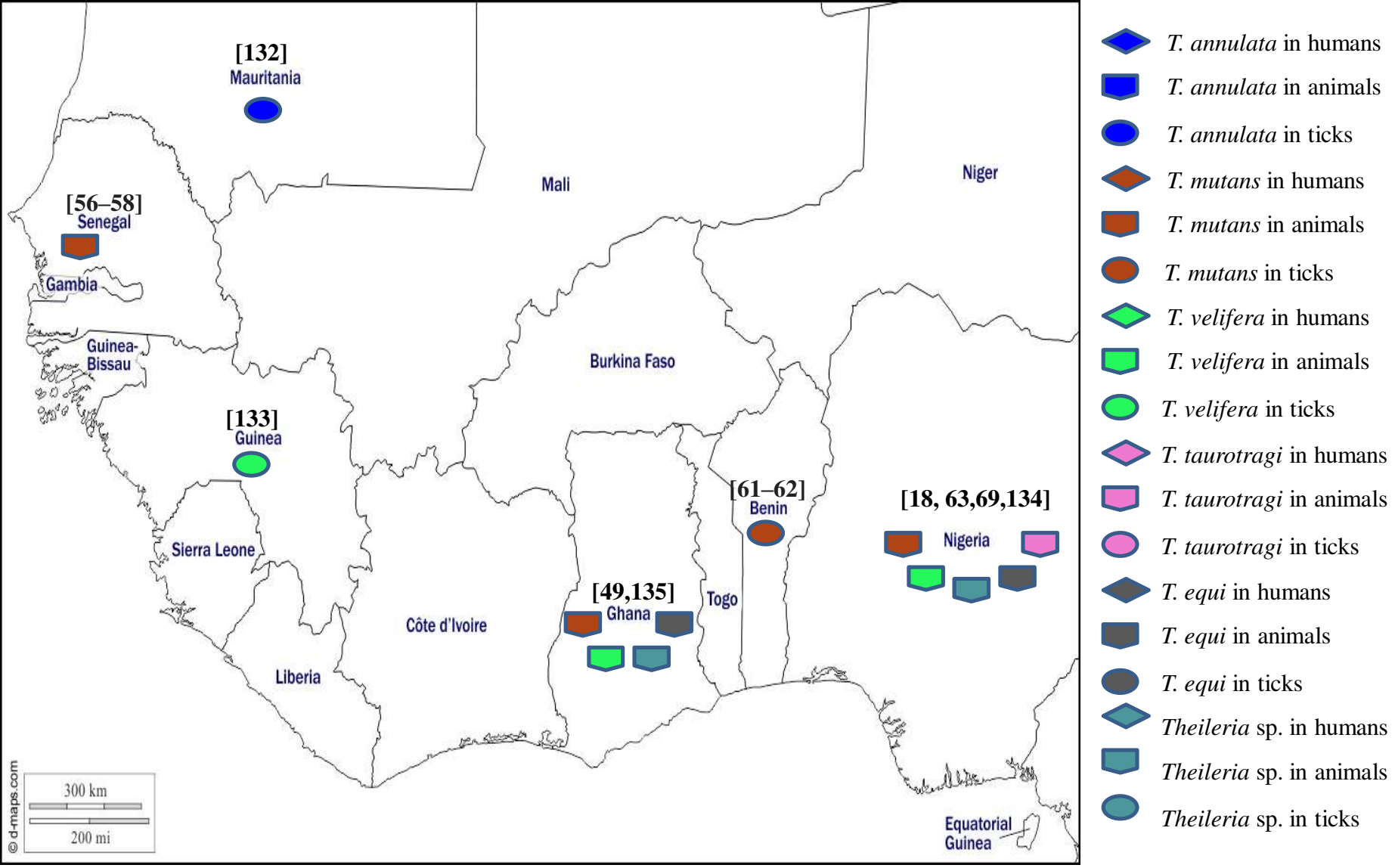
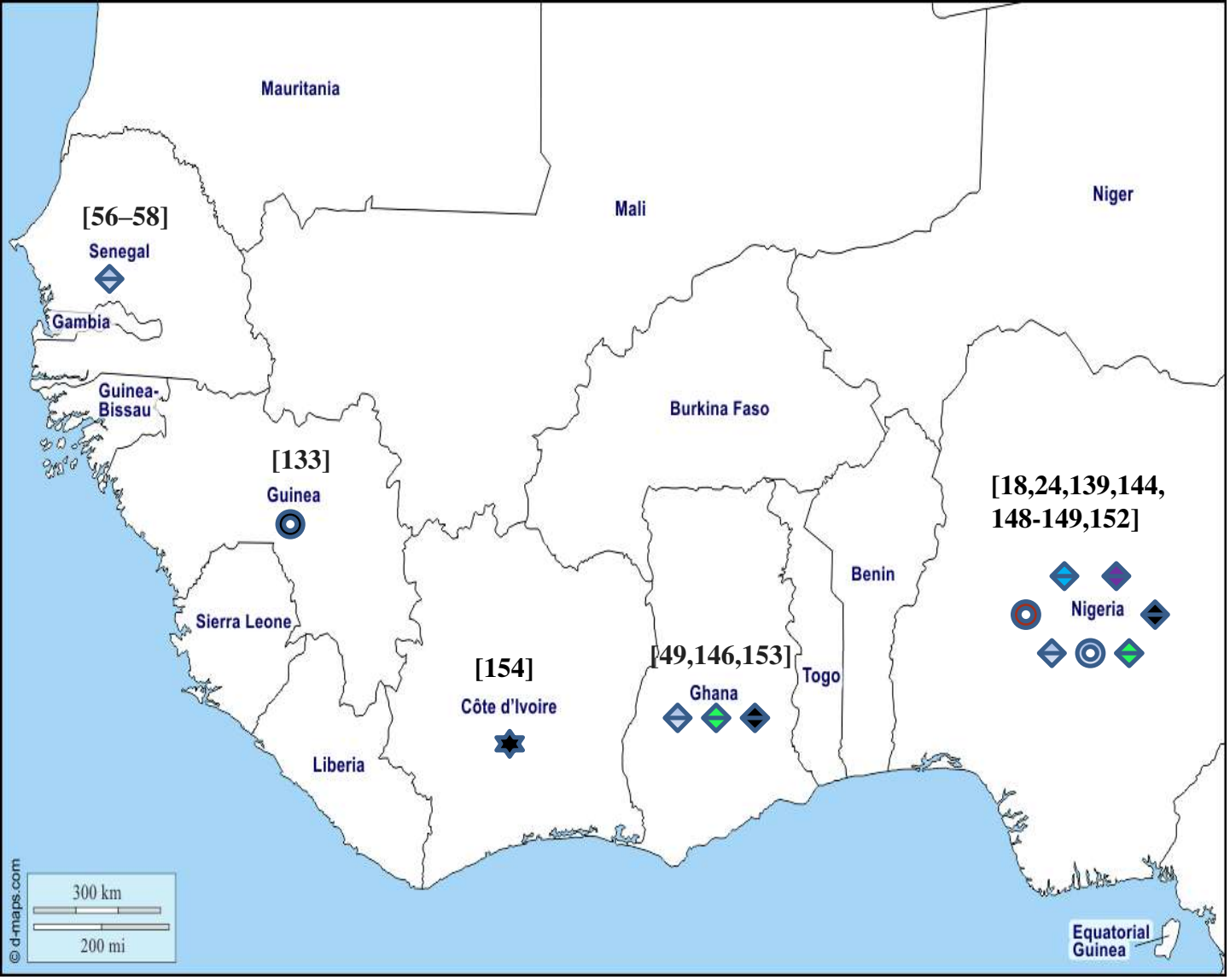


Fig 7: Different species of *Theileria* detected by PCR in humans, animals and ticks in West Africa



B. microti in humans
B. microti in animals
B. microti in ticks
B. divergens in humans
B. divergens in animals
B. divergens in ticks
B. bigemina in humans
B. bigemina in animals
B. bigemina in ticks
B. bovis in humans
B. bovis in animals
B. bovis in ticks
B. gibsoni in humans
B. gibsoni in animals
B. gibsoni in ticks
B. canis in humans
B. canis in animals
B. canis in ticks
Other *Babesia* in humans
Other *Babesia* in animals
Other *Babesia* in ticks

Fig 8: Different species of *Babasia* detected by PCR or microscopy in animals and ticks in West Africa

PREMIERE PARTIE :

Identification des arthropodes et leur repas de sang par la spectrométrie de masse MALDI-TOF MS

Préambule

L'identification des arthropodes est une étape cruciale dans la lutte contre les vecteurs et la documentation du risque de transmission des maladies vectorielles transmissibles. La méthode de référence est l'identification morphologique qui nécessite une expertise entomologique élevée et la disponibilité d'une documentation appropriée. Le faible nombre de spécialiste en entomologie, l'indisponibilité souvent de certaines clés d'identification, la ressemblance morphologique des espèces d'un même complexe et l'état ou le stade de l'arthropode sont des éléments qui rendent difficile et longue l'identification morphologique.

La biologie moléculaire a permis de contourner les limites de l'identification morphologique en permettant de différencier les espèces proches morphologiquement. Cependant le coût élevé, le temps nécessaire très long, l'absence de gène universel pour l'identification des arthropodes et le manque d'exhaustivité des bases de données sont les freins importants de cette méthode.

Le MALDI-TOF MS est une technique basée sur l'ionisation des molécules protéiques d'un organisme d'intérêt qui vont être propulsées dans un tube sous vide selon le ratio masse/charge et leur temps de vol sera détecté à la fin du tube. Chaque molécule détectée sera associée à un pic et le spectre global obtenu sera spécifique de l'échantillon analysé. Le MALDI-TOF MS étant une méthode est très rapide, fiable et moins couteux est aujourd'hui utilisé en routine dans les laboratoires de microbiologie clinique pour l'identification des bactéries, parasites et champignons.

Par la suite la spectrométrie de masse MALDI-TOF a été utilisé pour l'identification de nombreux arthropodes ayant un impact en santé humaine tels que les tiques, les puces, les moustiques, les punaises de lit et les phlébotomes. Des études ont aussi évalué la capacité du MALDI TOF à identifier le repas sanguin des moustiques et à pouvoir différencier les

arthropodes infectés des non infectés. Cependant, la technique de broyage, la méthode de conservation et le compartiment de l'arthropode utilisé peuvent influencer sur la qualité des résultats.

Dans le premier travail (*Article 2*) avons amélioré les conditions de préparation des échantillons de tiques en vue de leur identification par MALDI-TOF MS à partir d'échantillons de tiques collectées sur le terrain au Mali puis conservées dans l'éthanol, et d'évaluer la capacité de cette technologie à distinguer les tiques infectées et non infectées. Ce travail nous à permis de montrer que le MALDI-TOF MS permettait d'identifier correctement les tiques conservées en alcool mais qu'il fallait créer une base données spécifiques avec les spectres des tiques conservées en alcool pour avoir des scores d'identification optimum.

Dans le deuxième travail (*Article 3*) nous avons évalué la capacité du MALDI-TOF MS et démontré que le MALDI-TOF MS est un outil prometteur pour identifier les moustiques et leur repas de sang, lorsque les arthropodes sont collectés sur le terrain et conservés dans des tubes avec du silicagel à température ambiante.

J'ai aussi participé à un travail sur l'utilisation du MALDI-TOF MS pour identifier les moustiques collectés au Mali et leur repas de sang (*Article 6*). Ce travail ne sera pas discuté et ici et sera présenté en annexe.

Article 2

Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali.

Diarra Adama Zan, Almeras Lionel, Laroche Maureen, Berenger Jean Michel, Koné Abdoulaye Kassoum, Bocoum Zakaria, Dabo Abdoulaye, Doumbo Ogobara, Raoult Didier, Parola Philippe

PLoS Negl Trop Dis. 2017 Jul; 11(7): e0005762.

Résumé

Les tiques sont considérées comme le deuxième vecteur de maladies humaines et animales après les moustiques. Cependant, l'identification des tiques et des agents pathogènes associés est une étape importante dans la gestion de ces vecteurs. Ces dernières années, la spectrométrie de masse à temps de vol avec désorption / ionisation au laser assistée par matrice (MALDI-TOF MS) a été présentée comme une méthode prometteuse pour l'identification des arthropodes, y compris les tiques. L'objectif de cette étude était d'améliorer les conditions de préparation des échantillons de tiques en vue de leur identification par MALDI-TOF MS à partir d'échantillons maliens stockés dans l'éthanol et recueillis sur le terrain, et d'évaluer la capacité de cette technologie à distinguer les tiques infectées et non infectées.

Au total, 1 333 tiques ont été collectées chez des mammifères dans trois sites distincts du Mali. L'identification morphologique a permis de classer les tiques en 6 espèces, notamment *Amblyomma variegatum*, *Hyalomma truncatum*, *Hyalomma marginatum rufipes*, *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus evertsi evertsi* et *Rhipicephalus sanguineus* sl. Parmi ceux-ci, 471 tiques ont été sélectionnées au hasard pour des analyses moléculaires et protéomiques. Les tiques soumises à MALDI-TOF MS ont révélé une identification morpho / moléculaire concordante de 99,6%. L'inclusion dans notre base de données MALDI-TOF MS de spectres de référence d'arthropodes à partir d'échantillons de patte de tique préservés à l'éthanol était nécessaire pour obtenir une identification fiable. Lorsqu'ils ont été testés avec des outils moléculaires, 76,6%, 37,6%, 20,8% et 1,1% des échantillons testés étaient positifs en *Rickettsia* spp. *Coxiella burnetii*, des bactéries de la famille des *Anaplasmataceae* et des *Borrelia* spp. respectivement.

Ces résultats montrent que MALDI-TOF est un outil fiable d'identification des tiques conservées dans l'alcool et enrichissent les connaissances sur la diversité des espèces de tiques

et des agents pathogènes transmis par les tiques circulant au Mali. Ce travail a été publié dans Plos Negleted tropical diseases.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali

Adama Zan Diarra[1,2], Lionel Almeras[1,3], Maureen Laroche[1], Jean-Michel Berenger[1], Abdoulaye K. Koné[2], Zakaria Bocoum[4], Abdoulaye Dabo[2], Ogobara Doumbo[2], Didier Raoult[1], Philippe Parola[1]*

1 Aix Marseille Université, UM63, CNRS 7278, IRD 198, INSERM 1095, AP-HM, IHU - Méditerranée Infection, Marseille, France, **2** Malaria Research and Training Center, Département d'Epidémiologie des Affections Parasitaires, Faculté de Médecine et d'Odontostomatologie, Faculté de Pharmacie, USTTB, Bamako, Mali, **3** Unité de Parasitologie et Entomologie, Département des Maladies Infectieuses, Institut de Recherche Biomédicale des Armées, Marseille, France, **4** Laboratoire Central Vétérinaire, Bamako, Mali

* philippe.parola@univ-amu.fr

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Abstract

Ticks are considered the second vector of human and animal diseases after mosquitoes. Therefore, identification of ticks and associated pathogens is an important step in the management of these vectors. In recent years, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been reported as a promising method for the identification of arthropods including ticks. The objective of this study was to improve the conditions for the preparation of tick samples for their identification by MALDI-TOF MS from field-collected ethanol-stored Malian samples and to evaluate the capacity of this technology to distinguish infected and uninfected ticks. A total of 1,333 ticks were collected from mammals in three distinct sites from Mali. Morphological identification allowed classification of ticks into 6 species including *Amblyomma variegatum*, *Hyalomma truncatum*, *Hyalomma marginatum rufipes*, *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus evertsi evertsi* and *Rhipicephalus sanguineus sl*. Among those, 471 ticks were randomly selected for molecular and proteomic analyses. Tick legs submitted to MALDI-TOF MS revealed a concordant morpho/molecular identification of 99.6%. The inclusion in our MALDI-TOF MS arthropod database of MS reference spectra from ethanol-preserved tick leg specimens was required to obtain reliable identification. When tested by molecular tools, 76.6%, 37.6%, 20.8% and 1.1% of the specimens tested were positive for *Rickettsia* spp., *Coxiella burnetii*, *Anaplasmataceae* and *Borrelia* spp., respectively. These results support the fact that MALDI-TOF is a reliable tool for the identification of ticks conserved in alcohol and enhances knowledge about the diversity of tick species and pathogens transmitted by ticks circulating in Mali.

Author summary

Ticks are among the most important vectors and reservoirs of several animal and human pathogens such as viruses, bacteria and protozoa. However, very few studies have been done on ticks in Mali. At present, little information is available about tick species infesting

livestock or human tick-borne diseases transmitted in Mali. The identification of tick species and the determination of pathogens associated are essential to evaluate epidemiology and risks of human and animal diseases: the One Health approach. Current identification methods are time consuming, expensive and laborious. Previous studies have shown that MALDI-TOF mass spectrometry analyses may allow accurate tick species identification. A recent study suggested that it was possible to identify ticks preserved in alcohol by MALDI-TOF MS. The aim of the present study was to improve tick leg sample preparation conditions for their identification by MALDI-TOF MS from Malian ethanol-preserved specimens collected in the field. This study provided 99.4% concordance between morphological and MALDI-TOF identification. The detection of microorganisms was also performed by molecular biology revealing the presence of the presence of *Rickettsia* spp., *Coxiella burnetii*, *Borrelia* spp. and *Anaplasmataceae*. These results support the use of MALDI-TOF MS in entomology, tick diseases epidemiology and improve the knowledge of tick species-diversity and tick-borne pathogens circulating in Mali.

Introduction

Ticks are bloodsucking arthropods that parasitize most of the vertebrates in the world and occasionally bite humans [1]. About 900 tick species have been identified and classified worldwide [2]. In Africa, the number of tick species indexed is 223, including 180 hard and 43 soft ticks [2]. Currently, ticks are considered the second most important vector of human disease after mosquitoes and can transmit bacterial [1], viral [3] and protozoan pathogens [4]. A significant number of these pathogens are of exceptional importance, as they are responsible for high morbidity and mortality in humans and animals [1]. Identification of tick species is an important step in epidemiological studies, in order to establish tick species distribution maps and to characterize tick fauna and seasonal trends [5,6].

In Mali, a West African country, livestock farming is an essential economical factor. At present, there are few studies on tick species that infest cattle or tick-borne diseases transmitted in Mali. To date, 23 tick species belonging to six genera have been categorized in Mali [7–9]. Among them, *Amblyomma (Am.) variegatum*, *Rhipicephalus (Rh.)* spp. and *Hyalomma (Hy.)* spp. are the main ticks monitored by Malian veterinarians for their effects on livestock healthcare and productivity [10]. Other public health problems, such as tuberculosis, AIDS or malaria, take precedence over tick-borne diseases (TBDs), which are little explored by medical doctors.

Several bacteria were detected in ticks from Mali. Spotted fever group rickettsiae were detected, including *Rickettsia africae* in *Am. variegatum*, *R. aeschlimannii* in *Hy. marginatum rufipes*, and *R. massiliae in Rhipicephalus* spp., all three being human pathogens [11]. An *Ehrlichia* sp. of unknown pathogenicity, *Ehrlichia* Erm58, was detected in *Rh. mushamae* [11]. More recently, *Borrelia theileri*, the agent of bovine and equine borreliosis, and *B. crocidurae*, agents of relapsing fever in humans, have been detected in *Rh. geigyi* and *Ornithodoros sonrai*, respectively [12–14].

To study and control ticks and TBD transmission, accurate identification of tick species and determination of their infectious status are essential [1]. Currently, tick identification is principally conducted by observing morphological characteristics. However, it is limited by entomological expertise, dichotomous keys availability, tick integrity or engorged status [9]. Molecular tools have been used as an alternative to overcome the limitations of morphological identification [15]. Sequencing of several genes has been used, including ribosomal sub-units

(e.g., *12S*, *16S* or *18S*), the cytochrome c oxidase unit I (*COI*), or the internal transcribed spacer [16]. These techniques are generally time-consuming, laborious and can be expensive, preventing their use in large scale studies [17–20]. Moreover, the absence of a consensus gene target sequence for tick identification and/or the comprehensiveness of genomic databases are additional factors hampering their use [16].

Recently an alternative tool based on the analysis of protein profiles resulting from matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis has been explored to identify arthropods [21]. MALDI-TOF MS has been used to identify tick species [22–24] and to determine tick infectious status [25–27].

However, tick collection usually takes place far from analytical laboratories and therefore requires proper storage of samples. Ticks are generally stored either alive, at -20˚C, or in alcohol. Although alcohol storage is cheaper and easier, especially in African countries, previous studies reported that the use of fresh (i.e., recently dead) or frozen specimens led to more reproducible and better MS spectra compared to the alcohol preservation mode for ticks [24] [28], and also for other arthropod families[29,30]. In a recent study, it was demonstrated that long-term tick storage in alcohol altered MS profiles, which did not provide conclusive identification following in-house MS reference spectra database-querying containing MS spectra from counterpart fresh tick species. Nevertheless, the upgrading of the in-house MS reference spectra database of specimens stored in alcohol allowed correct identification of ticks at the species level, also underlining the reproducibility and specificity of MS profiles for tick specimens stored in alcohol [31].

The goal of the present work was to determine tick population diversity and associated pathogens from alcohol stored specimens collected on cattle in Mali by using MALDI-TOF MS and molecular approaches with specimens collected in the field. First, optimized sample preparation conditions for ticks stored in ethanol for MALDI-TOF MS analysis were established. Second, based on morphological and molecular identification of ticks, an MS reference spectra database was created and tested blindly using new tick specimens. In addition, tick-associated bacterial pathogens were screened by molecular biology on half-tick body parts and leg MS spectra from ticks mono-infected or not by bacterial pathogenic agents, and they were compared to assess the efficiency of this proteomic tool for classification of ticks according to their infectious status

Materials and methods

Ethical considerations

Tick collection protocols were developed as of a large study under the GIRAFE programme, UMI 3189 and MSHP-MRTC HFV project. The protocols were cleared by the FMPOS IRB in 2015 and 2016. Verbal informed consent was obtained from managers of the livestock selected for tick sampling directly on mammals. The collection of ticks on domestic animals did not involve national parks or other protected areas or endangered or protected species.

Study sites and collection period

Ticks were collected from three localities in Mali, including Bamako, Kollé and Bougoula Hameau, in September 2015 and August 2016 (Fig 1). Bamako, the capital city of Mali, is an urban area surrounded by hills. The climate is Sahelian-type with two distinct seasons, the dry season (i.e., from November to May) and the rainy season (i.e., from June to October). The total amount of precipitation was less than 900 milliliters in 2009. Kollé is a rural village located about 60 km southwest of the capital. Agriculture, livestock farming and small businesses are the main economic activities of the village. The village, located on a flat land with submersible

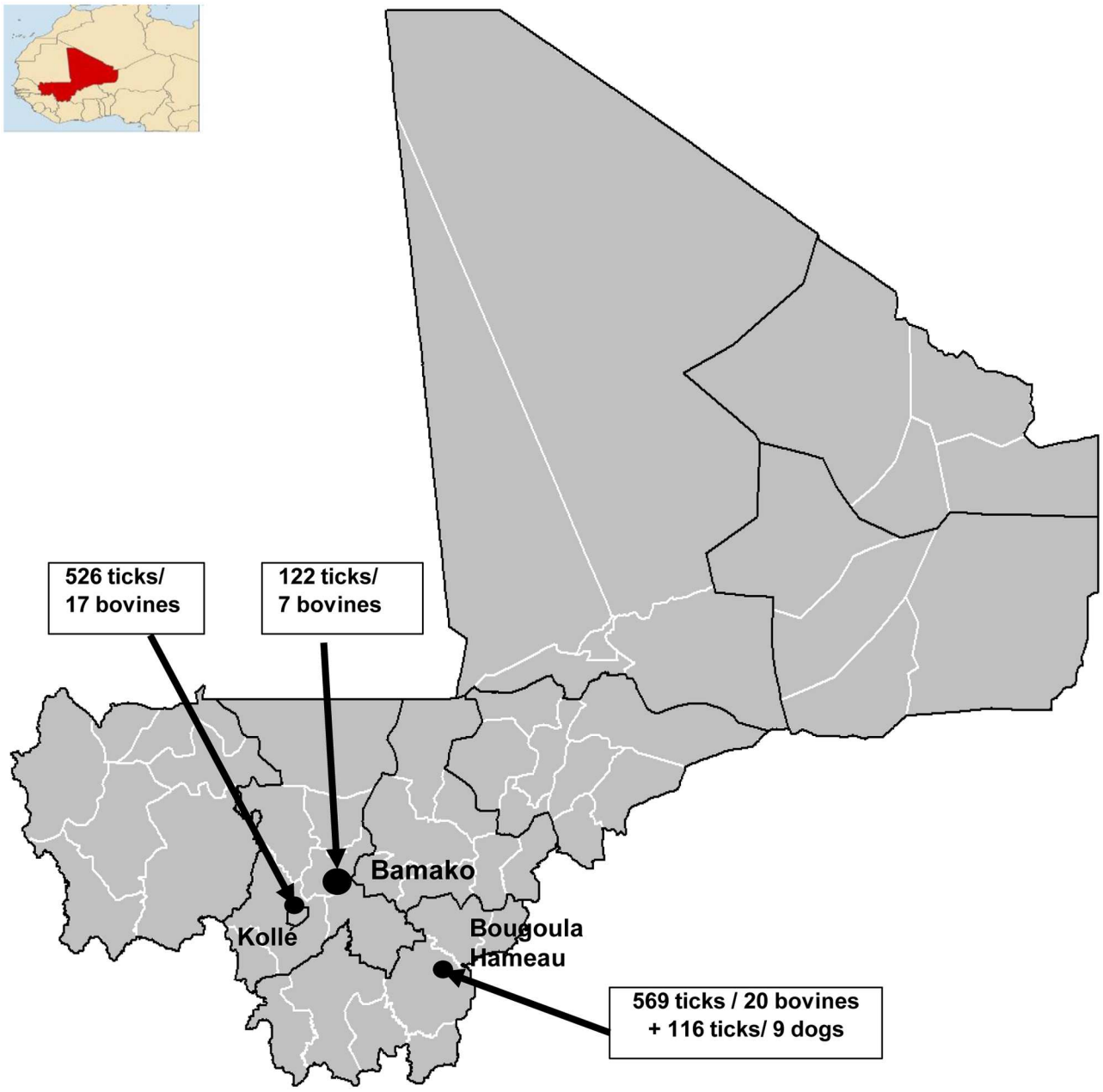

Fig 1. Map of Mali showing the sites where the ticks were collected for our study and number of ticks collected and number of cattle prospected per site.

https://doi.org/10.1371/journal.pntd.0005762.g001

and dry areas, presents a Sahelian-type climate with two distinct seasons, a rainy (i.e., from June to November with maximum rainfall in August-September of 350 to 400 milliliters) and a dry season (i.e., from December to May with a cool period in December- February and a warm period in March-May). The third site was Bougoula Hameau, a suburban village, located at 4 km of Sikasso town and it was situated at 374 km southeast of Bamako by road. The climate is of Sudanese type, under the influence of the humid forest with a rainy (i.e., from May to October) and a dry season (i.e., from November to April). The annual rainfall can vary from 1,200

to 1,800 milliliters, depending on the year. These climatic conditions are appropriate for agricultural and livestock farming.

Collection method

Ticks were collected from domestic animals and cattle. Examination of all body parts was conducted from the tail to the head of the animal to detect ticks on the skin. All ticks (engorged and non-engorged) were collected manually with forceps. The ticks of the same animal were counted, pooled in the same tube and stored at room temperature in 70% v/v ethanol (ticks collected in September 2015) or frozen at -20˚C (ticks collected in August 2016) until morphological, molecular and MALDI-TOF MS analyses. Ticks were transferred from MRTC (Bamako, Mali) to the URMITE laboratories (Marseille, France) for analysis.

Morphological identification

Ticks were identified morphologically to the species level firstly by a PhD student and then checked by expert tick entomologists using previously established taxonomic identification keys [9]. Tick identification and gender determination were performed under microscope at a magnification of ×56 (Zeiss Axio Zoom.V16, Zeiss, Marly le Roi, France). The tick genera, species, gender, host and animal number, collection site and date were codified to include this information on the tube.

Ticks dissection and sample preparation

Each tick was dissected with a new sterile surgical blade to remove the legs, which were used for MALDI-TOF MS analyses. The rest of the tick was longitudinally cut in two equal parts. The half part with legs cut off was immediately used for molecular biology, and the second half was stored frozen as a backup sample for any additional analysis.

DNA extraction

Each half-tick without legs was transferred to a 1.5 mL tube containing 180 μL of G2 lysis buffer and 20 μL proteinase K (Qiagen, Hilden, Germany), and incubated at 56˚C overnight. DNA extraction from the half-tick was performed with an EZ1 DNA Tissue Kit (Qiagen) according to manufacturer recommendations. The DNA from each sample was eluted with 100 μL of Tris-EDTA (TE) buffer (Qiagen) and was either immediately used or stored at -20˚C until use.

Molecular identification of ticks

Standard PCR, using an automated DNA thermal cycler amplifying a 405-base pair fragment of the mitochondrial 12S RNA gene (Table 1), was used for tick identification to the species level, as described previously [31]. The 16S RNA gene was used to confirm all *Rhipicepalus (Boophilus) microplus* identification. DNA from *Am. variegatum* specimens reared at the laboratory was used as positive control. PCR products of the positive samples were purified and sequenced as described previously [31]. The sequences were assembled and analyzed using the ChromasPro software (version 1.34) (Technelysium Pty. Ltd., Tewantin, Australia), and were then blasted against GenBank (http://blast.ncbi.nlm.nih.gov).

Detection of pathogens

Quantitative PCR was performed according to the manufacturer's protocol using a PCR detection system; a CFX Connect™ Real-Time (Bio-Rad) with the Eurogentec Takyon qPCR kit

Table 1. Primers and probes used for real-time quantitative and standard PCR in this study.

Microorganisms	Targeted sequence	Primers f, r (5'-3') and probes p (6FAM-TAMRA)	References
qPCR primers			
Rickettsia spp.	*gltA(RKND03)*	f_GTGAATGAAAGATTACACTATTTAT	[72]
		r_GTATCTTAGCAATCATTCTAATAGC	
		p_CTATTATGCTTGCGGCTGTCGGTTC	
R. africae	*poT15-dam2*	f_TGCAACACGAAGCACAAAAC	[32]
		r_CCTCTTGCGAAACTCTACTT	
		p_TGACGTGTGGATTCGAGCACCGGA	
Anaplasma spp.	*23SrRNA (TtAna)*	f_TGACAGCGTACCTTTTGCAT	[34]
		r_TGGAGGACCGAACCTGTTAC	
		p_GGATTAGACCCGAAACCAAG	
Borrelia spp.	*(Bor ITS4)*	f_GGCTTCGGGTCTACCACATCTA	[62]
		r_CCGGGAGGGGAGTGAAATAG	
		p_TGCAAAAGGCACGCCATCACC	
	(Bor_16S)	f_AGCCTTTAAAGCTTCGCTTGTAG	[73]
		r_GCCTCCCGTAGGAGTCTGG	
		p_CCGGCCTGAGAGGGTGAACGG	
C. burnetii	*(IS30A)*	f_CGCTGACCTACAGAAATATGTCC	[74]
		r_GGGGTAAGTAAATAATACCTTCTGG	
		p_CATGAAGCGATTTATCAATACGTGTATG	
Bartonella spp.	*(Barto ITS2)*	f_GATGCCGGGGAAGGTTTTC	[75]
		r_GCCTGGGAGGACTTGAACCT	
		p_GCGCGCGCTTGATAAGCGTG	
Standard PCR primers			
Rickettsia spp.	*gltA*	f_ATGACCAATGAAAATAATAAT	[33]
		r_CTTATACTCTCTATGTACA	
Anaplasma spp.	*23SrRNA*	f_ATAAGCTGCGGGGAATTGT	[34]
		r_TGCAAAAGGTACGCTGTCAC	
Borrelia spp.	*flaB*	f_TGGTATGGGAGTTTCTGG	[35]
		r_ TAAGCTGACTAATACTAATTACCC	

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(Takyon, Eurogentec, Belgium). The qPCR reaction contained 10 μl of Takyon Master Mix (Takyon, Eurogentec, Belgium), 3.5 μl sterile distilled water, 0.5 μl of each of the primers and probe and 5 μl of the DNA extract. A total of 471 samples were screened using primers and probes, targeting specific sequences of the following bacterial pathogens: *Rickettsia* spp., *Anaplasmataceae* spp., *Borrelia* spp., *Bartonella* spp. and *Coxiella burnetii* (Table 1). For *Borrelia* spp we used 2 genes, the 16S *Borrelia* gene first and all the ticks that were positive for this gene were retested by ITS4 for confirmation. Only samples positive for both genes (16S borrelia and ITS4) were considered positive. Positive samples for *Rickettsia* spp. were then submitted to a qPCR system specific for detecting *R. africae* [32]. Negative samples for *R. africae* but positive for *Rickettsia* spp. were submitted to *gltA* gene sequencing to determine *Rickettsia* species [33]. All ticks positive either for *Anaplasmataceae* spp. were submitted to amplification using standard PCR and sequencing to identify the bacteria species [34,35]. Ticks that were positive for Borrelia spp for both the 16S Borrelia gene and ITS4 were submitted to amplification using standard PCR and sequencing [33]. PCR tests were considered positive when the cycle threshold (Ct) was lower than 36 [36]. The DNA from *Rickettsia montanensis*, *Bartonella elizabethae*, *Anaplasma phagocytophilum*, *Coxiella burnetii* and *Borrelia crocidurae* was used as positive controls and mix as negative controls in PCR, respectively. All these bacteria come from the

strains of culture of our laboratory and *Borrelia crocidurae was* cultured in Barbour-Stoenner-Kelly (BSK-H) liquid medium supplemented with rabbit serum. Only samples considered as negative (i.e., Ct $\geq$ 36 for all bacteria tested), were submitted to 12 S tick gene amplification to control the correctness of DNA extraction.

MALDI-TOF MS analyses

Optimization of tick sample preparation prior to submission for MALDI-TOF MS. Three protocols were tested for tick leg sample preparation from specimens stored in 70% ethanol to determine the one exhibiting the best MS results (Fig 2). For each comparison, four legs from one side were cut off to test the first protocol, and the four other legs from the same tick were used to assess the second protocol. The criteria for protocol selection were by order of importance, intra-species MS spectra reproducibility and the simplicity of the protocol. The reference protocol, called "de-alcoholization" consisted of a 10-minute successive incubation washing of the whole tick (i.e., prior to dissection) in decreasing ethanol concentrations from 70% to 10% (v/v). A final wash in distilled water was conducted prior to sample drying on sterile filter paper and tick leg cutting as previously described [31]. The second protocol, called "direct-MS," consisted in drying the whole tick on filter paper, and the cutting of the four legs, which were directly homogenized for MALDI-TOF analysis. The third protocol, called "dry-MS", was similar to the "direct-MS" protocol, except those cuts off legs were dried overnight at 37˚C prior to homogenization. Whatever the protocol used, tick legs were homogenized with the same method. A pinch of glass powder (Sigma, Lyon, France) was added to the tick legs, plus 40 μL of a mix of 70% (v/v) formic acid and 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland). The legs were then homogenized using the TissueLyser apparatus (QIAGEN, Germany) with the following setup parameters as previously described [28]. First, the "de-alcoholization" protocol was compared to the "dry-MS" one, and then the "dry-MS" protocol was compared to the "direct-MS" protocol. The selected protocol was then applied to the other tick legs homogenized in the present study. The tick legs stored frozen were directly homogenized and used for MALDI-TOF MS.

Sample loading on the target plate

The homogenized tick legs were centrifuged at 2000 g for 30 seconds and 1 μL of the supernatant from each sample was carefully dropped onto the MALDI-TOF target plate as previously described [28]. Each spot was then recovered with 1 μL of CHCA matrix solution composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma, Lyon. France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Aldrich, Dorset, UK) and HPLC-grade water [16]. The target plate, after drying for several minutes at room temperature, was introduced into the Microflex LT MALDI-TOF Mass Spectrometer device (Bruker Daltonics, Germany) for analysis. The loading of the MS target plate, the matrix quality, and the performance of the MALDI-TOF were performed as previously described [28].

MALDI-TOF MS parameters

Protein mass profiles were obtained using a Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) using parameters previously described [31]. The spectrum profiles obtained were visualized with Flex analysis v.3.3 software and exported to ClinProTools software v.2.2 and MALDI-Biotyper v.3.0. (Bruker Daltonics, Germany) for analysis [25].

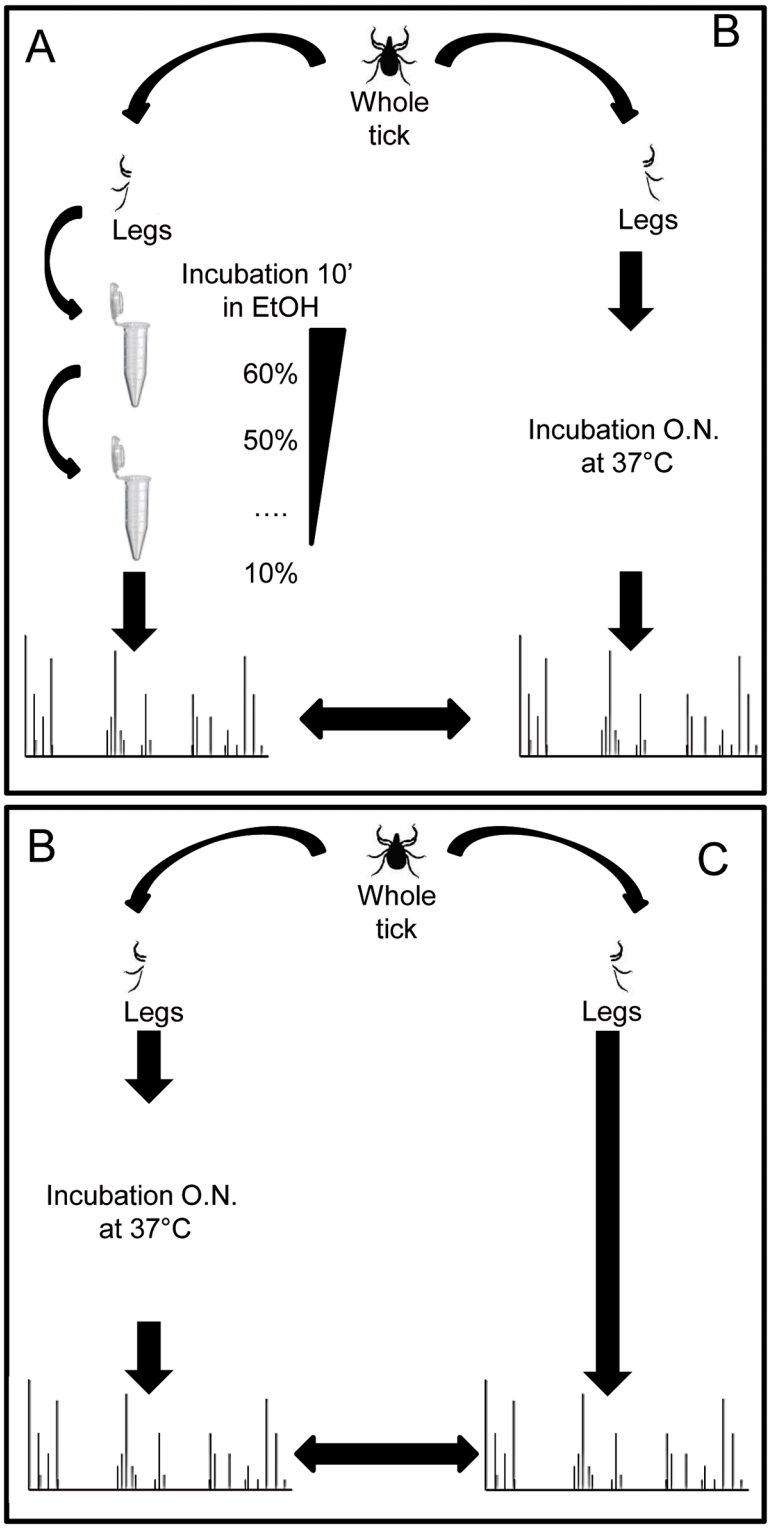

Fig 2. Protocol design of ticks treatment for MALDI- TOF MS analyses. "De-alcoholization" (A), "dry" (B) and "direct" (C) protocols for sample preparation were illustrated.

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Spectra analysis

The reproducibility of spectra was evaluated by analyzing ten *Am. variegatum* specimens from Kollé per sample preparation protocol as previously described [29]. The selected protocol was analyzed using an unsupervised statistical test classifying specimens according to MS spectra (i.e., Principal Component Analysis, PCA test, ClinProTools v2.2 software). The Composite Correlation Index (CCI) tool (MALDI-Biotyper v3.0. software, Bruker Daltonics), was used to assess spectra variations within each sample group according to protocol tested, as previously described [37]. CCI was computed using the standard settings of mass range 3000–12000 Da, resolution 4, 8 intervals and autocorrelation off. Higher the log score values (LSVs) and correlation values (expressed by mean ± standard deviation [SD]) reflect higher reproducibility of MS spectra and were used to determine the best protocol for sample preparation.

Reference database creation

Based on the correlation of morphological and molecular results of tick identification, two to five specimens per species were used to assess MS spectra reproducibility from specimens of the same tick species, and the MS spectra specificity from specimens of distinct tick species. These analyses were performed with the average spectral profiles (MSP, Main Spectrum Profile) obtained from the four spots of each individual tick specimen using Flex analysis v.3.3 and ClinProTools 2.2 softwares (Bruker Daltonics). Tick species exhibiting reproducible and specific MS spectra were then included in-house MS spectra reference database. To upgrade the database, MSP reference spectra were created using spectra from at least 2 specimens per species of both genders by the automated function of the MALDI-Biotyper software v3.0. (Bruker Daltonics). MS spectra were created with an unbiased algorithm using information on the peak position, intensity and frequency [38]. The spectra files are available on request and transferable to any Bruker MALDI-TOF device.

Blind tests

A blind test was performed with new tick specimens collected in Mali stored in 70% alcohol or frozen. A total of 451 MS spectra from tick legs, including 340 stored in alcohol and 111 frozen specimens were tested successively against the in-house MS reference spectra database (Database 1) and its upgraded version, which includes the 20 MS spectra from specimens of the 6 tick species collected in Mali and alcohol-preserved (Database 2). Among the 451 ticks tested 51 *Am. variegatum* and 23 *Rh (B) microplus* were fully engorged. Database 1 was composed of specimens of fresh or frozen arthropods (Table 2) [16,24,30,31,39]. Database 2 includes database 1 plus MS spectra of tick legs from 6 species stored in ethanol from the present study (Tables 3 and 4). The reliability of species identification was estimated using the LSVs obtained from the MALDI-Biotyper software v.3.3, which ranged from 0 to 3. These LSVs correspond to the degree of similarity between the MS reference spectra database and those submitted by blind tests. An LSV was obtained for each spectrum of the samples tested. Moreover, to decipher incoherent results obtained between morphological and MS identification, molecular identification of ticks was performed for the respective specimens.

Determination of tick infection status

These comparative analyses to determine the infectious status of ticks were made by ClinProTools v.2.2 software (Bruker Daltonics, Germany). Only tick leg MS spectra from species with at least five mono-infected or pathogen-free specimens were included in this analysis. The spectra of 30 specimens of *A. variegatum* infected by *R. africae* were compared to those of 12

Table 2. List of the arthropod species present in the MALDI-TOF MS database 1.

Ticks	*Amblyomma variegatum*, *Rh. sanguineus sl*, *Hyalomma marginatum rufipes*, *Ixodes ricinus*, *D. marginatus* and *D. reticulatus*, *Am. gemma*, *Am. cohaerens*, *Rh. e. evertsi*, *Rh. decoloratus*, *Rh. pulchellus*, *Rh. bergeoni*, *Rh. praetextatus*, *Hy. truncatum* and *Haemaphysalis leachi*
Mosquitoes	*Anopheles coluzzii* and *An. gambiae*, *An. funestus*, *An. ziemanni*, *An. arabiensis*, *An. wellcomei*, *An. rufipes*, *An. pharoensis*, *An. coustani*, *An. claviger*, *An. hyrcanus*, *An. maculipennis*, *Culex quinquefasciatus*, *Cx. pipiens*, *Cx. modestus*, *Cx. insignis*, *Cx. neavei*, *Ae. albopictus*, *Aedes excrucians*, *Ae vexans*, *Ae. rusticus*, *Ae. dufouri*, *Ae. cinereus*, *Ae. fowleri*, *Ae. aegypti*, *Ae. caspius*, *Mansonia uniformis*, *Orthopodomyia reunionensis*, *Coquillettidia richiardii* and *Lutzia tigripes*
Lice	*Pediculus humanus corporis*, *Damalinia bovis*, *D. caprae*, *D. ovis*, *Haematopinus eurysternus*, *Linognatus vituli*, *L. africanus*
Triatomine	*Triatoma infestans*,
Bedbugs	*Cimex lectularius*
Flea	*Ctenocephalides felis*, *Ct. Ct. canis*, *Archaeopsylla erinacei*, *Xenopsylla cheopis* and *Stenoponia tripectinata*

Mosquito, tick, triatomine, bedbug reference spectra were obtained from legs protein extracts. Flea reference spectra were obtained from the whole body without abdomen protein extracts. Sandfly reference spectra were obtained from thorax, wings and legs protein extracts. Louse reference spectra were obtained from half of the body protein extracts. These species include field specimens or from insectary breeding, but also specimens collected from patients.

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uninfected specimens from the same species. Moreover, MS spectra of 36 uninfected specimens of *Hy. truncatum* were also compared with the spectra of 23 specimens of *Hy. truncatum* infected by *C. burnetii*.

Results

Tick collection and morphological identification

A total of 1,333 ticks were collected from the three sites including 406 engorged (Fig 1). A total of 1,217 were found on 44 bovine specimens and 116 on 9 dogs. Nineteen engorged females of the *Hyalomma* genus (1.55% of ticks collected) were not morphologically identified to the species level. Morphologically, six distinct tick species belonging to three genera were identified among ticks collected in September 2015 (Table 3). *Am. variegatum* (n = 877, 71.79%) was the overall predominant tick species collected from different sites, followed by two species of the *Hyalomma* genus, *Hy. truncatum* (n = 260, 21.27%) and *Hy. m. rufipes* (n = 28, 2.29%). The three other tick species, *Rh. (Bo.) microplus*, *Rh. e. evertsi* and *Rh. sanguineus sensus lato*, represented less than 3.10% (n = 38). The five *Rh. sanguineus sl* [40] specimens were all collected on a dog. All 111 ticks collected in August 2016 were identified as *Rh. sanguineus sl*. Three hundred sixteen of the 1,222 ticks collected from three sites in 2015 and 111 ticks in 2016 had specimens of six species randomly selected for molecular and proteomic analyses (Table 3).

Validation of morphological identification by molecular tools on a subgroup of ticks

A total of 20 specimens, including 2 to 5 specimens per species and all specimens of *Rh (Bo.) microplus*, were randomly selected for molecular analysis. A GenBank query revealed that 12S gene sequences were available for the 6 tick species. BLAST analysis indicated high identity (i.e., a range from 99% to 100%) of 12S rRNA gene sequences among specimens classified per species according to morphological identification (Table 4). BLAST analysis revealed that

Table 3. Ticks collected per site and used for MALDI-TOF MS analyses.

Tick species	Bamako		Kollé		Bougoula-Hameau				Total number of specimens
	Number of specimens*	%	Number of specimens*	%	Number of specimens*	%	Number of specimens selected for MALDI-TOF MS	Number of MALDI-TOF MS specimens also selected for molecular analyses	
Am. variegatum	88 (39)	72.1	337 (182)	64.1	452 (208)	65.9	181	5	**877**
Hy. truncatum	8 (7)	6.6	164 (85)	31.2	88 (35)	12.8	122	5	**260**
Hy. m. rufipes	3 (0)	2.4	12 (5)	2.2	13 (3)	1.9	19	3	**28**
H. spp. [#]	0	0.0	11 (11)	2.1	8 (8)	1.2	0.0	0	**19**
Rh(Bo). microplus	16 (16)	13.1	2 (2)	0.4	8 (8)	1.2	26	3	**26**
Rh. e. evertsi	7 (2)	5.8	0	0	0	0	7	2	**7**
Rh. sanguineus sl	0	0	0	0	116 (52)	17	5+111[&]	2	**116**
Total	**122**	**100**	**526**	**100**	**685**	**100**	**471**	**20**	**1333**

*Females indicated in parentheses.

[#]Engorged specimens non-identifiable at the species level.

[&] Ticks collected in August 2016

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these 6 tick species and all specimen of *Rh (B) microplus* had high sequence identity with their respective homolog species available in GenBank (i.e., range 96.5% to 100%; Table 4).

Detection of bacteria in ticks

Among the ticks tested, 41.8% (197/471) were negative for the six bacteria tested, 37.4% (176/471) were positive for one bacterium and 20.8% (98/471) were found co-infected by two or

Table 4. Tick species selected to create a MALDI-TOF MS reference database, identified by molecular biology.

Morphological identification	Origin	Number of specimens tested	Intra-species similarity of 12S rRNA gene sequence (%)	Molecular identification by BLAST (Accession Number)	Query cover (%)	Similarity level with GenBank (%)
Am. variegatum	Bamako, Kollé, Bougoula	5	100%	*Am. variegatum* (JF949801.1)	100%	100%
Hy. truncatum	Bamako, Kollé, Bougoula	5	99–100%	*Hy. truncatum* (AF150031.1)	99–100%	96–97%
Hy. m. rufipes	Bamako, Kollé, Bougoula	3	100%	*Hy. m. rufipes* (KC817342.1)	100%	100%
Rh(Bo) microplus	Kollé, Bougoula	3	99–100%	*Rh(Bo) microplus* (DQ003008.1)	100%	99–100%
Rh. e. evertsi	Bamako	2	100%	*Rh. e. evertsi* (KU255856.1)	99%	100%
Rh. sanguineus sl	Bougoula	2	100%	*Rh. sanguineus* (KC817342.1)	100%	100%
Rh(Bo) microplus*	Kollé, Bougoula, Bamako	26	100%	*Rh. microplus* (KY020993.1)	100%	100%

*Ticks identified by 16S rRNA gene

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Table 5. Percentage of positive ticks detected by PCR.

Bacterium% (positives/tested)	*Am. variegatum*	*Hy. truncatum*	*Hy. m. rufipes*	*Rh (Bo) microplus*	*Rh. e evertsi*	*Rh. sanguineus sl*	Total
Rickettsia. Spp.	**92.2% (168/181)**	**20.5% (25/122)**	**68.4% (13/19)**	**7.7% (2/26)**	**14.3% (1/7)**	**0.8% (1/116)**	**76.6% (210/274)**
R. africae	92.2% (168/181)	9.8% (12/122)	5.2% (1/19)	7.7% (2/26)	14.3% (1/7)	-	87.6% (184/210)
R. aeschlimannii	-	10.6% (13/122)	52.6% (10/19)	-	-	0.8% (1/116)	11.4% (24/210)
R. mongolitimonae	-	1.6% (2/122)	-	-	-	-	1% (2/210)
Anaplasmataceae spp.	**8.3%(15/181)**	**10.6%(14/122)**	**10.5%(2/19)**	**80.7%(24/26)**	**42.8%(3/7)**		**20.8% (57/274)**
A. marginale	-	-	-	11.5% (3/26)	-		6% (3/50)
A. candidatus ivoriensis	-	-	-	0.5% (1/26)	-	-	2% (1/50)
E. ruminantium	8.3% (15/181)	2.4% (13/122)	10.5%(2/19)	42.3% (11/26)	28.5% (2/7)	-	86% (43/50)
Candidatus Ehrlichia rustica	-	0.8% (1/122)	-	-	-	-	2% (1/50)
Candidatus Ehrlichia urmitei				1.1% (2/181)			4% (2/50)
C. burnetii	**21.5% (39/181)**	**31.1% (38/122)**	**26.3% (5/19)**	**53.8% (14/26)**	**42.8% (3/7)**	**3.4% (4/116)**	**37.6% (103/274)**
Borrelia spp.	**1.1% (2/181)**	**0.8% (1/122)**	**-**	**-**	**-**	**-**	**1.1 (3/274)**

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three of the screened bacteria. Among the 274 specimens found positive for at least one bacteria tested, 76.6% (210/274) were infected by *Rickettsia* spp., among which *R. africae* was found in 87.6% (184/210) (Table 4). The amplification of the *ompA* fragment in the remaining ticks positive for *Rickettsia* spp. and negative for *R. africae* (n = 26) was used for identification of these *Rickettsia* spp. *R. aeschlimannii* and *R. mongolitimonae* were detected in 24 and 2 tick specimens, respectively (Table 5). Screening of all ticks for *Coxiella burnetii* revealed that 37.6% (103/274) of the specimens were positive (Table 4). Fifty-seven ticks, 20.8% (57/274) were positive in qPCR targeting the 23S rRNA of *Anaplasmataceae*. Among them, 23S rRNA amplification and sequencing was successful for 50 samples. The BLAST found broad agreement that 43 ticks were positive for *E. ruminantium* (GenBank accession number NR 077002.1), 2 ticks were positive for *Ehrlichia sp. urmitei* TCI148 (GenBank ACCN KT 364334.1) and 1 tick for *Ehrlichia sp. rustica* TCI141 (GenBank ACCN KT 364330.1). *A. marginale* was detected in 3 ticks and *A. sp. ivoriensis* TCI50 (GenBank ACCN KT 364336.1) in 1 specimen (Table 5). *Borrelia* spp. was detected in 1.1% (3/274) of ticks by qPCR. However, all standard PCR for determination of *Borrelia* species failed. No *Bartonella* spp. was detected in the ticks tested.

Sample preparation protocol optimization for MALDI-TOF MS tick species identification of specimens preserved in ethanol

A comparison of our current reference sample preparation method (i.e., "de-alcoholization") with the "dry" and "direct" methods was performed [31]. The best method was selected on the following criteria: reproducibility and intensity of MS spectra, low handling and simplicity of the protocol. To exclude inter-individual variability, protocols were successively compared by pairs, and then the four right legs were used for one protocol and the four left legs from the same tick for the other. Then, ten specimens of both genders tested per protocol, five males and five females, were included. For all these experiments, morphologically identified ticks from Kollé (*Am. variegatum)* were used. The first comparison concerned the "de-

alcoholization" and "dry" protocols (Fig 2A). The visual comparison of MS profiles between these two groups using the gel view tool and the superimposition of average MS profiles in each condition using ClinProTools software (Bruker) did not reveal differences in peak position between the two protocols (Fig 3A and 3B). This reproducibility of the profiles was analyzed using an unsupervised statistical test classifying specimens according to MS spectra (i.e., Principal Component Analysis, PCA test, ClinProTools software). The mixing of both groups on the graphical representation confirmed the absence of differences between both groups (Fig 3C). Thus, the "dry" protocol was preferred compared to the "de-alcoholization" protocol, the latter considered to be more time-consuming and fastidious. The second comparison concerned the "dry" and "direct" protocols, using ten *Am. variegatum* specimens from both genders (Fig 2B). The comparison of MS profiles between these two groups, either by gel view, superimposition or PCA (Fig 4A, 4B and 4C), could not determine the more relevant method. The Composite Correlation Index (CCI) tool revealed a higher CCI (LSV mean±SD: 0.783 ±0.101; Fig 4D) for the "dry" protocol compared to "direct" (LSV mean±SD: 0.755±0.175; Fig 4D). These results were in agreement with the gel view showing a higher visual homogeneity of the MS spectra from the "dry" group. Finally, the "dry" protocol appeared consistently to be the more reproducible and low-handling procedure for the preparation of ethanol-stored ticks for MS analysis, and was chosen for the next experiments of the present study.

Intra-species reproducibility and inter-species specificity of MS spectra

Twenty ticks, including several specimens per species coming from distinct localities, were identified by sequencing 12S tick gene. Their non-infected status was also controlled for the microorganisms tested in the present work by q PCR. These specimens were selected for evaluating intra-species reproducibility and inter-species specificity of MS spectra. Comparison of the MS spectra with Flex analysis software indicated reproducibility of the MS profiles between tick specimens from the same species (Fig 5A). Moreover, the visual comparison of MS profiles indicated a clear distinction of spectra according to species. To reinforce the specificity of MS profiles according to tick species, MS profiles from these 20 specimens were used to generate a dendrogram and PCA (Fig 5B and 5C). Clustering analysis revealed a gathering on distinct branches of ticks according to species. However, at the genus level, all specimens from the *Rhipicephalus* genus were not clustered in the same part of the dendrogram. The profile of the spectra of specimens preserved in alcohol was different from those of fresh specimens of the same species; this difference was also observed between manual sample homogenization and automated sample crushing using the TissueLyser apparatus.

MS reference spectra database upgrading and blind test for tick identification

To assess the efficacy of the in-house MS reference spectra database, named database 1 (DB 1) to correctly identify tick specimens preserved in alcohol, half of the MS spectra from ticks included in the present study were randomly selected. Then, MS spectra from 178 specimens including 60 *Am. variegatum*, 64 *Hy. truncatum*, 16 *Hy. m. rufipes*, 26 *Rh. (Bo.) microplus*, 7 *Rh. e. evertsi* and 5 *Rh. sanguineus sl* were queried against the DB 1 spectra database. The blind test against DB 1 revealed correct identification for some specimens of *Hy. truncatum* (n = 5) and *Hy. m. rufipes* (n = 5), with LSVs > 1.8 (Table 6). For the remaining ticks (n = 168), all LSVs were < 1.8 [24]. Tick MS spectra from 20 specimens, including 6 species identified morphologically and molecularly in this work, were added to DB 1, which was then renamed DB 2 (Table 4). Thereafter, the leg spectra of the 451 morphologically-identified ticks, including 340

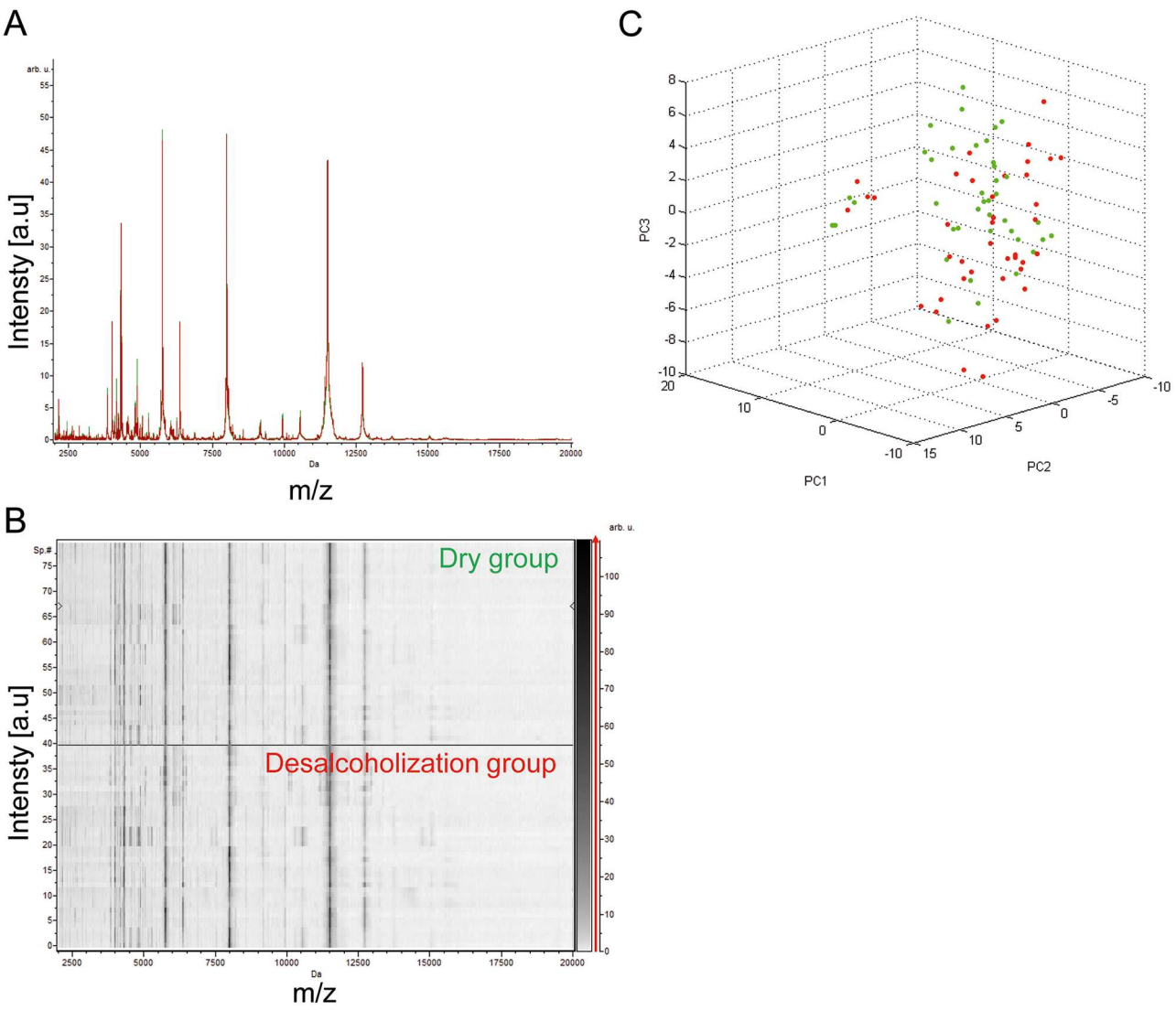

Fig 3. Comparison of MALDI-TOF MS spectra from legs of same tick between "de-alcoholization" and "dry" protocols. Representation of MS profiles by the superimposition of average MS profiles from legs of ticks treated by "de-alcoholization" and "dry" protocols (A), or the gel view tool (B). Tick legs MS spectra from "dry" (green dots) and "de-alcoholization" (red dots) protocols were compared by Principal Component Analysis (C); a.u., arbitrary units; m/z, mass-to-charge ratio.

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stored in alcohol and 111 frozen, were queried against DB 2. Among the 451 ticks tested 51 *Am. variegatum* and 23 *Rh (Bo.) microplus* were fully engorged.

The results of this second interrogation (blind test 2, BT2) showed 96.7% (325/340) concordance between morphological identification and MALDI-TOF MS identification. The percentage of concordant identification with morphology was 100% for *Rh (Bo) microplus*, *Rh. e. evertsi* and *Rh. sanguineus sl* stored in alcohol, with LSVs ranging from 1.89 to 2.71 (Table 6). A total of 15 specimens presented divergent identification between morphological and MALDI-TOF MS identification. To eliminate any doubt, these 15 specimens were submitted to molecular identification. Sequencing of the 12S gene confirmed the identification obtained by MALDI-TOF MS for 13 specimens (Table 6). The remaining 2 specimens identified as *Hy. m. rufipes* by MALDI-TOF MS were finally classified as *Hy. truncatum* by molecular biology,

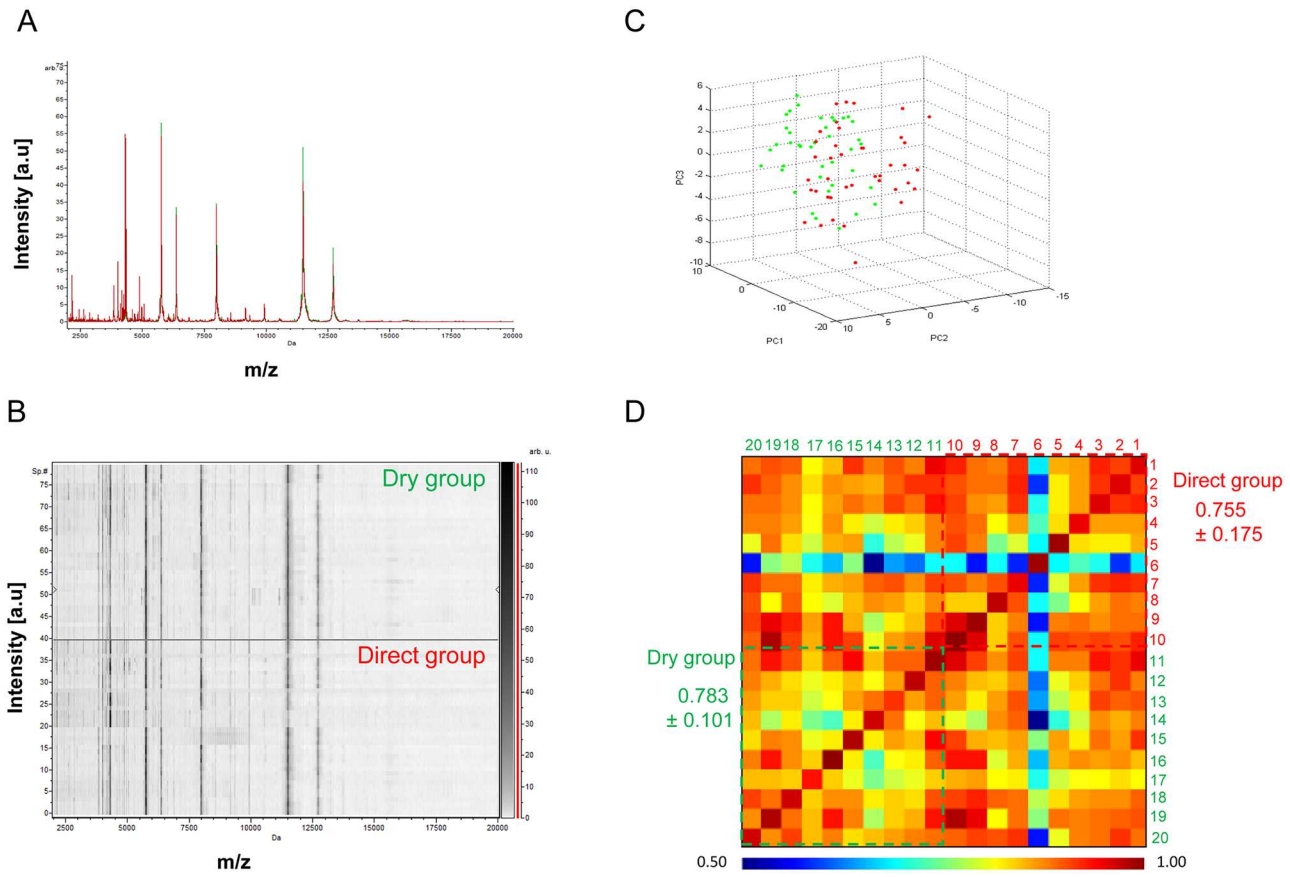

Fig 4. Comparison of MALDI-TOF MS spectra from legs of same tick using "dry" and "direct" protocol. Representation of MS profiles by the superimposition of average MS profiles of "dry" and "direct" protocol (A), the gel view tool of "dry" and "direct" protocol (B) and comparison by Principal Component Analysis between "dry" and "direct" protocol (C). Assessment of spectra reproducibility for two protocols using composite correlation index (CCI) (D): The rainbow colours indicate the degree of similarity between pair mass spectra comparisons ranging from red (very similar) to blue (very dissimilar). The numbers 1 to 10 are tick numbers treated by "direct" protocol and 11 to 20 those treated by "dry" protocol.

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confirming morphological identification. All fully engorged ticks were correctly identified by MALDI-TOF MS. The percentage of correct MALDI-TOF MS identification for all species was 99.6% (449/451) (Table 6).

Determination of tick bacterial infectious status by MALDI-TOF MS

The comparison of MS profiles between 30 *Am. variegatum* uninfected and 12 infected by *R. africae* using the gel view tool and Principal Component Analysis by ClinProTools software (Bruker), revealed no differences between the two groups (S1 Fig). The same observation was made by comparing of MS profiles of 36 *Hy. truncatum* uninfected and 23 *Hy. truncatum* infected by *C. burnetii* (S1C and S1D Fig).

Discussion

MALDI-TOF MS has revolutionized clinical microbiology by its use in the routine identification of bacteria [41,42] and archaea [43]. Even if the MALDI-TOF MS device acquisition could be expensive, its use for entomological analyzes induces low additional costs because reagents used for this high-throughput technique are economical and data analyses are simple

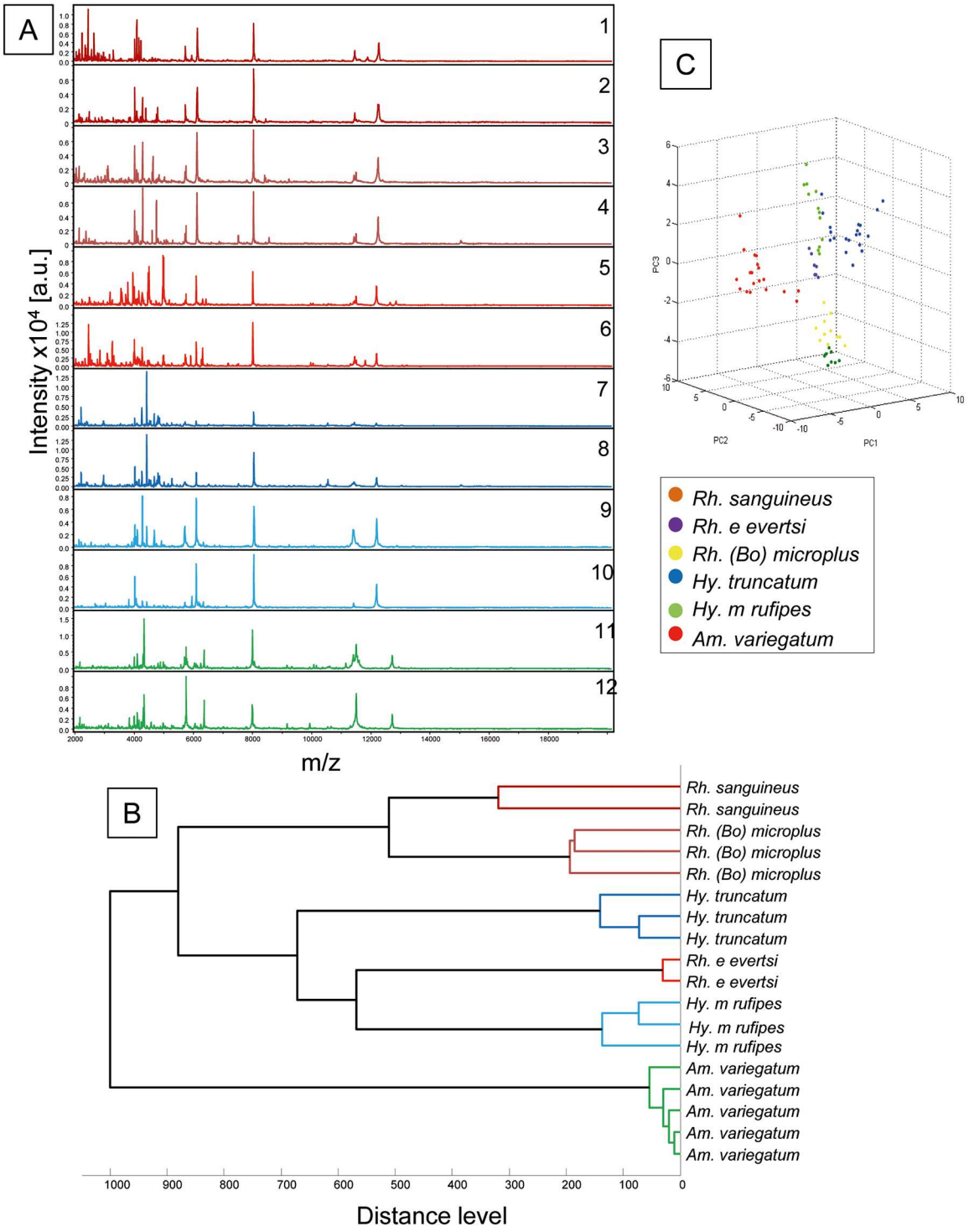

Fig 5. Specific MALDI-TOF MS spectra of six species of ticks using for database creation. (A) Representation of leg MS spectra from *Rh. sanguineus sl* (1, 2), *Rh. (B) microplus* (3, 4) *Rh. e. evertsi* (5, 6), *Hy. truncatum* (7, 8), *Hy. m. rufipes* 9, 10), *Am. variegatum* (11, 12). (B) Dendrogram constructed using 2 to 5 representative MS spectra from 6 distinct tick species. (C) Principal Component Analysis performed with 20 specimens of six tick species; a.u., arbitrary units; m/z, mass-to-charge ratio.

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Table 6. The number of ticks used to perform the blind test and percentage of correct identification.

Species	Number of specimens used for BT1	High LSVs obtained from BT1 against DB1[¤]	Number of concordant ID between morphology and MS[#]	Number of specimens added to DB1	Number of specimens used for BT2	High LSVs obtained from BT2 against DB2[¤]	Tick species ID by MS	Molecular ID[$]	Concordance of MS ID with morphology and molecular (%)[&]
Am. variegatum	60	*[1.10–1.73] (n = 60)*	n.d.	5	177	[1.81–2.63] (n = 173)	*Am. variegatum*	/	100
						[2.06–2.23] (n = 4)	*Hy. truncatum*	*Hy. truncatum*	
Hy. truncatum	64	[1.81–1.95] (n = 7)	5	5	117	[1.90–2.72] (n = 109)	*Hy. truncatum*	/	98.3
		[1.37–1.79] (n = 57)	n.d.			[1.98–2.35] (n = 5)	*Hy. m. rufipes*	*Hy. truncatum*	
						[1.87–2.35] (n = 3)	*Am. Variegatum*	*Am. Variegatum*	
Hy. m. rufipes	16	[1.81–1.91] (n = 5)	5	3	15	[2.10–2.77] (n = 12)	*Hy. m. rufipes*	/	100
		[1.54–1.79] (n = 11)	n.d.			[2.10–2.32] (n = 3)	*Hy. truncatum*	*Hy. truncatum*	
Rh (Bo) microplus[€]	26	*[1.20–1.72] (n = 26)*	n.d.	3	23	[1.91–2.71] (n = 23)	*Rh (Bo) microplus*	/	100
Rh. e. evertsi	7	*[1.51–1.61] (n = 7)*	n.d.	2	5	[1.90–2.49] (n = 5)	*Rh. e. evertsi*	/	100
Rh. sanguineus sl	5	*[1.20–1.48] (n = 5)*	n.d.	2	3	[1.89–2.10] (n = 3)	*Rh. sanguineus sl*	/	100
Rh. sanguineus sl	/	/	/		111	[1.90–2.29]	*Rh. sanguineus sl*	/	100
Total	**178**				**451**				**99.6**

[€]Tick species stored in alcohol not included in the DB1 [31]

*Tick specimens stored frozen.

[¤] The number of specimens included in each range of LSVs (above and *below* 1.8) are in parentheses.

[$]Molecular biology of tick species ID done only on discordant MS and morphological results.

[&]Percentages of tick species with ID concordance between MS and morphological results plus molecular determination.

BT, blind test; DB, database; ID, identification; LSVs, log score values; MS, mass spectrometry; n.d., not determined.

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and rapid compared to morphological and molecular methods [44]. This fast, economical and accurate proteomic tool has since been applied to the identification of arthropods: culicoides biting midges [45], mosquitoes[39,46,47], phlebotomine sand flies [48,49], fleas [30] and tsetse flies [50,51]. MALDI-TOF MS has also been proposed for identifying tick species which are laboratory-reared, collected in the field or on mammalian hosts, by analyzing whole specimens [22] or legs only [23,24]. More recently, preliminary studies have investigated the capacity of MALDI-TOF MS to differentiate ticks infected or not by *Borrelia spp*. or spotted fever group rickettsiae [25–27], and to detect the *Plasmodium* in anopheles [44]. However, tick collection is usually far from the analytical laboratories, requiring proper storage of samples. Although the alcohol storage mode is cheaper and easier, especially in African countries, previous studies reported that the use of fresh (i.e., recently dead) or frozen specimens led to more reproducible and better MS spectra, compared to the alcohol storage mode for ticks [24,28], and also for other arthropod families [29,30]. Recently, the application of MALDI-TOF MS for identification of ticks collected in the field in East Africa and preserved in alcohol has allowed reliable

identification [23]. More recently, the discriminatory power of MALDI MS-TOF for the correct identification of ixodid tick specimens collected in the field in Ethiopia, which were preserved in 70% ethanol for about two years, was reported [31].

In this study, the morphological identification of ticks revealed the presence of six species, including *Am. variegatum*, *Hy. truncatum*, *Hy. m. rufipes*, *Rh. (Bo) microplus* and *Rh. e. evertsi* that were collected from cattle and *Rh. sanguineus sl* from dogs. *Rh. e. evertsi* was found only in Bamako, while all other species of ticks were found on cattle in the three locations. In support of these morphological identification results, several studies have reported the presence of these tick species in Mali, except for *Rh. (Bo) microplus* [7–9]. *Rh. (Bo) microplus*, which is a southeast Asian tick, was introduced in the southeast of Africa (South Africa, Zambia, Tanzania and Malawi) by cattle from Madagascar [9]. It was reported in West Africa (Ivory Coast) for the first time in 2007 [52]. The presence of *Rh. (Bo) microplus* has only been found in three other countries of West Africa (Mali, Benin and Burkina Faso) [53]. Biguezoton et al (2016) and Boka et al (2017) found that *Rh (B) microplus* represent 70% and 63.2% of ticks in Burkina Faso and Benin and Ivory Coast respectively [54, 55]. Our study confirms the presence of this species in 3 localities in Mali, which could indicate its rapid spread and its probable installation in Mali. As expected, *Am. variegatum* was the most prevalent species in the three sites of the present study [10].

To confirm the morphological identification of tick specimens that were used for creating the MALDI-TOF MS database, sequencing of the 12S rRNA gene was performed. The 12S rRNA gene was chosen to validate identification because this gene is known as a reliable tool for molecular identification of ixodid ticks [16]. The coverage percentages and identity between the sequences of specimens of the same species were from 99 to 100% for all species of ticks. Percentages of identity and coverage of sequences *Am. variegatum*, *Hy. m. rufipes*, *Rh. (Bo) microplus*, *Rh. e. evertsi* and *Rh. sanguineus sl were* 99–100% with sequences of the same species available in GenBank. Interestingly, lower sequence identities (96–97%) of *Hy. truncatum* compared to the corresponding reference sequence in GenBank were observed. It could be hypothesized that the sequence differences could correspond to genetic variation within ticks of the same species adapted to different geographic regions of a country or countries, as previously described [56]. The difference between the sequences of 12S rRNA genes of *Hy. truncatum* collected in Mali and that available on GenBank tick collected in Zimbabwe [57] could explain these genetic variations. In the future, the sequencing of a second gene target, such as 16S or COI, could be performed to further study these variations [58].

In this study, DNA from *Rickettsia spp*. was detected in 76.6% of infected ticks collected from cattle, among which *R. africae* was found in 87.6% (184/210). *R. africae* was detected in 92.2% of *Am. variegatum*, a cattle tick found throughout sub-Saharan Africa. Such high prevalence of *R. africae* in *Am. variegatum* has already been reported [59–61]. *R. africae* was also detected in *Rhipicephalus* spp. and *Hyalomma* spp., respectively 7.9% and 9.2%. Other recent studies have detected *R. africae* in other tick genera, including *Rhipicephalus* and *Hyalomma* [59,62,63]. *R. africae* is the etiological agent of African tick-bite fever in humans (ATBF) [64]. *R. aeschlimannii* have been observed in *Hyalomma spp*., with 9% and 52.6% respectively in *Hy. truncatum* and *Hy. m. rufipes*. These data are comparable with those of previous studies that reported 45% to 55% of *Hy. m. rufipes* and 6% to 7% of *Hy. truncatum* were DNA carriers of *R. aeschlimannii* in Senegal [63], and 44% and 11% in Ivory Coast [59]. The sequences of *R. aeschlimannii* identified in our work were identical to those of *R. aeschlimannii*, previously detected in *Hy. truncatum* collected in Senegal (GenBank accession number HM050276.1). *R. aeschlimannii* is an agent of spotted fever in humans [64]. *R. aeschlimannii* is found in sub-Saharan Africa, North Africa, Europe and Asia [11,65]. Our results confirm a large prevalence of this pathogen in Mali.

For the first time, the presence of *R. mongolitimonae* was identified in *Hy. truncatum* from Mali. It had been previously detected in *Hy. truncatum* from the countries bordering Mali, including Niger [11] and Senegal [63]. *R. mongolitimonae* 12S sequence of the present study were 99% identical with the same sequence fragment of a strain previously isolated from a patient from Algeria (GenBank DQ097081.1).

Until now, two *Borrelia* species have been identified in Mali, *B. crocidurae* in the soft tick (*O. sonrai*) and *B. theileri* in the hard tick (*Rh. geigyi*) [12,13]. Our results show the presence of *Borrelia* spp. in 2 specimen of *Am. variegatum* and 1 of *Hy. truncatum* by qPCR using 16S *Borrelia* and ITS4 genes. Similarly, Ehounoud et al. previously reported the presence of *Borrelia spp*. in the same tick species in Ivory Coast [59]. Unfortunately, no PCR products using standard amplification were obtained for any of these ticks. This failing could be explained by the higher sensitivity of qPCR compared to standard PCR [66].

In the present work, *C. burnetii*, the agent of Q fever, was detected for the first time in ticks in Mali, with a prevalence of 33.4% in the six tick species identified. These results differ from those of Ehounoud et al. in Ivory Coast, who found only one tick infected with *C. burnetii* [59]. Q fever is a ubiquitous zoonotic disease caused by *C. burnetii*. It is poorly documented in Africa. A recent study conducted in febrile African patients found one male adult patient (0.3%) infected with *C. burnetii* in Algeria and six patients (0.5%) in Senegal [67]. However, in another study conducted in Senegal, *C. burnetii* was detected in humans as well as in ticks [68].

The *Anaplasmataceae* bacteria family was previously considered to be pathogens of veterinary importance [59]. However, in recent decades, many agents of this family have been described in humans [69]. Here, we reveal the presence of *A. marginale* in 11.5% of *Rh (Bo.) microplus*. This is the first demonstration of the presence of *A. marginale*, the agent of bovine anaplasmosis [70] in Mali. *A. marginale* is an intracellular bacterium responsible for bovine anaplasmosis which manifests with anemia and jaundice [64]. Also, *E. ruminantium* was found in *Am. variegatum*, *Hy. truncatum*, *Rh (Bo.) microplus*, and *Rh. e. evertsi*. The prevalence of *E. ruminantium* was 13.9% in ticks. Potential new species of *Ehrlichia* and *Anaplasma* (*E. sp urmitei* TCI148, *E. sp rustica* TCI141 and *A. sp ivoriensis* TCI50) have been detected in *Rh (Bo.) microplus* and *Hy. truncatum*. These bacteria had already been detected in ticks from Ivory Coast [59].

However, co-infections have been found in the ticks in this study. The percentage of co-infected ticks was 23.1% (109/471), and we describe for the first time multiple co-infections in ticks in Mali. Recently, multiple co-infections in ticks have been reported in Ivory Coast; these co-infections systematically involved *R. africae* [59]. The percentage of ticks co-infected was higher in our study than that obtained in Ivory Coast [59].

To avoid bias, we choose to query the MS spectra of 178 specimens of ticks, including 6 species against DB 1 which includes several families of arthropods, including mosquitoes. We constantly improve it with new specimens collected in the field and find it more relevant to carry out a total interrogation without the knowledge without any filter on a specific family. The results of the blind test revealed correct identification in 10 specimens only with high log score values, even though this database contained the same tick species that were also preserved in alcohol. This misidentification could be attributed to several factors: (i) the method used for sample crushing (initially manually, and here an automatic apparatus was used as previously described [28], (ii) the difference in storage time (6 months here vs 3 years in the previous study), (iii) the geographical distance (Mali vs. Ethiopia), which could have consequences on MS spectra profiles, as observed also at the genetic level. This last phenomenon had already been reported in other studies of sand flies [71], mosquito immature stages [46] and ticks [31]. Conversely, when database 1 was upgraded with 20 spectra of the six tick species of our study,

the blind test of all ticks revealed 95.60% (325/340) correct identification for tick species stored in alcohol. However, the remaining fifteen ticks (4.40%) with inconsistent identification between morphological and MALDI-TOF MS tools were subjected to molecular biology to determine the real identification of these specimens. The molecular biology results confirmed those of MALDI-TOF MS for 13 of these specimens. Two *Hy. truncatum* specimens were misidentified by MALDI-TOF MS. The reasons for the misidentification of the two specimens remain unknown. Additionally, all ticks frozenly stored were correctly identified by the blind test.

The results of this work show that MALDI-TOF MS is superior to morphological identification, as the correct identification percentage is 99.6% for all tested. It is also interesting because there are fewer entomologists able to identify ticks and the morphological identification keys are not always available. Another advantage of MALDI-TOF is that it can identify ticks that are completely engorged or damaged, for which morphological characteristics can be deformed or even disappear making morphological identification difficult or impossible. Conversely, the proteomic strategy proposed here, does not require specific skill or expertise, reagents are very cheap so the running cost is very low compared to a molecular biology. The current limiting factors of MALDI-TOF MS analysis are the small diversity of tick species included in the MS spectra reference database and the relative elevate cost to acquire the machine. Nevertheless, it high-throughput and large application for microorganisms identification either in research or medical diagnosis, do of this emerging tool a highly competitive method also for medical entomology studies. It is likely that MALDI-TOF MS will realize similar revolution in medical entomology as it was occurred in microbiology.

Our results confirm those of previous studies, according to which MALDI-TOF MS could be used for identification of ticks preserved in alcohol, but it requires the creation of a database with specimens stored in the same condition [31].

In our work, MALDI-TOF MS analysis was not able to differentiate ticks which were infected or not by the bacteria that were screened. However, preliminary studies from our laboratory seemed promising, as MALDI TOF analysis allowed differentiation of ticks infected or not by *Borrelia spp*. or spotted fever group rickettsiae [26,27]. The failing of bacteria-pathogen detection by MALDI-TOF MS could be attributed to several factors. The storage mode, fresh versus alcohol, might play a role. Moreover, the infectious status of these ticks was controlled against some bacteria pathogens, however, it was possible that they were infected by others pathogens not researched in the present study, which could impaired the determination of specific MS profiles for each associated pathogens. These factors could alter MS spectra profiles between uninfected and infected ticks. More studies are needed to explore the capacities of MALDI TOF to detect tick infectious status.

To conclude, the present work has confirmed that MALDI-TOF MS may represent a rapid and inexpensive alternative tool for accurate identification of ticks collected in the field and stored in alcohol. The recent demonstration of the use of MALDI-TOF MS for identification of ticks and associated pathogens requires further investigation.

Supporting information

S1 Fig. Comparison of MALDI-TOF MS spectra of ticks uninfected and infected. Representation of MS profiles of *Am. variegatum* unifected (Red) and infected by *R. africae* (Green) (A, B) and *Hy.truncatun* unifected (Red), infected by *C.burnetii* (Green) (C, D).
(TIF)

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Author Contributions

Conceptualization: Ogobara Doumbo, Didier Raoult, Philippe Parola.

Data curation: Ogobara Doumbo, Philippe Parola.

Formal analysis: Adama Zan Diarra, Lionel Almeras, Maureen Laroche.

Investigation: Adama Zan Diarra, Abdoulaye K. Koné, Zakaria Bocoum.

Methodology: Abdoulaye Dabo, Ogobara Doumbo, Philippe Parola.

Project administration: Abdoulaye Dabo, Ogobara Doumbo, Philippe Parola.

Resources: Jean-Michel Berenger, Didier Raoult, Philippe Parola.

Software: Lionel Almeras, Maureen Laroche.

Supervision: Abdoulaye Dabo, Philippe Parola.

Validation: Adama Zan Diarra, Lionel Almeras, Philippe Parola.

Writing – original draft: Adama Zan Diarra.

Writing – review & editing: Adama Zan Diarra, Lionel Almeras, Maureen Laroche, Abdoulaye Dabo, Ogobara Doumbo, Philippe Parola.

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Article 3

Use of MALDI-TOF MS for the Identification of Chad Mosquitoes and the Origin of Their Blood Meal

Diarra Adama Zan, Laroche Maureen, Berger Franck, Parola Philippe

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Résumé

La spectrométrie de masse à temps de vol de désorption / ionisation assistée par matrice (MALDI-TOF MS) est un outil de microbiologie clinique pour l'identification systématique de micro-organismes. Il a récemment été présenté comme un outil innovant pour l'identification rapide et précise des moustiques et de leur repas de sang. Pour évaluer la capacité de cet outil à identifier les moustiques collectés dans un environnement tropical et préservés avec du silicagel, nous avons analysé 188 moustiques de différentes espèces collectés au Tchad, qui ont été conservés avec du silicagel pendant 2 mois.

L'analyse par MALDI-TOF MS a correctement identifié 96% des moustiques et 37,5% de leurs repas de sang. A l'aide du MALDI-TOF MS et de la biologie moléculaire, huit espèces de moustiques ont été identifiées, notamment *Anopheles gambiae* s.l., *Anopheles rufipes*, *Culex quinquefasciatus*, *Culex neavei*, *Culex pipiens*, *Culex perexiguus*, *Culex rima* et *Culex watti*. L'identification du repas de sang a révélé que les moustiques se nourrissaient principalement d'humains, d'oiseaux et de vaches.

La spectrométrie de masse à temps de vol de désorption / ionisation assistée par matrice semble être un outil prometteur, rapide et fiable pour identifier les moustiques et l'origine de leur repas de sang pour les échantillons conservés avec du silicagel.

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Use of MALDI-TOF MS for the Identification of Chad Mosquitoes and the Origin of Their Blood Meal

Adama Zan Diarra,[1,2] Maureen Laroche,[1] Franck Berger,[3,4] and Philippe Parola[1]*

[1]*Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, IHU Méditerranée Infection, Marseille, France;* [2]*Department of Epidemiology of Parasitic Diseases, Malaria Research and Training Center, University of Science, Techniques and Technologies of Bamako, Bamako, Mali;* [3]*Aix Marseille Univ, Inserm, IRD, SESSTIM, Sciences Economiques & Sociales de la Santé & Traitement de l'Information Médicale, Marseille, France;* [4]*SSA, CESPA, Marseille, France*

Abstract. Matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a clinical microbiology tool for the systematic identification of microorganisms. It has recently been presented as an innovative tool for the rapid and accurate identification of mosquitoes and their blood meal. To evaluate the capacity of this tool to identify mosquitoes collected in a tropical environment and preserved with silica gel, we analyzed 188 mosquitoes of different species collected in Chad, which were preserved with silica gel for 2 months. The MALDI-TOF MS analysis correctly identified 96% of the mosquitoes and 37.5% of their blood meals. Using MALDI-TOF MS and molecular biology, eight mosquito species were identified, including *Anopheles gambiae* s.l., *Anopheles rufipes*, *Culex quinquefasciatus*, *Culex neavei*, *Culex pipiens*, *Culex perexiguus*, *Culex rima*, and *Culex watti*. Blood meal identification revealed that mosquitoes fed mainly on humans, birds, and cows. Matrix-assisted desorption/ionization time-of-flight mass spectrometry appears to be a promising, fast, and reliable tool to identify mosquitoes and the origin of their blood meal for samples stored with silica gel.

INTRODUCTION

Mosquitoes are the primary arthropod vectors of infectious diseases, posing serious economic and public health problems because of their role in the transmission of numerous human and veterinary pathogens.[1] To human, they are capable of transmitting not only parasitic diseases such as malaria and lymphatic filariasis but also serious arboviruses including yellow fever, dengue fever, chikungunya, Zika virus, and West Nile virus (WNV) infections.[2] Malaria, caused by several species of *Plasmodium* parasites (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale wallikeri*, *P. ovale curtisi*, *Plasmodium knowlesi*, and *Plasmodium simium*), is transmitted to humans by female *Anopheles* spp. mosquitoes.[3,4] According to the World Health Organization, approximately five million additional cases of malaria in 2016 compared with 2015 and 445,000 deaths were reported.[5]

Despite the availability of antimalarial treatments, vector control measures are needed to control the mosquito vectors.[6] Long-lasting insecticide-treated bed net, indoor residual spraying, larviciding, and community education to promote vector avoidance are commonly used approaches.[7] The implementation of vector control and surveillance strategies against mosquitoes requires entomological surveys including correct identification not only of the vectors but also of their blood meal for a better understanding of their biting behavior (endophilic or exophilic and anthropophilic or zoophilic).[8,9]

Mosquito identification is most often performed using morphological criteria using identification keys and/or molecular methods.[10] These methods, however, have limitations, which may be the absence of identification keys or specific documentation, expertise in entomology, and inability to differentiate species from the same complex for the morphological method. On the other hand, molecular approaches are time consuming, expensive, and limited by the completeness of online sequence databases.[10] Similarly, the origin of the blood meal is identified by several methods such as precipitin, enzyme immunoassay, and molecular tests.[11,12] However, these methods also have drawbacks, such as the difficulty of obtaining specific antisera against a wide variety of host species, the effect of blood meal digestion and DNA extraction protocol, the high cost, handling time, and the need for bulky equipment.[13,14]

Matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a clinical microbiology tool used for the systematic identification of bacteria,[15,16] archaea,[17] fungus, and parasites.[18] Recently, MALDI-TOF MS has been presented as an alternative tool for the rapid identification of many arthropods including mosquitoes[19–22] and the origin of their blood meal.[23,24] This involved different entomological studies using fresh samples from the laboratory and samples collected from the fields which were either frozen, preserved, preserved in alcohol, or crushed on Whatman paper.[23–26]

The aim of this study was to evaluate the ability of MALDI-TOF MS to identify mosquitoes and the origin of their blood meal using samples collected in the field in a tropical setting, preserved with silica gel, and sent to a place which has a MALDI-TOF MS device available.

MATERIALS AND METHODS

Mosquito collection. All mosquitoes were collected as part of an entomological survey made by the French Army Centre for Epidemiology and Public Health in Chad in October 2017. Mosquitoes were collected using BG sentinel traps (Biogents AG, Weißenburgstraße, Regensburg, Germany)[27] and CDC light traps (John W. Hock Company, Gainesville, États-Unis),[28] which were inspected every day between 6:00 AM and 7:00 AM and between 6:00 PM and 7:00 PM. The mosquitoes were morphologically identified every day to the genus and at the species level for *Anopheles* females, using morphological criteria.[29]

* Address correspondence to Philippe Parola, Aix Marseille University, Institut de recherche pour le devéloppement (IRD), Assistance publique-Hopitaux Marseille (AP-HM), Service de Santé des Armées (SSA), Vecteurs – Infections Tropicales et Méditerranéennes (VITROME), Institut hospitalo-universitaire (IHU) Méditerranée Infection, 19-21 Blvd. Jean Moulin, Marseille 13005, France. E-mail: philippe.parola@univ-amu.fr

A convenient sample of mosquitoes was selected for the present study. The mosquitoes were stored individually (for *Anopheles* spp.) or by a pool of 3–10 specimens (for *Culex* spp.) in a 1.5-mL Eppendorf tube with silica gel (Carl Roth GmbH, Karlsruhe, Germany) before being sent to Marseille, France, on November 3, 2017, for subsequent analysis.

Preparation of samples for MALDI-TOF MS analysis. *Matrix-assisted desorption/ionization time-of-flight mass spectrometry identification of mosquito samples.* Two months after being maintained at room temperature, the legs of each mosquito were removed and placed individually in 1.5 mL Eppendorf tubes with glass powder (Sigma, Lyon, France), 15 µL of 70% (v/v) formic acid (Sigma), and 15 µL of 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland). The samples were ground using a tissue lyzer (Qiagen, Hilden, Germany) over three cycles of 30 ms^{-1} for 60 seconds.[30] The samples were then centrifuged at 10.000 rpg for 1 minute, and 1 µL of supernatant of each homogenate was deposited on a MALDI-TOF MS target plate in quadruplicate (Bruker Daltonics, Wissembourg, France) and covered with 1 µL of α-cyano-4-hydroxycynnamic acid (CHCA) matrix solution composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Aldrich, Dorset, UK), and high-performance liquid chromatography (HPLC) grade water.[26,30] After drying for several minutes at room temperature, the target was introduced into the MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics, Breman, Germany) for analysis.

Matrix-assisted desorption/ionization time-of-flight mass spectrometry identification of blood meal sources. The engorged abdomen of each female mosquito was individually ground in an Eppendorf tube containing 50 µL of HPLC grade water. After centrifugation, 10 µL of the supernatant was used for 10 µL for the MALDI-TOF analysis, as previously described.[23] Ten microliter of the abdomen supernatant was mixed with 20 µL of 70% (v/v) formic acid and 20 µL of 50% (v/v) acetonitrile (Fluka) and then centrifuged at 10. 000 rpm for 20 seconds. One microliter of supernatant from each sample was placed on the MALDI-TOF target plate in quadruple (Bruker Daltonics) and recovered with 1 µL of CHCA matrix solution composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Aldrich), and HPLC grade water. After drying for several minutes at room temperature, the target was introduced into the MALDI-TOF Microflex LT mass spectrometer (Bruker Daltonics) for analysis.

Spectral analysis. Protein mass profiles were acquired using a Microflex LT MALDI-TOF mass spectrometer, with detection in the linear positive ion mode at a laser frequency of 50 Hz in a mass range of 2–20 kDa. The acceleration voltage was 20 kV and the extraction time was 200 ns. Each spectrum corresponds to the ions obtained from the 240 laser shots performed in six regions in the same location and acquired automatically using the AutoXecute Flex Control software v.2.4 (Bruker Daltonics). Spectrum profiles obtained from mosquito legs and engorged abdomens were visualized with FlexAnalysis software v.3.3, and low-quality spectra were excluded from the study based on their intensity, reproducibility, and noise. They were then exported to ClinProTools version v.2.2 (Bruker Daltonics) and MALDI-Biotyper v.3.0 (Bruker Daltonics) for data processing.

Blind tests for the identification of mosquitoes and blood meals. To determine the mosquito species and the origin of blood meals, MALDI-TOF MS spectra from the legs and abdominal protein extracts of blood-engorged females were queried against the homemade MS reference spectra database (Table 1) using the MALDI-Biotyper software v3.0. tool (Bruker Daltonics). The level of significance was determined using the log score values (LSVs) provided by the MALDI-Biotyper software v.3.3. corresponding to a matched degree of signal intensities of mass spectra of the query and the reference spectra. Log score values ranged from zero to three. The samples were correctly considered and significantly identified when the spectrum queried had an LSV ≥ 1.8.[23] After molecular identification, the reference spectra of *Culex perexiguus* (n = 1), *Culex watti* (n = 1), and *Culex rima* (n = 1) were added in the homemade MS reference spectra database and a second blind test was made against the new database.

Molecular identification. Mosquito samples with high-quality spectra and LSV ≥ 1.8 but showing discrepancies between morphological identification and MALDI-TOF MS, and those with high-quality spectra and LSV < 1.8 were all identified at the species level by molecular tools. Quality of spectra was evaluated based on overall intensity of peaks, absence of noise, and reproducibility among each species, and visualized on both FlexAnalysis and ClinProTools software. Besides, some randomly selected well-identified samples (LSV ≥ 1.8 with concordance between morphological identification and MALDI-TOF MS) were also identified at the species level by molecular tools. We used the same workflow for the molecular identification of blood meals.

DNA extractions from individual mosquito heads and thorax samples or 10 µL supernatant of engorged abdomen of females were performed using the EZ1 DNA Tissue kit (Qiagen) according to the manufacturer's recommendations. To determine the origin of the blood meal, we used primers that specifically amplified the vertebrate cytochrome c oxidase I gene (v*COI*) (v*COI*_long forward: 5′-AAGAATCAGAATARGTTG-3′; v*COI*_long reverse: 5′-AACCACAAAGACATTGGCAC-3′).[31] As for mosquitoes, a region of the cytochrome c oxidase I gene (m*COI*) was amplified using the following primers: (LCO1490 (before): 5′-GGTCAAC AAATCATAAGATATTGG-3′; HC02198 (reverse): 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′,[32] and the internal transcribed spacer 2 (ITS2) was amplified using the following primers: forward: 5′-ATCACTCGGCTCATGGATCG-3′; reverse: 5′-ATGCTTAAATTTAGGGGGTAGTC-3′.[33] Positive polymerase chain reaction (PCR) products were then purified and sequenced using the same primers with the BigDye version 1-1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA) and an ABI 3100 automated sequencer (Applied Biosystems). The sequences were assembled and analyzed using the ChromasPro software (version 1.34) (Technelysium Pty. Ltd., Tewantin, Australia) and the National Center for Biotechnology Information, Basic Local Alignment Search Tool (NCBI BLAST) website (http://blast.ncbi.nlm.nih.gov).

RESULTS

Mosquitoes collection and morphological identification. A total of 188 mosquitoes were selected randomly to have a varied number of species and sex, but not as representative of the entire collection during the entomological survey, which will be reported elsewhere. According to the morphological identification, the selected mosquitoes belonged to two genera: *Culex* spp. represented 112/188 (59.6%) of which 13 were males and 99 were females, and *Anopheles* spp. represented

TABLE 1
List of the arthropod species present in our homemade MALDI-TOF MS database

Mosquitoes	Imago: *Aedes aegypti*, *Aedes albopictus*, *Aedes alternans*, *Aedes australis*, *Aedes caspius*, *Aedes cinereus*, *Aedes dufouri*, *Aedes flavifrons*, *Aedes fowleri*, *Aedes multiplex*, *Aedes notoscriptus*, *Aedes polynesiensis*, *Aedes procax*, *Aedes vexans*, *Aedes vigilax*, *Aedes vittiger*, *Anopheles annulipes*, *Anopheles arabiensis*, *Anopheles claviger*, *Anopheles coluzzi*, *Anopheles coustani*, *Anopheles funestus*, *Anopheles gambiae* Giles, *Anopheles hyrcanus*, *Anopheles maculipennis*, *Anopheles pharoensis*, *Anopheles rufipes*, *Anopheles wellcomei*, *Anopheles ziemani*, *Coquillettidia richiardii*, *Coquillettidia xanthogaster*, *Culex annulirostris*, *Culex australicus*, *Culex insignis*, *Culex modestus*, *Culex molestus*, *Culex neavei*, *Culex orbostiensis*, *Culex pipiens*, *Culex quinquefasciatus*, *Culex perexiguus*, *Culex rima*, *Cx. sitiens*, *Culex watti*, *Culiseta longiareolata*, *Lutzia tigripes*, *Mansonia uniformis*, *Ochlerothatus rusticus*, *O. excrucians*, *Orthopodomyia reunionensis*, and *Verralina funerea* Larvae: *Ae. albopictus*, *Ae. aegypti*, *An. coluzzi*, *An. gambiae*, *Cx. molestus*, *Cx. pipiens*, and *Culiseta* sp.
Lice	*Pediculus humanus*, *Damalinia bovis*, *Damalinia caprae*, *Damalinia ovis*, *Haematopinus eurysternus*, *Linognatus vituli*, and *L. africanus*
Fleas	*Archaeopsylla erinacei*, *Ctenocephalides felis*, *Culex canis*, and *Xenopsylla chopis* *Pulex irritans*, *Stenoponia tripectinata*, *Nosopsyllus fasciatus*, and *Cx. canis*
Ticks	Legs: *Amblyomma variegatum*, *Dermacentor marginatus*, *D. marginatus*–infected with *R. slovaca*, *D. reticulatus*, *Haemaphysalis concinna*, *Haemaphysalis punctata*, *Hyalomma m. rufipes*, *Ixodes hexagonus*, *Ixodes ricinus*, *Rhipicephalus bursa*, *Rhipicephalus sanguineus*, *Rh. sanguineus*–infected with *Rickettsia conorii*, *Rh. sanguineus*–infected with *R. massiliae*, and *Rh. sulcatus* *Amblyomma gemma*, *Amblyomma cohaerens*, *Am. variegatum*, *Argas persicus*, *Haemaphysalis leachi*, *Hae. punctata*, *Haemaphysalis spinulosa*, *Hyalomma detritum*, *Hy. m. rufipes*, *Hyalomma truncatum*, *I. ricinus*, *Ornithodoros sonrai*, *Rhipicephalus annulatus*, *Rhipicephalus bergeoni*, *Rh. bursa*, *Rhipicephalus decoloratus*, *Rhipicephalus e. evertsi*, *Rhipicephalus microplus*, *Rhipicephalus praetextatus*, *Rhipicephalus pulchellus*, and *Rh. sanguineus* Hemolymph: *Am. variegatum*–infected with *Rickettsia africae*, *D. marginatus*, *Hy. m. rufipes*, *Rh. bursa*, and *Rh. sanguineus*
Bed bugs	*Cimex lectularius* and *Cimex hemipterus*
Triatominae	*Eratyrus mucronatus*, *Panstrongylus geniculatus*, *Rhodnius prolixus*, *Rhodnius pictipes*, *Rhodnius robustus*, and *Triatoma infestans*
Sand flies	*Phlebotomus papatasi*, *Phlebotomus longicuspis*, *Phlebotomus perfiliewi*, *Phlebotomus perniciosus*, *Phlebotomus sergenti*, *Sergentomyia minuta*
Mite	*Leptotrombidium chiangraiensis*, *Leptotrombidium imphalum*, and *Leptotrombidium deliense*
Blattidae	*Supella longipalpa*, *Periplaneta americana*, *Blatta orientalis*, *Blatella germanica*, and *Blaptica dubia*
Flies	*Melophagus ovinus* and *Hippobosca equina*
Abdomen of mosquitoes engorged	*An. gambiae* Giles fed on *Homo sapiens*, *Equus caballus*, *Ovis aries*, rabbit, Balb/*C. mouse*, *Rattus norvegicus*, *Canis familiaris*, *Bos taurus*, *Capra hircus*, *Gallus gallus*, *Equus asinus*, *Tapirus indicus*, *Tapirus terrestris*, *Carollia perspicillata*, *Thraupis episcopus*, *Erythrocebus patas*, and *Callithrix pygmaea* blood *Ae. albopictus* fed on *H. sapiens* blood

76/188 (40.4%) of which 44 were males and 32 were females (Table 2). A total of 62.5% (20/32) of *Anopheles* females were morphologically identified as belonging to *Anopheles gambiae* s.l. and 37.5% (12/32) to *Anopheles rufipes* species.

Matrix-assisted desorption/ionization time-of-flight mass spectrometry and molecular identification of mosquitoes. Among the 188 mosquitoes that were preserved with silica gel, 169/188 (89.9%) had at least four legs and were selected for MALDI-TOF MS analysis (Table 2). Of these 169 mosquitoes, 104 (61.5%) provided good-quality MS spectra and were included for further MS analysis.

The spectra obtained from the legs of these 104 mosquitoes were then queried against the in-lab MS arthropod database. A total of 93.3% (97/104) were identified with LSVs ranging from 1.84 to 2.427 (average: 2.119). The other including six *Culex* spp. (females only) and one *Anopheles* spp. male had LSV less than 1.8 (Table 3).

Regarding the molecular identification, the seven mosquitoes that had LSVs less than 1.8 and a high-quality spectra, and 26 mosquitoes that had LSVs greater than 1.8 and discrepancies with morphological identification (or obtained from specimen with identification to the genus only) were subjected to standard PCR and sequencing. Among the mosquitoes identified with LSVs greater than 1.8, 22/26 (84.6%) were definitively confirmed by molecular biology with unambiguous similarities with the *COI* gene of the corresponding species (Table 3). For 4/26 (15.4%) morphologically identified as male *Anopheles* spp. and as *An. rufipes* by MS, they showed 99.7% identity with *Anopheles* sp. M36YA (GenBank accession number: KU187107.1), and 98.8% identity with *An. rhodesiensis* and *An. rufipes*, (GenBank accession numbers: KU187106.1 and KJ522838.1), with the *COI* gene. Samples that could not be identified using the *COI* gene (not enough divergence between *An. rhodesiensis* and *An. rufipes*) were sequenced using the ITS2 gene and revealed 100% identity with *An. rufipes* reference sequences (GenBank accession number: KJ522822.1). Therefore, MALDI-TOF identification of selected mosquitoes has been confirmed by molecular biology.

Partial COI gene sequences were obtained from the seven mosquitoes that had LSVs less than 1.8: one had 99% identity with *An. rufipes* (GenBank accession number: KJ522838.1), three were identified as *Cx. perexiguus* (100% identity; GenBank accession number: KU380423.1), two as *Cx. watti* (99.2%; GenBank accession number: KU187063.1), and one as *Cx. rima* (99.6%; GenBank accession number: KU187034.1). Our homemade MS reference spectra database did not contain any of the *Cx. rima*, *Cx. perexiguus*, and *Cx. watti* spectrum. The spectra of the new species not present in our in-lab database before this study (*Cx. rima*, *Cx. watti*, and *Cx. perexiguus*) have been added.

After the molecular identification and upgrade of the database with the reference spectra of *Cx. rima*, *Cx. perexiguus*, and *Cx. watti* from Chad, the second blind test against this updated database identified another *Cx. perexiguus* that had not been identified during the first blind test with LSV greater than 1.8. After the second blind test analysis, the percentage of MS correct identification reached 96.4%.

Identification of blood meals. A total 59 abdomens of engorged female mosquitoes (58 *Culex quinquefasciatus* and

TABLE 2

The number of mosquitoes collected in Chad preserved on silica gel by sex and gender, with the legs, those with the good matrix-assisted desorption/ionization time-of-flight mass spectrometry spectra, and the percentage of identification after the first blind test

Mosquitos species or genus	Number collected	Number with legs	Number of spectra of good quality	Percentage of identification to the species level
Anopheles sp. ♂	44	38 (62.3%)	18 (47%)	94.4% (17/18)
Anopheles gambiae ♀	22	18 (81.8%)	11 (61.1%)	100% (11/11)
Anopheles rufipes ♀	10	5 (50%)	1 (20%)	100% (1/1)
Culex sp. ♂	13	11 (84.6%)	7 (63.6%)	100% (7/7)
Culex sp. ♀	99	97 (97.9%)	67 (69%)	91% (61/67)
Total	188	169 (89.9%)	104 (61.5%)	93.3% (97/104)

one *An. gambiae* s.l.) were used for MALDI-TOF MS analysis. Of these, 24/59 (40.7%) samples had good quality MS spectra. A total of 9/24 (37.5%) were identified as human blood with LSVs between 1.901 and 2.308. The remaining 15 samples were not realibily identified (LSVs between 1.222 and 1.681, Because of low LSVs, these identifications were considered unreliable[23] (Table 4). The abdomens of engorged mosquitoes with MS spectra of good quality were subjected to sequencing using the *COI* gene vertebrate to determine the origin of the blood meal (Table 4). Of these, nine that had already been identified as human blood by MALDI-TOF MS with LSVs greater than 1.8 were confirmed by molecular biology with identities ranging from 99.7% to 100% (GenBank accession numbers: MF621085.1, MG970575.2, MG272704.1, MH161386.1, and MF696131.1). Among the 15 samples with low LSVs, sequencing showed that eight had identities ranging from 99.8% to 100% with human blood reference sequences (GenBank accession numbers: MF621085.1, MG970575.2, MG272704.1, MH161386.1, and MF696131.1), two had identities ranging from 95.3% to 96.4% with the European roller (*Coracias garrulous*) blood reference sequences (GenBank accession number: GQ481616.1), and one had 98.2% identity with domestic goat (*Capra hircus*) blood reference sequence (GenBank accession number: KX845672.1). No sequence was obtained for three samples.

DISCUSSION

Matrix-assisted desorption/ionization time-of-flight mass spectrometry, a widely used tool for the identification biomolecules, is based on the acidic extraction and ionization of

TABLE 3

Molecular identification of mosquitoes collected in Chad in October 2017 randomly selected for confirmation of MALDI-TOF MS identification

Number of samples	Morphological identification	Log score value	MALDI-TOF MS identification	Molecular identification (COI gene)	Molecular identification (internal transcribed spacer 2 gene)
632	*Anopheles* sp. ♂	1.79	/	*Anopheles rhodesiensis/Anopheles rufipes**	*An. rufipes*
595	*Anopheles* sp. ♂	1.84	*An. rufipes*	*An. rufipes*	*An. rufipes*
657	*Anopheles* sp. ♂	1.84	*An. rufipes*	*An. rufipes*	–
693	*Anopheles* sp. ♂	1.854	*An. rufipes*	*An. rhodesiensis/An. rufipes**	*An. rufipes*
599	*Anopheles* sp. ♂	1.869	*An. rufipes*	*An. rufipes*	–
698	*Anopheles* sp. ♂	1.879	*An. rufipes*	*An. rhodesiensis/An. rufipes**	*An. rufipes*
621	*Anopheles* sp. ♂	1.89	*An. rufipes*	*An. rufipes*	–
677	*Anopheles* sp. ♂	1.91	*An. rufipes*	*An. rufipes*	–
587	*Anopheles* sp. ♂	1.977	*An. rufipes*	*An. rufipes*	–
585	*Anopheles* sp. ♂	2.005	*An. rufipes*	*An. rhodesiensis/An. rufipes**	–
583	*Anopheles* sp. ♂	2.023	*An. rufipes*	*An. rufipes*	–
563	*Anopheles* sp. ♂	2.065	*An. rufipes*	*An. rufipes*	–
487	*Anopheles* sp. ♂	2.11	*An. rufipes*	*An. rufipes*	–
491	*Anopheles gambiae* ♀	2.126	*An. gambiae*	*An. gambiae*	–
593	*An. rufipes* ♀	2.128	*An. rufipes*	*An. rufipes*	–
297	*An. gambiae* ♀	2.23	*An. gambiae*	*An. gambiae*	–
572	*An. gambiae* ♀	2.261	*An. gambiae*	*An. gambiae*	–
533	*Culex* sp. ♀	1.037	/	*Culex watti*	–
694	*Culex* sp. ♀	1.452	/	*Culex rima*	–
618	*Culex* sp. ♀	1.513	/	*Culex perexiguus*	–
374	*Culex* sp. ♀	1.635	/	*Cx. perexiguus*	–
556	*Culex* sp. ♀	1.679	/	*Cx. perexiguus*	–
720	*Culex* sp. ♀	1.73	/	*Cx. watti*	–
624	*Culex* sp. ♀	1.956	*Culex pipiens*	*Cx. pipiens*	–
521	*Culex* sp. ♂	2.131	*Culex quinquefasciatus*	*Cx. quinquefasciatus*	–
522	*Culex* sp. ♂	2.139	*Cx. quinquefasciatus*	*Cx. quinquefasciatus*	–
740	*Culex* sp. ♀	2.145	*Cx. pipiens*	*Cx. pipiens*	–
705	*Culex* sp. ♀	2.161	*Cx. pipiens*	*Cx. pipiens*	–
715	*Culex* sp. ♀	2.161	*Cx. pipiens*	*Cx. pipiens*	–
568	*Culex* sp. ♀	2.253	*Cx. quinquefasciatus*	*Cx. quinquefasciatus*	–
682	*Culex* sp. ♀	2.267	*Cx. quinquefasciatus*	*Cx. quinquefasciatus*	–
523	*Culex* sp. ♂	2.314	*Cx. quinquefasciatus*	*Cx. quinquefasciatus*	–
520	*Culex* sp. ♀	2.337	*Cx. pipiens*	*Cx. pipiens*	–
592	*Culex* sp. ♀	2.427	*Cx. quinquefasciatus*	*Cx. quinquefasciatus*	–

MALDI-TOF MS = Matrix-assisted desorption/ionization time-of-flight mass spectrometry.
* Non-discriminative results (identical cover and identity values).

TABLE 4
MALDI-TOF MS and molecular identification of blood meal origin from abdomens of engorged females

Sample numbers	Log score value	MALDI-TOF MS identification	Molecular identification	% Identity	GenBank accession number
762	1.222	/	No sequence	/	–
605	1.267	/	*Coracias garrulus*	95.27	GQ481616.1
506	1.271	/	*Coracias garrulus*	96.40	GQ481616.1
681	1.290	/	*Capra hircus*	98.17	KX845672.1
623	1.309	/	*Homo sapiens*	100	MF621085.1
671	1.334	/	*H. sapiens*	99.68	MH378688.1
646	1.339	/	*H. sapiens*	100	MH378688.1
691	1.343	/	No sequence	/	–
670	1.377	/	No sequence	/	–
714	1.421	/	*H. sapiens*	99.07	MF621085.1
550	1.438	/	No sequence	/	–
673	1.453	/	*H. sapiens*	99.68	MG936624.1
761–3	1.457	/	*H. sapiens*	99.69	MF621085.1
505	1.509	/	*H. sapiens*	99.37	MF621085.1
716	1.681	/	*H. sapiens*	99.69	MH378688.1
695	1.901	Crushed abdomen of *Aedes albopictus* containing human blood	*H. sapiens*	100	MH378688.1
663	1.929	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	99.84	MH161386.1
717	1.953	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	99.68	MH378688.1
761-7	2.019	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	99.84	MF696131.1
644	2.078	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	100	MG272704.1
680	2.102	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	99.37	MH378688.1
727	2.147	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	100	MF621085.1
627	2.148	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	100	MH378688.1
537	2.308	Crushed abdomen of *Ae. albopictus* containing human blood	*H. sapiens*	100	MH378688.1

MALDI-TOF MS = Matrix-assisted desorption/ionization time-of-flight mass spectrometry. Results below the threshold of reliable identification (< 1.8) are not reported.

the proteins of an organism of interest. The extract is deposited on a steel target, covered with a MALDI matrix, and then dried at room temperature until co-crystallization. The crystallized target is then introduced into the apparatus, where the crystal is irradiated with laser pulses, performing desorption and "soft" ionization.[10] These desorbed and ionized molecules are accelerated in an electric field and separated by a flight tube in the linear or reflectron mode according to their mass–load ratio until they reach a detector.[34] Thus, the mass/charge values and intensities, that is, the so-called mass fingerprint of a generated sample, are then compared with a database containing species reference mass fingerprints for species identification.[35] Matrix-assisted desorption/ionization time-of-flight mass spectrometry has revolutionized clinical microbiology by its use in the systematic identification of bacteria,[15,16,36] archaea,[17] parasites, and fungi.[18] Recently, it has been introduced in medical entomology as a tool for the rapid and accurate identification of arthropods, detection of the origin of their blood meal, and detection of associated microorganisms.[23,24,26,37–41] In entomology, adjustments such as arthropod-based body selection and sample crushing protocol are required for proper MALDI-TOF MS identification of arthropods or detection of associated pathogens.[10,26,38,39]

The method of conservation may also be a limiting factor of this method. Generally, arthropods are collected in the field, far from the laboratories. Therefore, they are usually stored dry with silica gel, in 70% ethanol at +4°C or –20°C for transport to laboratories.[25] It is easier and less expensive to transport samples preserved with silica gel or in 70% ethanol than samples preserved at –20° C, and these methods are widely used in African countries.[25] However, it was reported that storage for a long time in 70% alcohol may impact MALDI-TOF MS profiles, resulting in lower intensity and lower overall quality than those of fresh or frozen samples.[25,30,42] No MALDI-TOF MS study has yet been conducted on arthropods stored with silica gel despite the advantages of this method such as limited cost and simplicity.

In this study, 96% of the mosquito legs with good spectra were correctly identified with LSV ≥ 1.8 by the MALDI-TOF MS. This confirms the reliability of this method for mosquito identification, as long as the spectra are of good quality. This allowed us to confirm the morphological identification of the female *Anopheles* spp. up to the species level and of the *Culex* spp. up to the genus level. It helped us to identify not only male *Anopheles* spp. but also minority species such as *Cx. rima*, *Cx. perexiguus*, and *Cx. watti* which were not included in our database before this work. The results of this study can be considered robust and reliable as they have been confirmed by molecular biology.

The two species of *Anopheles* identified in this study, that is, *An. gambiae* s.l. and *An. rufipes*, have already been reported in Chad.[43] *Anopheles gambiae* s.l. were the most abundant mosquitoes included in our study and considered the main vector of malaria in Chad.[43] *Anopheles rufipes* is a mosquito that rests frequently in human habitation but feeds on domestic animals and accidentally on humans.[29] In this study, we have also identified several *Culex* species such as *Cx. quinquefasciatus*, *Culex pipiens*, *Cx. perexiguus*, *Cx. watti*, and *Cx. rima*. Many of these species have been implicated or

suspected in the transmission of parasitic or viral pathogens. The *Cx. pipiens* species consists of two morphologically identical subspecies, that is, *Cx. pipiens pipiens* and *Cx. pipiens molestus*, with distinct trophic preferences: *Cx. pipiens pipiens* feeds on birds, whereas *Cx. pipiens molestus* prefers mammals.[44,45] *Culex pipiens* has been identified as the most important vector species of WNV in the United States because of their vectorial competence and summer abundance.[46] Presently, only one study has reported the presence of *Cx. pipiens* in Chad.[47] *Culex quinquefasciatus*, known vector of lymphatic filariasis and arboviruses including WNV, has been reported in some African countries.[48–50] *Culex perexiguus*, considered a potential vector for WNV transmission in birds and horses, has recently been reported in Mali.[24] *Culex watti* has been reported in Madagascar and is believed to have played a role in the WNV transmission.[51] As for *Cx. rima*, its presence has already been reported in some African countries,[52–54] but its vector role remains unknown to this day. We found that the fragment of the *COI* gene amplified in this study could not distinguish *An. rufipes* and *An. rhodesiensis*. This limitation of molecular biology had already been reported.[55] Therefore, MALDI-TOF MS could be an alternative tool to meet this challenge because this tool has been proven relevant to discriminate cryptic mosquito species.[30]

Only 40.7% of abdomens engorged had good quality MS spectra and 37.5% were correctly identified as human blood. *Niare* et al.[56] had shown that identification of the origin of the blood meal was relevant up to 24 hours because of blood digestion altering the resulting spectrum. We support the idea that this low percentage of good spectra could be explained by the fact that the Sella score to describe the digestive state of blood, from engorged mosquitoes from zero (non-nourished mosquitoes) to seven (females without visible blood and fully developed eggs in their abdomen), was poorly appreciated visually.[14]

CONCLUSION

The present study demonstrated that MALDI-TOF MS appears to be a promising tool for identifying mosquitoes stored in silica gel and moderately the origin of their blood meal. Although the number of samples used in our study is relatively small, the results obtained are robust and reliable. Thus, it opens the way for future studies with a large number of samples to confirm these preliminary results.

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Authors' addresses: Adama Zan Diarra, Maureen Laroche, and Philippe Parola, Aix Marseille University, Institut de recherche pour le devéloppement (IRD), Assistance publique-Hopitaux Marseille (AP-HM), Service de Santé des Armées (SSA), Vecteurs – Infections Tropicales et Méditerranéennes (VITROME), Institut hospitalo-universitaire (IHU) Méditerranée Infection, Marseille, France, E-mails: adamazandiarra@gmail.com, maureen.laroche972@gmail.com, and philippe.parola@univ-amu.fr. Franck Berger, Aix Marseille University, Institut national de la santé et de la recherche médicale (INSERM), Institut de recherche pour le devéloppement (IRD), Sciences Economiques & Sociales de la Santé & Traitement de l'Information Médicale (SESSTIM), Marseille, France, Service de Santé des Armées (SSA), Centre d'épidémiologie et de santé publique des armées (CESPA), Marseille, France, E-mail: franck1.berger@intradef.gouv.fr.

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DEUXIEME PARTIE :

Détection des microorganismes associés aux animaux et leurs ectoparasites (arthropodes)

Préambule

Les animaux sauvages et domestiques sont connus comme des réservoirs jouant un rôle important dans le maintien et la circulation de nombreux agents pathogènes infectieux transmis aux humains, notamment des bactéries telles que *Rickettsia* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *Leptospira* spp.; des eucaryotes tels que *Leishmania* spp., *Plasmodium* spp., *Theileria* spp., *Babesia* spp. , *Trypanosoma* spp.; et des virus tels que l'hantavirus, le virus fièvre hémorragique de Crimée-Congo et le virus Lassa . La transmission d'agents infectieux d'un animal à un autre y compris l'homme, peut avoir lieu directement par morsure ou par contact avec leurs excrétions, ou indirectement par l'intermédiaire d'arthropodes vecteurs (moustiques, tiques, puces, phlébotomes, glossines).

L'essor de la biologie moléculaire ces dernières années a permis de détecter un grand nombre de pathogènes vectorisés aussi bien chez l'hôte comme chez les arthropodes vecteurs. Mais la détection des pathogènes chez l'arthropode ne signifie pas que l'arthropode en question est un vecteur efficace car il peut être simplement porteur transitoire de l'agent infectieux au moment où il est testé.

Le premier travail (Article 1) a porté sur la recherche des bactéries dans 471 tiques colletées dans trois localités du Mali. Nous avons trouvé que 76,6 %, 37,6 %, 20,8 % et 1,1 % des tiques étaient positifs pour *Rickettsia* spp., *Coxiella burnetii*, *Anaplasmataceae* et *Borrelia* spp. respectivement. Ce travail a été publié dans PLOS Neglected Tropical Diseases.

Le second travail (*Article 4*) a consisté étudier et actualisé le répertoire des micro-organismes transmis par les tiques chez 120 tiques et 87 les animaux domestiques dans le nord de l'Algérie, à l'aide des méthodes de la biologie moléculaire. Nous avons détecté de nombreux micro-organismes chez les tiques et les animaux dont certains ont été rapportés pour la première fois. Ce travail est en Révision pour une publication dans Ticks and Tick-Borne Diseases.

Enfin nous avons aussi utilisé les outils moléculaires pour la détection des micro-organismes chez les petits mammifères et leurs ectoparasites au Mali (Article 5). Ainsi nous avons détecté *Bartonella* sp. et *Coxiella burnetii* dans les petits mammifères, *Coxiella burnetii*, *Rickettsia africae* et *Ehrlichia ruminantium* dans tiques. Ce travail est revision à American Journal of Tropical Medicine and Hygiene.

Article 4

Molecular identification of protozoal and bacterial organisms in domestic animals and their infesting ticks from north-eastern Algeria

Sadeddine Rima, Diarra Adama Zan, Laroche Maureen, Mediannikov Oleg, RIGHI Souad, Benakhla Ahmed, Dahmana Handi, Raoult Didier, Parola Philippe

En revision dans Ticks Tick Borne Dis

Résumé

Une étude moléculaire a été entreprise pour déterminer la présence de micro-organismes chez 120 tiques et 87 échantillons de sang prélevés sur des mammifères dans le nord-est de l'Algérie. Morphologiquement, huit espèces de tiques ont été identifiées, dont 70 *Rhipicephalus* (*Boophilus*) *annulatus*, 23 *Rhipicephalus bursa*, cinq *Rhipicephalus sanguineus* sensus lato, 11 *Hyalomma impeltatum*, cinq *Hyalomma scupense*, deux *Hyalommma marginatum*, un *Hyalomma anatolicum* et trois *Ixodes ricinus*.

Le screening par qPCR des tiques a montré que *Theileria annulata*, «*Candidatus* Ehrlichia urmitei», *Theileria buffeli* et *Anaplasma platys* ont été détectés dans *Rh. annulatus*. *Rickettsia massiliae* et *Anaplasma ovis* ont été détectés dans *Rh. sanguineus s.l.* et *Rh. bursa*. *Rickettsia aeschlimannii* détécté chez *Hy. marginatum*, *Hy. scupense* et *Hy. impeltatum*. Enfin, «*Candidatus* Rickettsia barbariae» a été détecté dans *Rh. bursa*. Dans les échantillons de sang nous avons détécté *Theileria equi*, *T. annulata*, *T. buffeli*, *Babesia bovis*, *Anaplasma marginale*, *A. ovis* et *Borrelia* spp. chez les bovins. *Theileria ovis*, *T. annulata* et *A. ovis* ont été détectés chez des ovins. En fin, *A. ovis* et *T. equi* ont été détectés respectivement chez les caprins et les équidés. Dans cette étude, *T. equi* et «*Candidatus* Rickettsia barbariae» ont été identifiés pour la première fois en Algérie, ainsi que de nouvelles espèces potentielles d'*Ehrlichia* et d'*Anaplasma*.

Bien que la détection moléculaire n'indique pas la compétence du vecteur surtout lorsque les tiques sont gorgées et collectés sur des animaux, cette étude augment le nombre de microorganismes détectés chez les tiques dans le nord-est de l'Algérie.

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Abstract

A molecular survey was undertaken to determine the presence of protozoal and bacterial organisms in 120 ticks and 87 blood samples collected from mammals in north-eastern Algeria. Eight tick species were morphologically identified including 70 Rhipicephalus (Boophilus) annulatus, 23 Rhipicephalus bursa, five Rhipicephalus sanguineus sensu lato, 11 Hyalomma impeltatum, five Hyalomma scupense, two Hyalommma marginatum, one Hyalomma anatolicum and three Ixodes ricinus. Quantitative PCR screening of the ticks showed that Theileria annulata, “Candidatus Ehrlichia urmitei”, Theileria buffeli and Anaplasma platys were detected in Rh. annulatus. Rickettsia massiliae and Anaplasma ovis were detected in Rh. sanguineus s.l. and Rh. bursa. Rickettsia aeschlimannii was detected in Hy. marginatum, Hy. scupense and Hy.nimpeltatum. Finally, “Candidatus Rickettsia barbariae” was detected in Rh. bursa. In the screening blood samples, Theileria equi, T.annulata, T. buffeli, Babesia bovis, Anaplasma marginale, A. ovis and Borrelia spp. were detected in cattle. Theileria ovis, T. annulata, and A. ovis were detected in sheep. In addition, A. ovis and T. equi were detected in goats and equidea respectively. In this study, T. equi and “Candidatus Rickettsia barbariae” were identified for the first time in Algeria as well as potential new species of Ehrlichia and Anaplasma. Although molecular detection does not indicate vector/reservoir competence when investigating ticks removed from animals, this study expands the knowledge of the microorganisms detected in ticks in north-east of Algeria.

Keywords	Piroplasmorida ;bacteria; animals; Ticks; PCR; Algeria

Corresponding Author	Phillipe Parola
Order of Authors	Rima SADEDDINE, Adama Z DIARRA, Maureen Laroche, Oleg MEDIANNIKOV, Souad Righi, Ahmed Benakhla, handi dahmana, Didier RAOULT, Phillipe Parola

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Molecular identification of protozoal and bacterial organisms in domestic animals and their infesting ticks from north-eastern Algeria

Rima SADEDDINE[1,2*], Adama Zan DIARRA[2,3,4*], Maureen LAROCHE[2], Oleg MEDIANNIKOV[3,5], Souad RIGHI[1], Ahmed BENAKHLA[1], Handi DAHMANA[3,5], Didier RAOULT[3,5], Philippe PAROLA[2,3]

1. Université Chadli Bendjdid, Département des Sciences Vétérinaires, El Tarf, 36000, Algeria
2. Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France
3. IHU Méditerranée Infection, Marseille, France
4. Department of Epidemiology of Parasitic Diseases, Malaria Research and Training Center, University of Science, Techniques and Technologies of Bamako, Bamako, Mali
5. Aix Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France

* Equal contributors

✧ Corresponding author: Philippe PAROLA, Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, IHU-Méditerranée Infection, Marseille, France, philippe.parola@univ-amu.fr.

Abstract

A molecular survey was undertaken to determine the presence of protozoal and bacterial organisms in 120 ticks and 87 blood samples collected from mammals in north-eastern Algeria. Eight tick species were morphologically identified including 70 *Rhipicephalus* (*Boophilus*) *annulatus*, 23 *Rhipicephalus bursa*, five *Rhipicephalus sanguineus* sensu lato, 11 *Hyalomma impeltatum*, five *Hyalomma scupense*, two *Hyalommma marginatum*, one *Hyalomma anatolicum* and three *Ixodes ricinus*. Quantitative PCR screening of the ticks showed that *Theileria annulata*, "*Candidatus* Ehrlichia urmitei", *Theileria buffeli* and *Anaplasma platys* were detected in *Rh. annulatus*. *Rickettsia massiliae* and *Anaplasma ovis* were detected in *Rh. sanguineus* s.l. and *Rh. bursa*. *Rickettsia aeschlimannii* was detected in *Hy. marginatum*, *Hy. scupense* and *Hy.nimpeltatum*. Finally, "*Candidatus* Rickettsia barbariae" was detected in *Rh. bursa*. In the screening blood samples, *Theileria equi*, *T.annulata*, *T. buffeli*, *Babesia bovis*, *Anaplasma marginale*, *A. ovis* and *Borrelia* spp. were detected in cattle. *Theileria ovis*, *T. annulata*, and *A. ovis* were detected in sheep. In addition, *A. ovis* and *T. equi* were detected in goats and equidea respectively. In this study, *T. equi* and "*Candidatus* Rickettsia barbariae" were identified for the first time in Algeria as well as potential new species of *Ehrlichia* and *Anaplasma*.

Although molecular detection does not indicate vector/reservoir competence when investigating ticks removed from animals, this study expands the knowledge of the microorganisms detected in ticks in north-east of Algeria.

Key words: Piroplasmorida , Tick-associated bacteria; Domestic animals; Ticks; PCR and sequencing; Algeria

1. Introduction

Ticks are obligate hematophagous arthropods known to be important vectors of a wide variety of protozoa, fungi, bacteria, viruses and filarial worms of medical and veterinary importance (Aydin et al., 2015; Pereira et al., 2016; Remedio et al., 2015). In Algeria, Mediterranean spotted fever caused by *Rickettsia conorii* is known to be endemic, and other rickettsial pathogens have been detected in ticks, including *R. aeschlimannii*, *R. massiliae*, *Rickettsia helvetica*, *Rickettsia monacensis*, *Rickettsia slovaca*, *Rickettsia africae* and *Rickettsia sibirica mongolitimonae* (Kernif et al., 2012). In addition, other tick-borne pathogens such as *Borrelia* spp., *Anaplasma* spp., and *Coxiella burnetii*, have also been detected in ticks and/or cattle (Aouadi et al., 2017; Boucheikhchoukh et al., 2018; Dahmani et al., 2015; Leulmi et al., 2016; Rjeibi et al., 2016; Ziam et al., 2015). Very few studies have been conducted on *Theileria* spp. and *Babesia* spp. in cattle and ticks from Algeria (Aouadi et al., 2017; Ziam et al., 2015). These studies reported the presence of *Theileria orientalis, T. annulata*, *T. ovis*, *Babesia ovis*, and *B. bovis* in cattle and ticks (Aouadi et al., 2017; Ziam et al., 2015). These apicomplexan protozoa are causative agents of piroplasmoses which are among the most economically important haemoparasitic tick-borne diseases of ruminants worldwide (Adjou Moumouni et al., 2015; Aydin et al., 2015; Toma et al., 2017; Dib et al., 2008; Pereira et al., 2016).

The aim of this study was to update the repertoire of protozoan and bacterial diseases in domestic animals and their infesting ticks in northern Algeria, using molecular methods.

2. Materials and methods

2.1. Study area, tick collection and blood sampling

From March 2016 to February 2017, 303 ticks and 87 blood samples were collected from cattle in the provinces of Guelma (36 ° 27 '0 "N 7 ° 25' 59.999" E), Annaba (36 ° 53 '60" N 7 ° 46 '0.001 "E) and El-Tarf (36 ° 46' 1.2" N 8 ° 19 '1.2 "E) in north-eastern Algeria (Figure 1).

Blood samples were collected and stored in EDTA tubes at -20°C and ticks were removed from analyzed animals and stored at room temperature in 70% ethanol. The morphological identification of ticks was carried out using a microscope and identification key (Walker, 2003). The sample collection was authorised by the Animal Ethics Committee of El-Tarf University (Law No. 88-08 of 26 January 1988 on the activities of veterinary medicine and the protection of animal health). Molecular analysis was performed on 120 selected ticks and all blood samples.

2.2. Detection of microorganisms

DNA was extracted individually from each sample (tick and blood) using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany). Both sample types were screened by quantitative real-time PCR (qPCR) for *Rickettsia* spp., *Bartonella* spp., *Anaplasmataceae* spp., *C. burnetii*, *Borrelia* spp., *Theileria* spp. and *Babesia* spp., by using specific primers and probes Table1. All the genus-qPCR positive samples for the different microorganisms were then tested using specific qPCR and/or subjected to standard PCR prior to sequencing to identify the pathogen species (Dahmani et al., 2015; Diarra et al., 2017; Tahir et al., 2016). Phylogenetic trees were drawn based on the alignment of the different genes using Bioedit and TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, United Kingdom) using the Tnr + Γ substitution model. The nucleotide sequences were compared with GenBank entries using BLASTn and filed in GenBank under the following accession numbers: MH319801, MH327771, MH327772, MH327773, MH327774, MH321192, MH321193, MH321194, MH321195 and MH321197.

3. Results

3.1. Tick identification and sample collection

A total of 120 ticks belonging to eight species were morphologically collected and identified, including *Rh. annulatus*, which was the most prevalent with 58.3% (70/120), followed by *Rh. bursa* (23/120), *Hy. impeltatum* with (11/120), *Hy. scupense* (5/120), *I. ricinus* (3/120), *Rh. sanguineus* s.l. (5/120), *Hy. marginatum* (2/120) and *Hy. anatolicum* (1/120) (Table 1). Of the 87 blood samples, 35, 27, 17 and 8 were collected from cattle, sheep, horses and goats respectively.

3.2. Detection of microorganisms

Piroplasmorida were detected in seven (7/70; 10 %) *Rh. annulatus* ticks and fourteen blood samples. Sequencing showed that six out of seven sequences obtained from ticks were 99.42% identical to *T. annulata* (KX273857) and one was 99.24% identical to *T. buffeli* (HM538197). For the blood samples the sequencing showed that five of fourteen sequences were 99.15-99.89% identical to *T. annulata* (KX273857, KT367878), four were 99.89-100% identical to *T. ovis* (FJ603460), three were 99.04% identical to *T. equi* (KY952232), one was 99.25% identical to *T. buffeli* (HM538197) and one was 99.35% identical to *B. bovis* (EF601930) (Table 2). The phylogenetic position of Piroplasmida identified in our study is illustrated in Figure 2.

Similarly, three *Rh. annulatus*, one *Rh. sanguineus* s.l., one *Rh. bursa* and nine blood samples were positive for *Anaplasma* spp. The sequences obtained from *Rh. sanguineus* s.l. and *Rh. bursa* were 100% identical to *A. ovis* (KY498325). Two of the three sequences obtained from *Rh. annulatus* were 98.07% identical to "*Candidatus* Ehrlichia urmitei" (KM021422) and one was 97.69%, identical to *A. platys* (KM021414). In animals, four sequences were 99.57-100% identical to *A. marginale* (CP006847, KY498335) and five were 99.5-100% identical to *A. ovis* (KM021411) (Table 2). The phylogenetic tree based on the 23S gene (485 bp) shows the position of the Anaplasmatacae genotypes of this study compared to the other genotypes in supplementary Figure 1.

Rickettsia massiliae was identified in two *Rh. sanguineus* s.l. and two *Rh. bursa* and *R. aeschlimannii* in two *Hy. scupense*, one *Hy. impeltatum* and one *Hy. marginatum* by specific qPCR. The sequencing of three ticks which were positive using pan-rickettsial qPCR and negative using specific qPCR allowed us to obtain a sequence only from *Rh. bursa*, which was 99.28% identical to "*Candidatus* Rickettsia barbariae" (KU645284). All blood samples were negative for *Rickettsia* spp. (Table 2).

All tested ticks were negative for *Borrelia* spp. using qPCR. Three blood samples were positive for *Borrelia* spp. and sequencing showed that the sequences were 99.5% identical to "uncultured *Borrelia* sp." detected in *Haemaphysalis megaspinosa* and *Haemaphysalis japonica* from Japan (LC170035 and AB897891). The phylogenetic tree based on the 16S rRNA gene (1355 bp) shows the position of the *Borrelia* sp. of this study compared to the other *Borrelia* spp. in Supplementary Figure 2.

All tested samples were found to be negative for *C. burnetii* and *Bartonella* spp. (Table 2).

4. Discussion

In this study we detected the presence of several pathogens in domestic animals and their infesting ticks using robust molecular approaches including appropriate controls. *Theileria equi* is an obligate haemoparasite transmitted by ticks that can cause equine piroplasmosis characterised by fever, anaemia, jaundice, hepatosplenomegaly, intravascular haemolysis and haemoglobinuria, or even death (Malekifard et al., 2014). In this study, *T. equi* was detected in blood of cattle and equids for the first time in Algeria. In Africa, *T. equi* had been detected in Tunisia (Ros-García et al., 2013), Kenya (Hawkins et al., 2015), Egypt (Mahmoud et al., 2016) Nigeria and South Africa (Adamu et al., 2014; Rosa et al., 2014). *Theileria annulata* is an obligate intracellular protozoan parasite infecting monocytes/macrophages and B cells (Glass et al., 1989). Here, *T. annulata* was identified in *Rh. annulatus* ticks and in the blood of

145 cattle and sheep. The presence of *T. annulata* in cattle was previously detected in eastern and
146 northern central Algeria, in Egypt, Ethiopia, Tunisia and Sudan (Elsify et al., 2015;
147 Gebrekidan et al., 2014; M'ghirbi et al., 2008; Taha et al., 2013; Ziam and Benaouf, 2004;
148 Ziam et al., 2015). *Theileria buffeli* is known as a widely distributed benign haemoparasite
149 of cattle usually transmitted by *Haemaphysalis punctata* ticks throughout the Mediterranean
150 basin (Grech-Angelini et al., 2016). In our study, *T. buffeli* was detected in cattle and *Rh.*
151 *annulatus* ticks. *Theileria buffeli* was reported for the first time in Algeria by Ziam et al.
152 (2015). It has also been found in cattle from Tunisia (M'ghirbi et al., 2008) and in buffalo
153 from South Africa and Mozambique (Chaisi et al., 2013). *Theileria ovis* was detected only in
154 sheep, in line with previous studies reporting the detection of this pathogen in sheep, goats
155 and *Rhipicephalus* ticks in Algeria (Aouadi et al., 2017). It is a causative agent of subclinical
156 infection in small ruminants and is widespread throughout in the world (Jalali et al., 2014).

157 *Babesia bovis*, the agent responsible for bovine babesiosis, is a highly prevalent protozoan
158 intra-erythrocyte parasite of economic importance in cattle and is transmitted by ticks
159 (Mtshali and Mtshali, 2013). In this study, *B. bovis* was found in cattle from eastern Algeria,
160 which was consistent with previous results reported by Ziam and Benaouf (2004). It has also
161 been detected in cattle from Kenya (Adjou Moumouni et al., 2015).

162 In this study, we detected several *Anaplasma* species, including *A.marginale*, an obligate
163 intracellular bacterium responsible for bovine anaplasmosis worldwide, manifested by
164 anaemia and jaundice (Parola and Raoult, 2001). As previously reported in Algeria by Ziam
165 and Benaouf (2004), this bacterium was detected in cattle. In addition, we detected *A. ovis* in
166 the blood of small ruminants, *Rh. sanguineus* s.l. and *Rh. bursa*. Previous studies had already
167 reported the presence of *A. ovis* in these ticks (Aouadi et al., 2017; Aubry and Geale, 2011;
168 Dahmani et al., 2017). *Anaplasma ovis* is the causative agent of anaplasmosis in small
169 ruminants and is known to be transmitted by several tick species (Dumler et al., 2001).

Anaplasma ovis has already been reported in small ruminants and ticks from Algeria and Tunisia (Aouadi et al., 2017; Belkahia et al., 2015, 2017; Ben Said et al., 2015) and in ticks from Ethiopia (Teshale et al., 2016). Interestingly, we identified a new *Anaplasma* genotype close to *A. platys*, the agent of canine anaplasmosis which exclusively infects platelets and periodically causes deep thrombocytopenia in dogs (Nair et al., 2016), transmitted by *Rh. sanguineus* s.l. ticks (Sanogo et al., 2003). Strains of *A. platys* which are genetically close to *A. platys* have already been reported in cattle in Tunisia (Belkahia et al., 2015; Ben Said et al., 2017, 2018). Further analyses are needed, to know whether it is specifically *A. platys* or genetically-related strains. We also found a potential new species of *Ehrlichia* which is phylogenetically close to "*Candidatus* Ehrlichia urmitei" in *Rh. annulatus*. Little is known about this species since it was recently detected for the first time in *Amblyomma variegatum*, *Rhipicephalus microplus* and *Hyalomma truncatum* collected from cattle in Côte d'Ivoire (Ehounoud et al., 2016). More recently, it has also been detected in *Rh. microplus* and *Rh. bursa* collected in France and Mali respectively (Dahmani et al., 2017; Diarra et al., 2017).

In this study, *R. massiliae* was detected in *Rh. bursa* and *Rh. sanguineus* s.l., thus confirming its presence in the country. Similarly, in our study, *R. aeschlimannii* DNA was found in *Hy. impeltatum*, *Hy. scupense* and *Hy. marginatum*. In Algeria, previous studies have reported the presence of *R. aeschlimannii* in several tick species of the *Hyalomma* genus (Bitam et al., 2006, 2009; Djerbouh et al., 2012; Leulmi et al., 2016). Both *R. massiliae* and *R. aeschlimannii* are spotted fever group rickettsial, known to be agents of emerging rickettsioses in humans. "*Candidatus* Rickettsia barbariae" was also found in one *Rh. bursa*. This bacterium was first detected in *Rh. sanguineus* group ticks collected from Italian sheep (Mura et al., 2008). Since then, several studies have reported the presence of "*Candidatus* Rickettsia barbariae" in ticks and fleas from Europe and Asia (Chochlakis et al., 2012; Socolovschi et al., 2012; Zhao et al., 2016). Our study is the first to report the presence of

195 "*Candidatus* Rickettsia barbariae" in Africa. The pathogenicity for animals or humans of this
196 bacterium remains unknown.

197 The spirochaete *Borrelia theileri*, the causative agent of bovine borreliosis in cattle,
198 associated with fever and anaemia (Smith et al., 1985), was detected in two cattle blood
199 samples. In Algeria, this species has been reported in blood from sheep and goats (Aouadi et
200 al., 2017), but no studies have thus far indicated its presence in Algerian cattle. In Africa, *B.*
201 *theileri* has also been reported in *Rhipicephalus geigyi* collected from cattle in Mali (McCoy
202 et al., 2014)

203 **Conclusion**

204 In this study, we reported the presence of numerous pathogens in mammals and their ticks in
205 north-eastern Algeria. Although, we contribute towards broadening the knowledge of the
206 repertoire of microorganisms, our results do not show infection status of the region due to the
207 small amount of material collected. Also, it is not possible to draw conclusion on the
208 competence of these vectors/reservoirs, because these data do not suggest that the tick species
209 mentioned in the document can serve as a competent vector for all the pathogens detected in
210 this study. Nevertheless, other studies are needed to show the exact distribution of ticks and to
211 appreciate the extent of the distribution of microorganisms in Algeria.

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214 various institutions for the collection of field samples and laboratory analysis.

215 **List of figures**

216 **Figure 1**: Map of Algeria with sample collection sites

Figure 2: Phylogenetic tree showing the relationships between Piroplasmida identified in the present study relative to other species based on a comparison of a 965 bp fragment of the 18S gene.

GenBank access numbers are shown at the beginning and the strains are shown at the end. The 18S gene sequences of Piroplasmida were aligned using CLUSTALW for multi-sequence alignment implemented using Bioedit software, and phylogenetic inferences obtained using ML phylogenetic analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) in the integrated ML application, using the Tnr +G+ Γ substitution model. The numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree (only the values equal to or greater than 80 have been retained).

Supplementary Figure 1: Phylogenetic tree showing the position of amplified Anaplasmataceae species in this study compared to other species based on a 485 bp portion of a 23S gene sequence.

GenBank access numbers are shown at the beginning and the strains are shown at the end. The 23S gene sequences were aligned using CLUSTALW for multi-sequence alignment implemented using Bioedit software, and phylogenetic inferences were obtained using ML phylogenetic analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) in the integrated ML application, using the Tnr +G+ Γ substitution model. The numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree (only the values equal to or greater than 80 have been retained).

Supplementary Figure 2: Phylogenetic tree highlighting the position of *Borrelia* sp. identified in this study compared to other strains based on a 1,355 bp portion of a 16S gene sequence.

GenBank access numbers are shown at the beginning and the geographic origin of the species is given at the end. The 16S rRNA gene sequences were aligned using CLUSTALW for multi-sequence alignment implemented using Bioedit software, and phylogenetic inferences obtained using ML phylogenetic analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) in the integrated ML application, using the Tnr +G+ Γ substitution model. The numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree (only the values equal to or greater than 80 have been retained).

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441 **Supplementary Table 1. Sequences of primers and probes used for quantitative real-time PCRs (qPCR) and standard PCRs in this**

442 **study.**

Target microorganisms	Target gene	Primers (5'-3') and probes (6FAM–TAMRA)		References
		Quantitative real-time PCRs (qPCRs)		
Rickettsia spp.	*gltA* ("RKND03")	F_GTGAATGAAAGATTACACTATTTAT R_GTATCTTAGCAATCATTCTAATAGC 6FAM-CTATTATGCTTGCGGCTGTCGGTTC-TAMRA	166pb	(Ehounoud et al., 2016) (Aouadi et al., 2017)
Rickettsia aeschlimannii	SCA1	F_AAGCGGCACTTTAGGTAAAGAAA R_CATGCTCTGCAAATGAACCA 6FAM- TGGGGAAATATGCCGTATACGCAAGC-TAMRA	110pb	(Ehounoud et al., 2016)
Rickettsia conorii	RC0743	F_TTGGTAGGCAAGTAGCTAAGCAAA R_GGAAGTATATGGGAATGCTTTGAA 6FAM- GCGGTTATTCCTGAAAATAAGCCGGCA-TAMRA	116pb	(Sokhna et al., 2013)
Rickettsia massiliae	hypothetical protein	F_CCAACCTTTTGTTGTTGCAC R_TTGGATCAGTGTGACGGACT 6FAM- CACGTGCTGCTTATACCAGCAAACA- TAMRA	127pb	(Ehounoud et al., 2016)

		R_GTTTAATGGGCATTGGGCTA		
		6FAM- CCTGTGTAGACGATTCAAGAGGGATGA-TAMRA	169bp	
Anaplasmataceae	23S rRNA	F_TGACAGCGTACCTTTTGCAT		(Dahmani et al., 2015)
		R_GTAACAGGTTCGGTCCTCCA		
		6FAM-CTTGGTTTCGGGTCTAATCC-TAMRA		
Piroplasmida spp.	5.8S rRNA	F_ AYYKTYAGCGRTGGATGTC	85pb	Dahmana et al., (*unpublished data*)
		R_ TCG CAG RAG TCT KCA AGT C		
		FAM-TTYGCTGCGTCCTTCATC GTTGT-MGB		
Borrelia spp.	Intergenic spacer (ITS4)	F_GGCTTCGGGTCTACCACATCTA		(Ehounoud et al., 2016)
		R_CCGGGAGGGGAGTGAAATAG	200bp	
		6FAM- TGCAAAAGGCACGCCATCACC-TAMRA		
Bartonella spp.	16S/23S rRNA intergenic spacer (ITS2)	F_GGGGCCGTAGCTCAGCTG		
		R_TGAATATATCTTCTCTTCACAATTTC	104bp	(Aouadi et al., 2017)
		6FAM- CGATCCCGTCCGGCTCCACCA-TAMRA		
Coxiella burnetii	Intergenic spacer	F_ CGCTGACCTACAGAAATATGTCC		(Ehounoud et al.,

	"IS30a"	R_ GGGGTAAGTAAATAATACCTTCTGG 6FAM- CATGAAGCGATTTATCAATACGTGTATGC-TAMRA	164bp	2016) ; (Aouadi et al., 2017)
		Standard PCR		
Anaplasmataceae	23S rRNA	F_ATAAGCTGCGGGGAGTTGTC R_TGCAAAAGGTACGCTGTCAC	485bp	(Dahmani et al., 2015)
Borrelia spp.	16S rRNA	BF1_GCTGGCAGTGCGTCTTAAGC BR1_GCTTCGGGTATCCTCAACTC	1355bp	(Aouadi et al., 2017)
Rickettsia spp.	*ompA*	F_ATGGCGAATATTTCTCCAAAA R_GTTCCGTTAATGGCAGCATCT	630bp	(Tahir et al., 2016)
Piroplasmida spp.	18S rRNA	F1_ GCGAATGGCTCATTAIAACA F3_ GTAGGGTATTGGCCTACCG R3_ AGGACTACGACGGTATCTGA R4_ TTTCAGMCTTGCGACCATACT	965bp	Dahmana et al., (*unpublished data*)

443

444 **Table 1: Numbers of ticks used for pathogens identification**

Tick species	Number collected from cattle	Number collected from sheep	Number collected from goats	Number collected from equidae	Total
Rhipicepalus annulatus	29	14	7	20	70
Rhipicepalus bursa	0	14	9	0	23
Hyalomma impeltatum	8	3	0	0	11
Hyalomma scupense	0	1	4	0	5
Ixodes ricinus	0	2	1	0	3
Rhipicephalus sanguineus s.l.	0	2	3	0	5
Hyalomma marginatum	0	1	1	0	2
Hyalomma anatolicum	0	0	1	0	1
Total	37	37	26	20	120

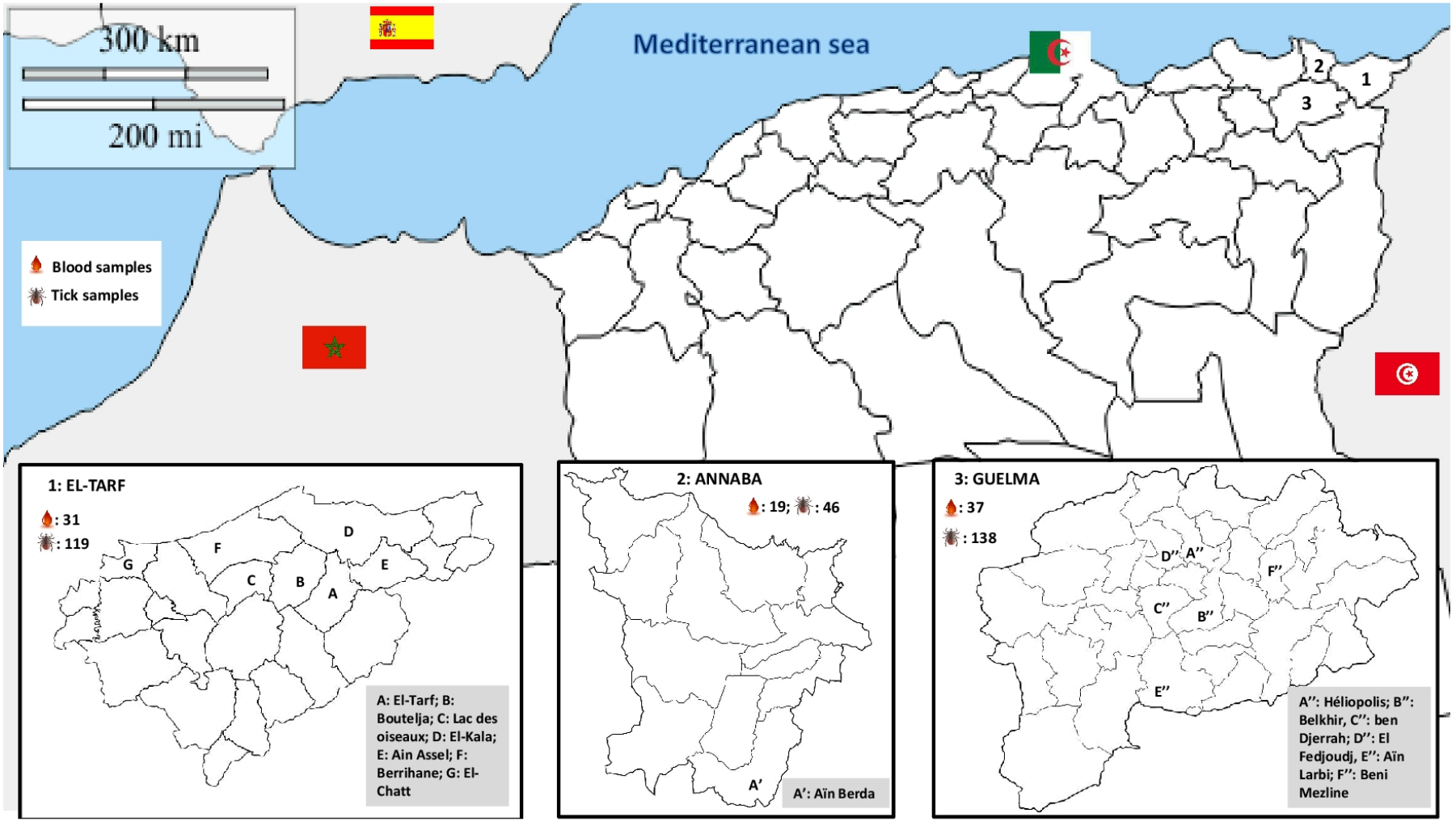
445 **Table 2: Numbers of ticks or blood samples which tested positive for various pathogens analysed in the present study**

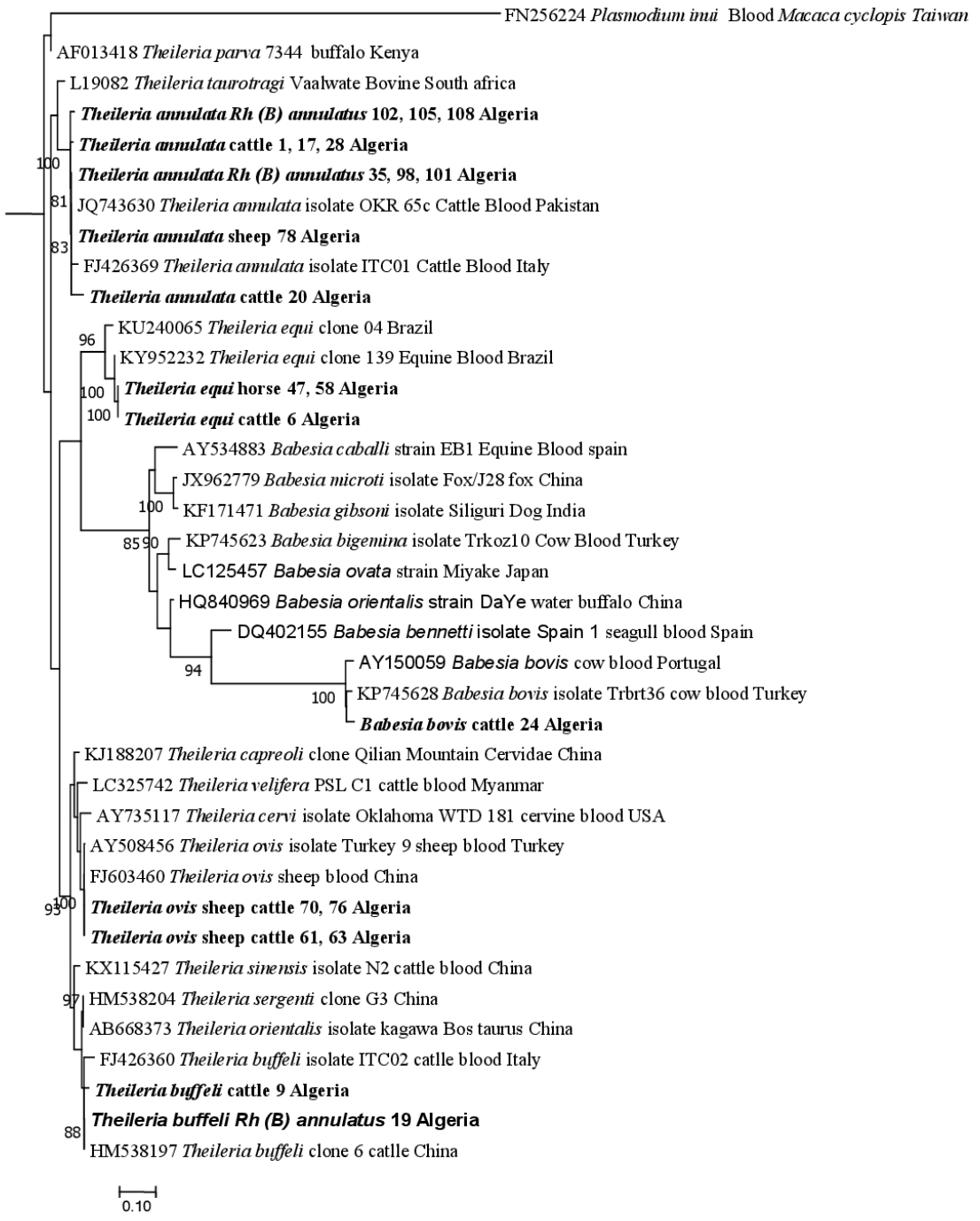
446

		Numbers of samples positive for													
Samples	**Total tested**	*R. massiliae*	*R. aeschlimannii*	Other *Rickettsia*	*A. marginale*	*A. ovis*	*A. patys*	*Candidatus* Ehrlichia urmitei	*T. annulata*	*T. equi*	*T. ovis*	*T. buffeli*	*B. bovis*	*Borrelia* spp.	*Bartonella* spp.	*C. burnetii*
Ticks	120	2 *Rh. bursa*, 2 *Rh. sanguineus* s.l.	1 *Hy. impeltatum*, 2 *Hy. scupense*, 1 *Hy. marginatum*	1 *Rh. bursa*		1 *Rh. sanguineus* s.l., *1 Rh. bursa*	1 *Rh. annulatus**	2 *Rh. annulatus**	4 *Rh. annulatus**, 2*Rh. annulatus*	-	-	1 *Rh. annulatus**	-	-	-	-
Blood	87	-	-		4 Cattle	3 Goat, 1 Sheep, 1 Cattle	-	-	4 Cattle, 1 Sheep	1 Cattle, 2 Equidea	4 Sheep	1 Cattle	1 Cattle	2 Cattle	-	-

447

448 * Engorged tick specimen


300 km
200 mi
Mediterranean sea
Blood samples
Tick samples
1
2
3
1: EL-TARF
: 31
: 119
A
B
C
D
E
F
G
A: El-Tarf; B: Boutelja; C: Lac des oiseaux; D: El-Kala; E: Ain Assel; F: Berrihane; G: El-Chatt
2: ANNABA
: 19; : 46
A'
A': Aïn Berda
3: GUELMA
: 37
: 138
A''
B''
C''
D''
E''
F''
A'': Héliopolis; B'': Belkhir, C'': ben Djerrah; D'': El Fedjoudj, E'': Aïn Larbi; F'': Beni Mezline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AF013418 Theileria parva 7344 buffalo Kenya
L19082 Theileria taurotragi Vaalwate Bovine South africa
Theileria annulata Rh (B) annulatus 102, 105, 108 Algeria
Theileria annulata cattle 1, 17, 28 Algeria
Theileria annulata Rh (B) annulatus 35, 98, 101 Algeria
JQ743630 Theileria annulata isolate OKR 65c Cattle Blood Pakistan
Theileria annulata sheep 78 Algeria
FJ426369 Theileria annulata isolate ITC01 Cattle Blood Italy
Theileria annulata cattle 20 Algeria
KU240065 Theileria equi clone 04 Brazil
KY952232 Theileria equi clone 139 Equine Blood Brazil
Theileria equi horse 47, 58 Algeria
Theileria equi cattle 6 Algeria
AY534883 Babesia caballi strain EB1 Equine Blood spain
JX962779 Babesia microti isolate Fox/J28 fox China
KF171471 Babesia gibsoni isolate Siliguri Dog India
KP745623 Babesia bigemina isolate Trkoz10 Cow Blood Turkey
LC125457 Babesia ovata strain Miyake Japan
HQ840969 Babesia orientalis strain DaYe water buffalo China
DQ402155 Babesia bennetti isolate Spain 1 seagull blood Spain
AY150059 Babesia bovis cow blood Portugal
KP745628 Babesia bovis isolate Trbrt36 cow blood Turkey
Babesia bovis cattle 24 Algeria
KJ188207 Theileria capreoli clone Qilian Mountain Cervidae China
LC325742 Theileria velifera PSL C1 cattle blood Myanmar
AY735117 Theileria cervi isolate Oklahoma WTD 181 cervine blood USA
AY508456 Theileria ovis isolate Turkey 9 sheep blood Turkey
FJ603460 Theileria ovis sheep blood China
Theileria ovis sheep cattle 70, 76 Algeria
Theileria ovis sheep cattle 61, 63 Algeria
KX115427 Theileria sinensis isolate N2 cattle blood China
HM538204 Theileria sergenti clone G3 China
AB668373 Theileria orientalis isolate kagawa Bos taurus China
FJ426360 Theileria buffeli isolate ITC02 catlle blood Italy
Theileria buffeli cattle 9 Algeria
Theileria buffeli Rh (B) annulatus 19 Algeria
HM538197 Theileria buffeli clone 6 catlle China
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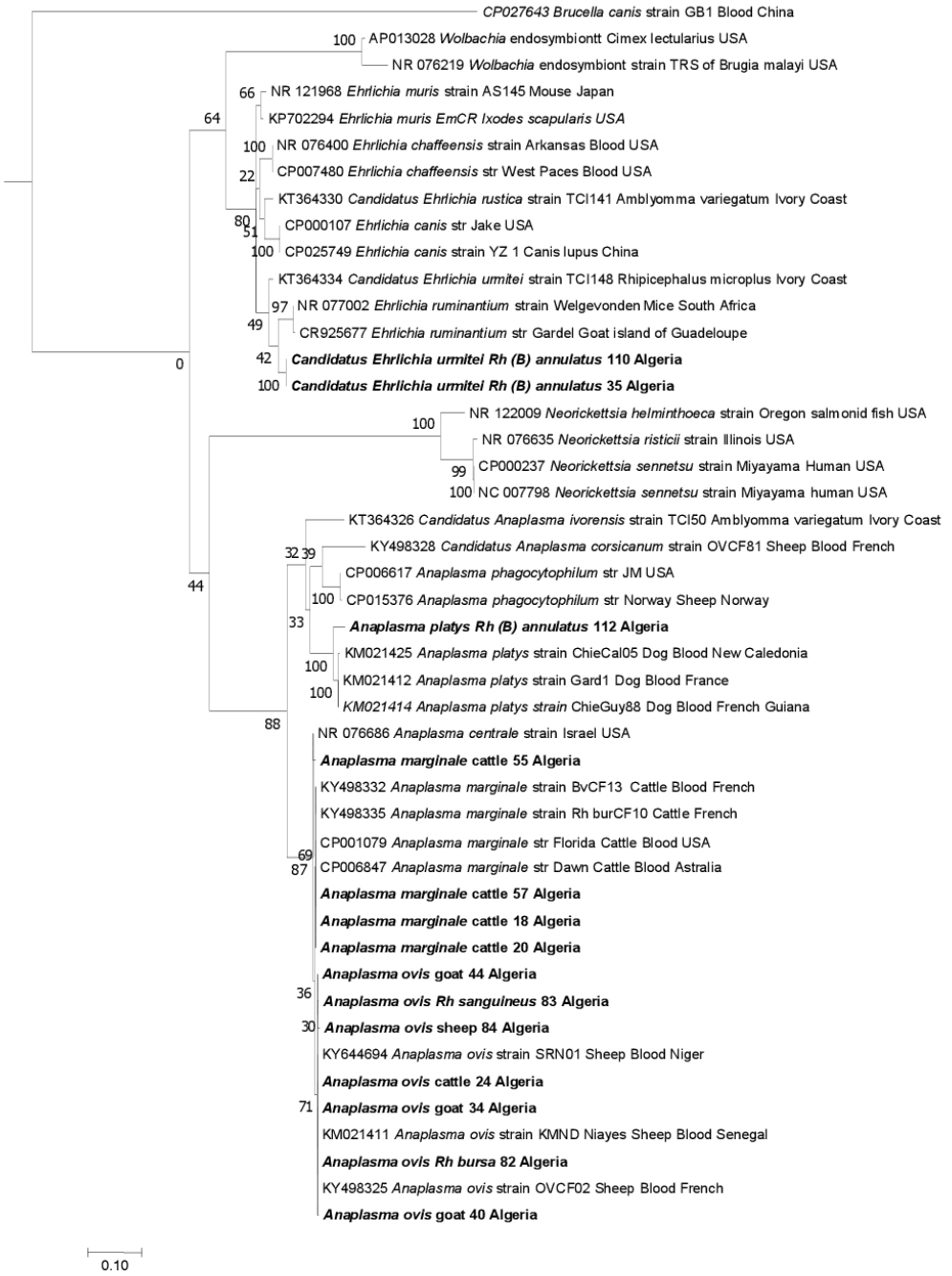
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NR 076219 Wolbachia endosymbiont strain TRS of Brugia malayi USA
66
NR 121968 Ehrlichia muris strain AS145 Mouse Japan
64
KP702294 Ehrlichia muris EmCR Ixodes scapularis USA
100
NR 076400 Ehrlichia chaffeensis strain Arkansas Blood USA
22
CP007480 Ehrlichia chaffeensis str West Paces Blood USA
KT364330 Candidatus Ehrlichia rustica strain TCI141 Amblyomma variegatum Ivory Coast
80
51
CP000107 Ehrlichia canis str Jake USA
100
CP025749 Ehrlichia canis strain YZ 1 Canis lupus China
KT364334 Candidatus Ehrlichia urmitei strain TCI148 Rhipicephalus microplus Ivory Coast
97
NR 077002 Ehrlichia ruminantium strain Welgevonden Mice South Africa
49
CR925677 Ehrlichia ruminantium str Gardel Goat island of Guadeloupe
42
Candidatus Ehrlichia urmitei Rh (B) annulatus 110 Algeria
0
100
Candidatus Ehrlichia urmitei Rh (B) annulatus 35 Algeria
100
NR 122009 Neorickettsia helminthoeca strain Oregon salmonid fish USA
NR 076635 Neorickettsia risticii strain Illinois USA
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CP000237 Neorickettsia sennetsu strain Miyayama Human USA
100
NC 007798 Neorickettsia sennetsu strain Miyayama human USA
KT364326 Candidatus Anaplasma ivorensis strain TCI50 Amblyomma variegatum Ivory Coast
32
39
KY498328 Candidatus Anaplasma corsicanum strain OVCF81 Sheep Blood French
CP006617 Anaplasma phagocytophilum str JM USA
44
100
CP015376 Anaplasma phagocytophilum str Norway Sheep Norway
33
Anaplasma platys Rh (B) annulatus 112 Algeria
100
KM021425 Anaplasma platys strain ChieCal05 Dog Blood New Caledonia
100
KM021412 Anaplasma platys strain Gard1 Dog Blood France
KM021414 Anaplasma platys strain ChieGuy88 Dog Blood French Guiana
88
NR 076686 Anaplasma centrale strain Israel USA
Anaplasma marginale cattle 55 Algeria
KY498332 Anaplasma marginale strain BvCF13 Cattle Blood French
KY498335 Anaplasma marginale strain Rh burCF10 Cattle French
CP001079 Anaplasma marginale str Florida Cattle Blood USA
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87
CP006847 Anaplasma marginale str Dawn Cattle Blood Astralia
Anaplasma marginale cattle 57 Algeria
Anaplasma marginale cattle 18 Algeria
Anaplasma marginale cattle 20 Algeria
Anaplasma ovis goat 44 Algeria
36
Anaplasma ovis Rh sanguineus 83 Algeria
30
Anaplasma ovis sheep 84 Algeria
KY644694 Anaplasma ovis strain SRN01 Sheep Blood Niger
Anaplasma ovis cattle 24 Algeria
71
Anaplasma ovis goat 34 Algeria
KM021411 Anaplasma ovis strain KMND Niayes Sheep Blood Senegal
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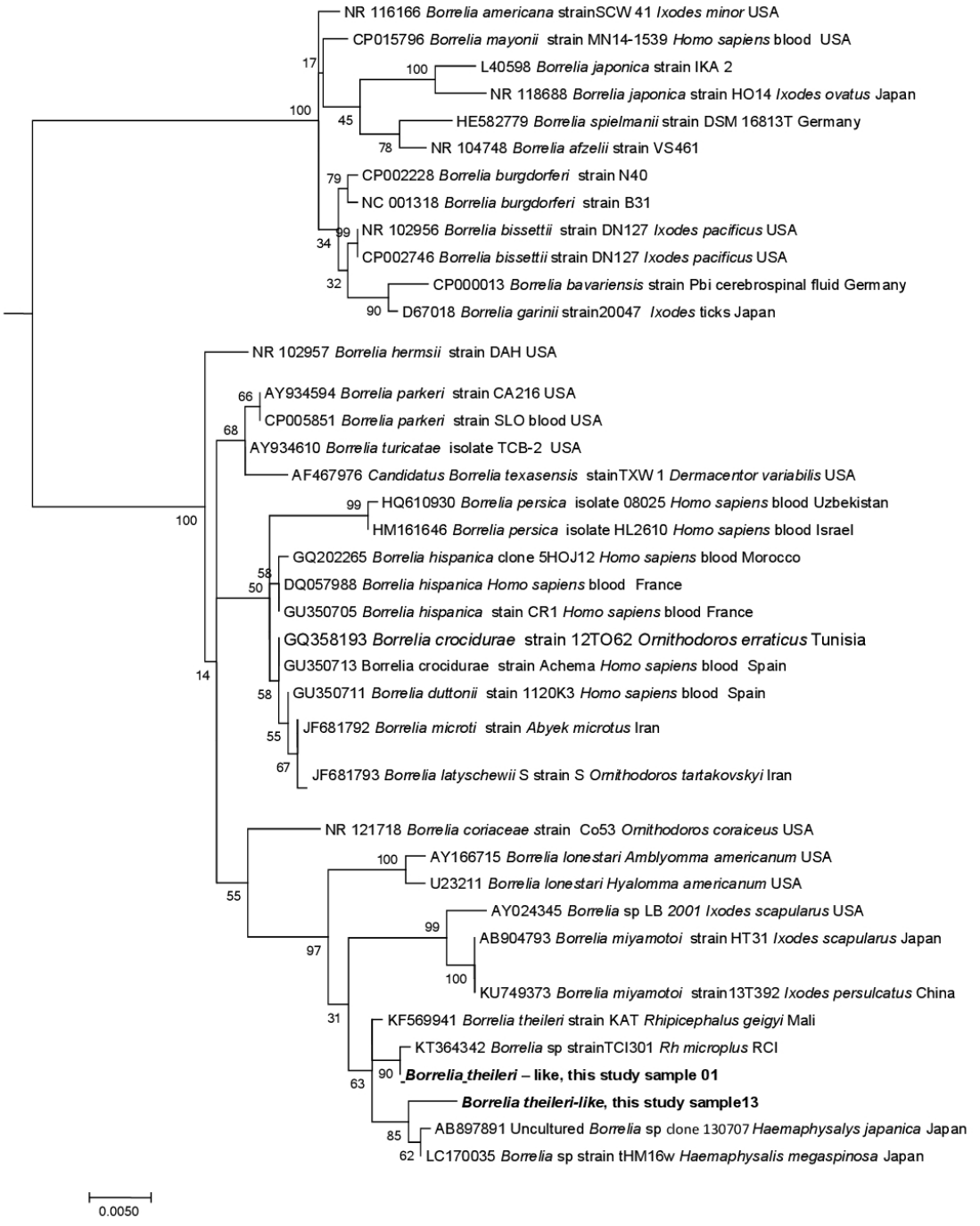
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NR 118688 Borrelia japonica strain HO14 Ixodes ovatus Japan
HE582779 Borrelia spielmanii strain DSM 16813T Germany
NR 104748 Borrelia afzelii strain VS461
CP002228 Borrelia burgdorferi strain N40
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NR 102956 Borrelia bissettii strain DN127 Ixodes pacificus USA
CP002746 Borrelia bissettii strain DN127 Ixodes pacificus USA
CP000013 Borrelia bavariensis strain Pbi cerebrospinal fluid Germany
D67018 Borrelia garinii strain20047 Ixodes ticks Japan
NR 102957 Borrelia hermsii strain DAH USA
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AY934610 Borrelia turicatae isolate TCB-2 USA
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HM161646 Borrelia persica isolate HL2610 Homo sapiens blood Israel
GQ202265 Borrelia hispanica clone 5HOJ12 Homo sapiens blood Morocco
DQ057988 Borrelia hispanica Homo sapiens blood France
GU350705 Borrelia hispanica stain CR1 Homo sapiens blood France
GQ358193 Borrelia crocidurae strain 12TO62 Ornithodoros erraticus Tunisia
GU350713 Borrelia crocidurae strain Achema Homo sapiens blood Spain
GU350711 Borrelia duttonii stain 1120K3 Homo sapiens blood Spain
JF681792 Borrelia microti strain Abyek microtus Iran
JF681793 Borrelia latyschewii S strain S Ornithodoros tartakovskyi Iran
NR 121718 Borrelia coriaceae strain Co53 Ornithodoros coraiceus USA
AY166715 Borrelia lonestari Amblyomma americanum USA
U23211 Borrelia lonestari Hyalomma americanum USA
AY024345 Borrelia sp LB 2001 Ixodes scapularus USA
AB904793 Borrelia miyamotoi strain HT31 Ixodes scapularus Japan
KU749373 Borrelia miyamotoi strain13T392 Ixodes persulcatus China
KF569941 Borrelia theileri strain KAT Rhipicephalus geigyi Mali
KT364342 Borrelia sp strainTCI301 Rh microplus RCI
Borrelia_theileri – like, this study sample 01
Borrelia theileri-like, this study sample13
AB897891 Uncultured Borrelia sp clone 130707 Haemaphysalys japanica Japan
LC170035 Borrelia sp strain tHM16w Haemaphysalis megaspinosa Japan
0.0050

Article 5

Molecular detection of microorganisms associated with small mammals and their ectoparasites in Mali

Diarra Adama Zan, Kone Abdoulaye Kassoum, Doumbo Safiatou Niare, Laroche Maureen, Diatta Georges, Ag Atteynine Souleymane, Coulibaly Maïmouna, Sangare Abdoul Karim, Kouriba Bouréma, Djimde Abdoulaye, Dabo Abdoulaye, Sagara Issaka, Davoust Bernard, Ranque Stephane, Thera Mahamadou A, Didier Raoult, Doumbo Ogobara K, Parola Philippe

Préparation pour soumission dans Am J Trop Med Hyg

Résumé

Les petits mammifères sont les réservoirs naturels de plusieurs agents pathogènes zoonotiques, notamment des bactéries, des parasites et des virus. Dans cette étude, nous avons évalué la prévalence d'agents pathogènes chez les petits mammifères et leurs ectoparasites au Mali à l'aide d'outils moléculaires.

Les petits mammifères piégés avec des pièges BTS ont été identifiés morphologiquement. Sang, organes et ectoparasites ont été recueillis. Au total, 130 petits mammifères appartenant à 10 espèces différentes ont été capturés, dont 74 (56,92%) étaient infestés par des ectoparasites, dont 248 *Lealaps echidnina*, 64 *Xenopsylla cheopis*, 62 nymphes *Haemaphysalis* spp. et trois nymphes *Amblyomma* spp. L'ADN de *Bartonella* a été trouvé dans 23/130 (17,69%) des petits mammifères, 33/94 (35,10%) des acariens et 24/64 (37,5%) des puces. Les séquences partielles des gènes *gltA*, *ftsZ* et *rpoB* de *Bartonella* détectées chez les petits mammifères étaient proches de *Bartonella mastomydis*, de *Bartonella elizabethae* et de *Bartonella* spp. L'ADN de *Coxiella burnetii* a été détecté chez 32/65 (49,23%) des tiques, 3/130 (2,3%) des petits mammifères et 1/94 (1,06%) des acariens. Nous avons trouvé l'ADN des Anaplasmatacae dans les puces et l'ADN de *Rickettsia africae* et d'*Ehrlichia ruminantium* dans les tiques.

Les résultats de notre étude montrent que plusieurs espèces de petits mammifères pourraient être considérées comme des réservoirs potentiels de *Bartonella* spp. susceptibles de jouer un rôle majeur dans le maintien, la circulation et la transmission de *Bartonella* spp. au Mali. Cependant, la pathogénicité de ces bactéries chez les humains ou chez les animaux reste à démontrer.

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	Medicine and dentistry/ UMI 3189 "Environnement, Santé, Sociétés"/University of Science, Techniques and Technologies of Bamako (USTTB) DABO, Abdoulaye; 3. Malaria Research and Training Center (MRTC), Department of Epidemiology of Parasitic Diseases (DEAP)/ Faculty of Medicine and dentistry/ UMI 3189 "Environnement, Santé, Sociétés"/University of Science, Techniques and Technologies of Bamako (USTTB) Sagara, Issaka; 3. Malaria Research and Training Center (MRTC), Department of Epidemiology of Parasitic Diseases (DEAP)/ Faculty of Medicine and dentistry/ UMI 3189 "Environnement, Santé, Sociétés"/University of Science, Techniques and Technologies of Bamako (USTTB) Davoust, Bernard; 2. IHU, Méditerranée Infection; 6. Aix Marseille Univ, IRD, AP-HM, MEPHI Ranque, Stéphane; 1. Aix Marseille Univ, IRD, AP-HM, SSA, VITROME; 2. IHU, Méditerranée Infection Thera, Mahamadou; 3. Malaria Research and Training Center (MRTC), Department of Epidemiology of Parasitic Diseases (DEAP)/ Faculty of Medicine and dentistry/ UMI 3189 "Environnement, Santé, Sociétés"/University of Science, Techniques and Technologies of Bamako (USTTB) Raoult, Didier; 2. IHU, Méditerranée Infection; 6. Aix Marseille Univ, IRD, AP-HM, MEPHI Doumbo, Ogobara; 3. Malaria Research and Training Center (MRTC), Department of Epidemiology of Parasitic Diseases (DEAP)/ Faculty of Medicine and dentistry/ UMI 3189 "Environnement, Santé, Sociétés"/University of Science, Techniques and Technologies of Bamako (USTTB) Parola, Philippe; 1. Aix Marseille Univ, IRD, AP-HM, SSA, VITROME; 2. IHU, Méditerranée Infection
Key Words:	Bartonella, Small mammals, Ectoparasites, Molecular Biology, Mali

Molecular detection of microorganisms associated with small mammals and their ectoparasites in Mali

Adama Zan DIARRA[1,2,3✧], Abdoulaye Kassoum KONE[3✧], DOUMBO Safiatou NIARE[3], Maureen LAROCHE[1,2], Georges DIATTA[4], Souleymane AG ATTEYNINE[5], Maïmouna COULIBALY[3], Abdoul Karim SANGARE[3], Bouréma KOURIBA[3], Abdoulaye DJIMDE, Abdoulaye DABO[3], Issaka SAGARA[3], Bernard DAVOUST[2,6], Stephane RANQUE[1,2], Mahamadou A THERA[3], Raoult DIDIER[2,6], Ogobara K DOUMBO[3], Philippe PAROLA[1, 2*]

1. Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France
2. IHU-Méditerranée Infection, Marseille, France
3. Malaria Research and Training Center (MRTC), Department of Epidemiology of Parasitic Diseases (DEAP)/ Faculty of Medicine and dentistry/ UMI 3189 "Environnement, Santé, Sociétés"/University of Science, Techniques and Technologies of Bamako (USTTB), Bamako, Mali
4. VITROME Dakar, Campus International IRD-UCAD Hann, Dakar, Senegal
5. Institut de Recherche pour le Développement (IRD) BP. 2528, Bamako, Mali
6. Aix Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France.

*Corresponding author: Philippe PAROLA, VITROME, IHU Méditerranée Infection, 19-21 Boulevard Jean Moulin 13005 Marseille, France, E-mail, philippe.parola@univ-amu.fr.

✧ These authors contributed equally to this work.

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Abstract

Small mammals are the natural reservoirs for several zoonotic pathogens, including bacteria, parasites and viruses. In this study, we assessed the prevalence of pathogens in small mammals and theirs ectoparasites in Mali using molecular tools. Small mammals trapped alive with BTS traps were morphologically identified. Blood, organs and ectoparasites were collected. A total of 130 small mammals belonging to 10 different species were captured, of which 74 (56.92%) were infested by ectoparasites, including 248 *Lealaps echidnina* mites, 64 *Xenopsylla cheopis* fleas, 62 *Haemaphysalis* spp. and three *Amblyomma* spp. nymphs. Using molecular tools, the DNA of *Bartonella* was found in 23/130 (17.69%) of the small mammals, 33/94 (35.10%) of the mites and 24/64 (37.5%) of the fleas. Partial *gltA*, *ftsZ* and *rpoB* genes sequences of *Bartonella* DNA from small mammals were close to sequences of *Bartonella mastomydis*, *Bartonella elizabethae*, and uncultured *Bartonella* spp. *Coxiella burnetii* DNA was detected in 32/65 (49.23%) of ticks, 3/130 (2.3%) of small mammals and 1/94 (1.06%) of mites. We have found DNA of Anaplasmataceae in fleas and DNA of *Rickettsia africae* and *Ehrlichia ruminantium* in ticks. The results of our study show that several small mammal species could be considered as potential reservoirs of *Bartonella* spp., likely to play a major role in the maintenance, circulation and transmission of this pathogen in Mali. The pathogenicity of these bacteria for humans or animals remains to be demonstrated.

INTRODUCTION

There are nearly 2,277 known species of rodents belonging to 33 families, with distribution which is global with the exception of Antarctica and some isolated islands[1]. Small mammals are known to play a role in the maintenance and circulation of many zoonotic pathogens including bacteria such as *Yersinia pestis*, *Rickettsia* spp., *Bartonella* spp., *Borrelia* spp., *Leptospira* spp.; eukaryotes such as *Leishmania* spp., *Trypanosoma* spp.; and viruses such as *Hantavirus* and Lassa virus[2-9]. Transmission of infectious agents from small mammals to other animals, including humans, can occur directly through bites or contact with their excretions, or indirectly through arthropod vectors, such as fleas[10, 11].

Rat fleas are involved in the transmission and spread of several pathogens such as *Yersina pestis,* the agent of plague[12], *Rickettsia typhi,* the agent of murine typhus[13], and several *Bartonella* spp., including *Bartonella tribocorum*, *B. elizabethae*, *B. queenslandensis* and *B. Rochalimae*.[14] *Rickettsia felis,* an emerging agent of spotted fever rickettsiosis, has been identified in various fleas species, including rodent fleas (*Xenopsylla cheopis* and *X. brasiliensis*).[13] Soft ticks of *Ornithodoros* genus that infesting small mammals are known vectors of relapsing fever caused by *Borrelia crocidurae, B. duttonii* and *B. hispanica* which are responsible for a significant proportion of so-called fevers of unknown origin in tropical areas[14]. *Laelaps echidnina* is another very common ectoparasite in domestic rats and mice in most tropical, subtropical and temperate regions of the world but his vector role of *L. echidnina* is poorly known.[15]

In Mali, Western Africa, zoonoses involving rodents and insectivores or their ectoparasites have been poorly studied. However, twenty-eight species of small mammals including rodents and insectivores have been reported in Mali.[14, 16-18] A study showed that *Mastomys natalensis* rats from the village of Soromba carried a genetically unique strain of Lassa virus[19], and the seroprevalence of IgG against Lassa virus in humans was 44.0% in

Bamba, 41.0% in Soromba and 14.5% in Banzana.[20] Recently, LASV RNA was detected in blood samples from 2 febrile patients from Bamako in Mali.[21] A study conducted in 20 villages in southern Mali also reported the presence of *B. crocidurae*, by agent of tick-borne relapsing fever in West Africa, in rodents, insectivores and ticks collected rodent burrows.[17] Two other studies have reported the presence of *Borrelia* spp. in small mammals, and *Trypanosoma* spp. in rodents and rodent fleas collected in Mali.[18, 22] The objective of our study was to use molecular tools to detect bacteria and eukaryotes in small mammals and their ectoparasites in three locations in Mali and to assess the prevalence of these pathogens in different locations.

MATERIALS AND METHODS

Ethical considerations

This work was included in a protocol entitled "Investigation of prevalence, investigative sufficiency of emerging and re-emerging viral diseases and infectious causes of fever in Mali" which was reviewed and approved by the ethical committee of the Faculty of Medicine, Pharmacy and Dentistry, University of Science, Techniques and Technologies of Bamako (USTTB) under the number N° 2016/113/CE/FMPOS before starting the study. This study was conducted in three locations in Mali: Faladjè and its surroundings (13° 00'N-8°20'W and 13°08'N-8°20'W) which is located in a rural area, Bougouni (11°25'N-7 °29'W and 11 °25'N-7°28'W) which is located in a semi-urban areas, and the Bamako district (12°39'0N-8°0'0W), an urban area (Fig 1) between 11 and 18 December 2016. The village of Faladjè and the city of Bougouni are 77 km and 160 km from Bamako respectively. No protected animals were captured during this study.

Small mammals and their ectoparasites

The animals were captured alive using BTS-style metal mesh traps containing onion or peanut paste as bait. In the wild, the traps were arranged in a line of 20 traps for one or two nights with an inter-trap distance of 10 metres. One to two traps were placed inside granaries, warehouses and bedrooms.[22] The trapping was performed at night with traps installed in the evening and retrieved the next morning. All captured animals were morphologically identified, as previously described[23] and a catch number was assigned chronologically to each small mammal captured. For each trapped animal, a cardiac puncture was performed after the animal was killed by cervical dislocation or chloroform inhalation. Finally, the body of the animal was combed with a fine comb to dislodge and collect the different ectoparasites described above.[18] The liver, spleen and droppings of each animal were collected by laparotomy using dry aseptic scissors. Two drops of blood were put on Whatman blotting paper. Samples of blood and dung were kept at -20°C, Whatman paper with dried blood at room temperature with silica gel. The livers, spleens and ectoparasites were kept in alcohol at 70 ° C until they were transported to Marseille, France. Once there, ectoparasites (fleas, ticks and mites) were morphologically identified at the species and/or genus level the appropriate keys[24-26], using the microscope at a magnification of x56 (Zeiss Axio Zoom.V16, Zeiss, Marly le Roi, France).

Molecular analyses

DNA was extracted from 113 small cuts of spleen, 94 randomly selected mites, 65 ticks, 64 fleas and 17 filter papers containing dried blood. All samples were individually incubated at 56°C overnight in 1.5 ml tubes containing 200 µL G2 lysis buffer and 20 µL proteinase K (Qiagen, Hilden, Germany). After centrifugation at 1000 rpm for 30 seconds, 200ul of the supernatant was used to extract the DNA using the EZ1 DNA Tissue Kit (Qiagen), according

to manufacturer's recommendations. The DNA from each sample was eluted with 100 µL of Tris-EDTA (TE) buffer (Qiagen) and was either immediately used or stored at -20°C until use.

Quantitative PCRs were performed to individually screen all samples using previously reported primers and probes for *Rickettsia* spp., *Borrelia* spp., *Bartonella* spp., Anaplasmataceae, *Coxiella burnetii*, *Leishmania* spp. and *Leptospira* spp.[27, 28] All the sequences of primers and probes as well as their respective sources used in this study are presented in Table 1. The various qPCRs were carried out using a CFX96 Real-Time system (Bio-Rad, Marnes-la-Coquette, France) and the Light Cycler[R] 480 Probes Master mix (Indianapolis, USA). The DNA of *Rickettsia montanensis*, *Bartonella elizabethae*, *Anaplasma phagocytophilum*, *Coxiella burnetii*, *Borrelia crocidurae*, *Leishmania infantum* and *Leptospira inadai* were used as a positive control. The mixture without DNA was used as negative control for each test. The samples were considered to be positive when the cycle threshold (Ct) was below 35 Ct.

Samples which were positive for *C. burnetii* for the IS30a gene were subjected to a second qPCR using the IS1111 gene to confirm the results [27] and samples positive for *Rickettsia* spp. were then subjected to a specific qPCR system for the detection of *R. africae*. [29] Samples which were positive for *Bartonella* spp. in qPCR were subjected to standard PCR followed by sequencing for species identification using primers allowing the amplification of 850 bp, 292 bp and 200 bp fragments of the *Bartonella rpoB*, *ftsZ* and *gltA* genes respectively. [30] The partial sequences of the *gtA*, *ftsZ* and *rpoB* genes of *Bartonella* spp. obtained were used for the construction of phylogenetic trees. Tick sample that were positive using 23S rRNA Anaplasmataceae qPCR were subjected to a specific qPCR targeting the 23S rRNA gene of *Wolbachia* spp. These samples were also subjected to standard PCR using primers amplifying a 485bp fragment of the 23S rRNA encoding gene and 310 bp fragment of the 16S *Ehrlichia*

rRNA gene of Anaplasmataceae followed by sequencing.[31] All ticks and fleas that tested positive for *Bartonella* spp. were subjected to standard PCR and sequencing to determine the species using 16S ticks and *ITS2* genes respectively.[29,32] The sequences obtained were assembled and analyzed using the ChromasPro software (version 1.34) (Technelysium Pty. Ltd., Tewantin, Australia), and were then blasted against GenBank (http://blast.ncbi.nlm.nih.gov). The partial nucleotide sequences of the *gltA*, *ftsZ* and *rpoB* gene of *Bartonella* spp. and 23S Anaplasmataceae and 16S *Ehrlichia* rRNA gene obtained in this study were deposited in the NCBI GenBank database.

Phylogenetic analysis

The sequences of our study and those extracted on GenBank have been aligned with CLUSTALW for multi-sequence alignment using Bioedit software. The aligned sequences were submitted TOPALi software 2.5 (Biomathematics and Statistics Scotland, Edinburgh, United Kingdom) for the selection of the best tree model. The phylogenetic trees were performed using the software TOPALi 2.5 by choosing the proposed model. Node numbers are percentages of the bootstrap values obtained by repeating the analysis of 100 repetitions to generate a majority consensus tree (only those with a value equal to or greater than 80 have been retained).

RESULTS

Small mammal sampling

Overall, 130 small mammals including nine different species were captured in the three localities (Fig1). Of these, 75/130 (57.70%) were captured at Faladjè, 48/130 (36.92%) at Bougouni and 7/130 (5.38%) at Bamako. Five species of rodents and one insectivore were captured in Faladjè (*Crocidura cf olivieri*, *Gerbilliscus gambianus*, *Mastomys erythroleucus*, *Mastomys natalensis*, *Praomys daltoni* and *Taterillus gracilis*), two species of insectivores

and four other rodents in Bougouni (*Atelerix cf albiventris*, *Crocidura* spp., *M. erythroleucus*, *M. natalensis*, *P. daltoni* and *Rattus rattus*) and two species in Bamako respectively 1 insectivore and 1 rodent (*Crocidura* spp., *Rattus norvegicus*) (Fig 1). *M. erythroleucus* was the most abundant species with 44.61% (58/130), followed by *M. natalensis* with 20% (26/130), *R. rattus* with 13.85% (18/130) and other species with 21.54% (28/130). *C. cf olivieri*, *G. gambianus* and *T. gracilis* were found only in Faladjè, *A. cf albiventris* and *R. rattus* only in Bougouni, and *R. norvegicus* in Bamako.

From 130 trapped small mammals, 130 livers, 113 spleens, 109 blood samples, 26 droppings and 123 Whatman paper with dried blood samples were collected (Table 2). Seventy-two rodents (55.33%) and two insectivores (1.54%) were infested with ectoparasites. A total of 377 ectoparasites, including 248 *Lealaps echidnina* mites, 64 *Xenopsylla cheopis* fleas, 62 *Haemaphysalis* spp. nymph ticks and three *Amblyomma* spp. nymph ticks (Table 2) were identified.

Molecular analysis

- **Detection of pathogens**

Bartonella DNA was detected by qPCR in 23/130 (17.69%) of the small mammals organs, 33/94 (35.10%) of the mites and 24/64 (37.5%) of the fleas. In small mammals, the prevalence of *Bartonella* DNA was 14/23 (60.87%), 6/23 (26.07%) and 3/23 (13.04%) in Faladjè, Bougouni and Bamako respectively. *Bartonella* DNA was found in 12/23 (52.17%) of *M. erythrolucus*, 6/23 (26.08%) of *M. natalensis*, 3/23 (13.04%) of *R. norvegicus*, 1/23 (4.34%) of *C. cf olivieri* and *P. daltoni*. For ectoparasites *Bartonella* DNA was detected in 31/33 (93.94%) and 2/33 (6.06%) in the mites at Faladjè and Bougouni respectively: it was 58.33% (14/24) and 41.67% (10/24) in these two localities. A total of 9/33 (27.27%) mites

193 and 11/24 (45.83%) fleas positive for *Bartonella* sp. were collected from positive small
194 mammals.

195 Standard PCR prior to sequencing the 23 rodent DNA samples that were positive for
196 *Bartonella* spp. qPCR provided 23, 21 and 18 informatif partial sequences for *gltA*, *ftsZ* and
197 *rpoB* genes respectively.

198 The BLAST analysis of the 23 *Bartonella gltA* gene sequences allowed us to obtain a total of
199 six groups of different sequences. The first group consisted of nine identical sequences from
200 nine different samples (*Bartonella* sp. Mali1) sharing 98.64% identity with *B. mastomydis*
201 detected in *M. erythroleucus* from Senegal (KY555066). The second group consisted of five
202 identical sequences (*Bartonella* sp. Mali2) which had 94.50% identity to *Bartonella* sp.
203 B28303 detected in insectivore *C. olivieri* from Kenya (KM233490), the closest described
204 species being *B. acomydis* (AB444979; 93.15% identity). The third group comprised three
205 identical sequences (*Bartonella* sp. Mali 3) which were 99.09% identical to "Uncultivated
206 *Bartonella* sp." detected in *R. rattus* from Uganda (KC763960), with the closest *B.*
207 *mastomydis* of the known species (KY555066; 97.74% identity). The fourth group consisted
208 of 3 identical sequences (*Bartonella* sp. Mali4) with 96.82% identity with *Bartonella* sp.
209 RT91YN detected in rats from China (FJ492788) with the closest known species, *B.*
210 *coopersplainsensis* (HQ444160, identity at 95.45%). The fifth group also included two
211 identical sequences (*Bartonella* sp. Mali 5) which were 100% identical with "Uncultured
212 *Bartonella* sp." detected in *Xenopsylla cheopis* from Uganda (MF443365) with the closest
213 known species being *B. taylorii* (AB779517; 94.97% identity). Finally, the sixth group
214 contained one sequence (*Bartonella* sp. Mali6) which had 100% identity with *Bartonella* sp.
215 B28303, detected in insectivore *C. olivieri* from Kenya (KM233490), the closest described
216 species being *B. florencae* (HM622142; 97.48% identity) (Table 3). The phylogenetic
217 position of the *Bartonella* species identified by *gltA* gene in our study is presented in Fig 2.

The sequences of the *gltA* gene obtained in this study were deposited in the GenBank database under following access numbers: MK902921, MK902922, MK902923 and MK902924 (S1 Table).

Similarly, for the 21 sequences of the *ftsZ* gene, BLAST analysis showed that these sequences formed six different groups. The first group consisted of six identical sequences (*Bartonella* sp. Mali1) from six different samples which were 100% identical with *B. mastomydis* (KY555065) detected in *M. erythroleucus* from Senegal. The second group consisted of five identical sequences (*Bartonella* sp. Mali2) was 92.49% identical to *B. florencae* (HM622141), detected in *Crocidura russula* from France. The third group consisted of three identical sequences (*Bartonella* sp. Mali3) with 98.97% identity with *B. mastomydis* (KY555065) detected in *M. erythroleucus* from Senegal. The fourth group consisted of two sequences (*Bartonella* sp. Mali4) which were 97.94% identical with *Bartonella* sp. (KJ361691) detected in rodent from China, and *B. japonica* (AB440633) detected in *Apodemus argenteus* from Japan. The fifth group consisted of two sequences (*Bartonell*a sp. Mali5) which were 99.31% identical with *B. mastomydis* (KY555065) detected in *M. erythroleucus* from Senegal. The sixth group consisted of three sequences (*Bartonella* sp. Mali6) that were 100% identical with *B. elizabethae* (AF467760) (Table 3). The phylogenetic position of the *Bartonella* species identified by *ftsZ* gene in our study is presented in Fig 3. The sequences of the *ftsZ* gene obtained in this study were deposited in the GenBank database under following access numbers: MK892984, MK892985, MK892986 and MK892987.

For the 18 sequences of the *rpoB* gene, the BLAST analysis showed that they formed six distinct groups, the first group of which consisted of five sequences (*Bartonella* sp. Mali1) which were 99.65% with uncultivated *Bartonella* sp. (JQ425631), detected in *Stenocephalemys albipes* from Ethiopia nearest known species of which was *B. mastomydis* (KY555068; identity 99.17%). The second group, consisted of three sequences (*Bartonella* sp.

Mali2) and was 97.10% identical to *Bartonella* sp. (KT881102) detected in the spleen of *Mastomys* sp. from Benin, with the nearest known species being *B. mastomydis* (KY555068; identity 96.17%). The third group consisted of three sequences (*Bartonella* sp. Mali3) which were 99.18% identical with uncultivated *Bartonella* sp. (JQ425631), detected in *Stenocephalemys albipes* from Ethiopia with *B. mastomydis* as the closest known species (KY555068; identity 98.59%). The fourth group contained two sequences (*Bartonella* sp. Mali4) which were at 97.53% identical to uncultivated *Bartonella* sp. (JQ425631) detected in *S. albipes* from Ethiopia with *B. mastomydis* as the closest known species (KY555068; identities 97.29%). The fifth group also consisted of two sequences (*Bartonella* sp Mali5) were 99.66% identical to uncultivated *Bartonella* sp. (GU143503) detected in *R. rattus brunneusculus* from Nepal with *B. mastomydis* as the closest known species (KY555068; identity 99.06%). Finally, the sixth group consisted of three sequences (*Bartonella* sp. Mali6) which were 100% identical to *B. elizabethae* (JX158367) (Table 3). The phylogenetic position of the *Bartonella* species identified by *rpoB* gene in our study is presented in Fig 4. The sequences of the *rpoB* gene obtained in this study were deposited in the GenBank database under following access numbers: MK913650, MK913651, MK913652 and MK913653 (S1 Table).

The phylogenetic position of the *Bartonella* species identified in our study is presented in Fig 5 in a concatenated phylogenetic tree made from the genes *gltA* (200 bp), *ftsZ* (292 bp) and *rpoB* (850 bp) for the samples for which we have the sequences of the three genes.

Not all arthropod specimens (mites and fleas) that were qPCR-positive for *Bartonella* spp. yielded exploitable sequences after standard PCR and sequencing because they contained approximately 15-20% double peaks.

In Faladjè, *C. burnetii* DNA was detected in 49.23% (32/65) of ticks, 2.30% (3/130) of rodents (2 *M. erythroleucus* and 1 *P. daltoni*) and 1.06% (1/94) of mites using both genes. All ticks positive in *C. burnetii* were collected in one rodent infected by *C. burnetiid* and only one rodent was co-infected with *Bartonella* sp. and *C. burnetii*.

Anaplasmataceae DNA was found in 15.62% (10/64) of the *X. cheopis* fleas and 33.33% (1/3) of *Am. variegatum* by qPCR targeting the 23S rRNA Anaplasmataceae gene (Table 3). All samples of fleas were positive using the 23S *Wolbachia* gene by qPCR. Conventional PCR and sequencing using Anaplasmataceae 23S primers showed that all the sequences (named *Wolbachia* sp. Mali Fleas) obtained were similar to one another and were 95.56% identical to the "*Candidatus* Wolbachia ivorensis strain TCI113" detected in *Rhipicephalus microplus* ticks from Côte d'Ivoire (KT364329). Using the 16S *Ehrlichia* gene, the BLAST analysis showed that the sequences were 99.34% identical to the *Wolbachia* endosymbiont of the *Pentalonia nigronervosa* clone PnGu1 (KJ786950) reference sequences. The BLAST analysis of sequence obtained from tick positive after conventional PCR and sequencing using Anaplasmataceae 23S primers showed that was 98.95% identical *Ehrlichia ruminantium* (NR_077002). The sequences of the 23S rRNA Anaplasmataceae and 16S *Ehrlichia* gene obtained in this study were deposited in the GenBank database under following access numbers: MK911751, MK911752, MK911753, MK911754, MK911755, MK911756, MK911757, MK920301, MK920302, MK920303, MK920304, MK920305, MK920306 and MK920307 (S1 Table).

Two ticks (*Am. variegatum*) were positive for Rickettsia spp. and have been confirmed as *R. africae* by specific qPCR. All samples that were tested were negative for *Borrelia* spp., *Leptospira* spp., and *Leishmania* spp.

- **Identification of ectoparasites**

The BLAST analysis of sequences obtained from fleas showed that all were 98.26% identical to *X. cheopis* (KX982860). For the 65 ticks subjected to standard PCR and sequencing we obtained 56 sequences of good quality. The BLAST analysis showed that 39 sequences were 99.12 to 92.75 % identical to *Hae. histricis* (LT593110, KC170733), 12 sequences were 99.62% identical to *Rh. sanguineus* (KT382448), three sequences were 99.75% identical to *Am. variegatum* (KU130401) and two were 94.62% identical to *Rh. congolensis* (MK894257).

Discussion

This study is the first molecular survey of the prevalence of bacteria in small mammals and their ectoparasites in Mali. We morphologically identified 7 different species of rodents and two of insectivore. All these species of small mammals had already been described in Mali.[14, 16-18] In this study, *M. erythroleucus* was the most abundant species with 44.61% (58/130) followed by *M. natalensis* with 20% (26/130). However, in the previous study in Mali the most prominent species was *M. natalensis* followed by either *M. erythroleucus* or by *C. Olivieri*.[17, 22] These results contribute towards an increase in the repertoire of rodent species identified in Mali.

More than half of the small mammals (56.92%) were infested with ectoparasites, from which we morphologically identified *L. echidnina*, *X. cheopis*, *Haemaphysalis* spp. nymphs and *Amblyomma* spp. nymphs. *Laelaps echidnina* are well known as ectoparasites of domestic rodents in most tropical, subtropical and temperate regions.[15] *Laelaps echidnina* cannot break the intact skin of the host, but most often attacks previously abraded or damaged areas in order to soak up blood. However, it is able to attack humans. In rats, *L. echidnina* is able to transmit the apicomplexan *Hepatozoon muris* and the Junin virus.[15] *Xenopsylla cheopis* fleas are cosmopolitan because of their main association with commensal rats, including the

Norwegian rat (brown), *R. norvegicus*, and the black rat *R. rattus*.[33] It is widespread in Africa and includes an East African subspecies parasitizing small mammals in domestic habitats.[33, 34] *Xenopsylla cheopis* has been found in many countries of West and North Africa[18, 35], including Mali.[18] In Mali, in addition to *X. cheopis*, *X. nubica*, which lives in desert and semi-desert environments, has also been found in domestic rats, with multiple specimens (*M. natalensis*) living in human dwellings.[18] However in this study we found no *X. nubica*.

We also found immature stages (nymphs) identified morphological as *Haemaphysalis* spp. and *Amblyomma* spp. ticks on small mammals. However, molecular biology has allowed us to identify these ticks as *Am. variegatum, Rh. sanguineus*, *Rhipicephalus* sp. and *Haemaphysalis* sp. among which the first two species have already been found in Mali.[29] Although these ticks were not identified to the species level, ticks belonging to these genera have already been described as feeding on rodents.[36, 37]

Bartonella spp. are gram-negative bacteria that parasitize erythrocytes and endothelial mammalian hosts that can infect a variety of domestic and wild mammal hosts, including rodents, insectivores, rabbits, cats, dogs, humans and bats.[38] Many species have been detected in arthropods such as sand flies, lice, ticks and fleas, although these arthropods' role as vectors has not been definitively demonstrated for most of them.

Many *Bartonella* species such as *B. rattimassiliensis*, *B. tribocorum*, *B. grahamii, B. birtlesii, B. acomydis, B. rattaustraliani, B. phoceensis*, *B. doshiae*, *B. tamiae*, *B. elizabethae*, *B. queenslandensis*, *B. silvatica, B. japonica, B. coopersplainsensis, B. vinsonii subsp. yucatanensis, B. vinsonii subsp. arupensis, B. taylorii, B. rochalimae*, *B. henselae* and *B. clarridgeiae* have been associated with small mammals.[39, 40] Of these, some are responsible for diseases in humans such as *B. tamiae*, *B. elizabethae*, *B. rochalimae*, *B.*

henselae, *B. clarridgeiae*, *B. grahamii* and *B. vinsonii subsp. Arupensis*.[39] The pathogenetic role of other species remains unknown in humans.

In our study *Bartonella* spp. DNA was found in 17.69% of small mammals, 35.10% of mites and 37.5% of fleas by qPCR. Analysis of the DNA sequences obtained with the *gltA*, *ftsZ* and *rpoB* genes showed us that these sequences could be divided into six groups. The first group was close to *B. mastomydis*. *B. mastomydis* was discovered for the first time in rodents of the genus *Mastomys* in Cotonou, Benin.[41] It has also recently been identified in *M. erythroleucus* rodents in the region of Sine-Saloum in Senegal, a country neighbouring Mali.[5] For the first time, we identified *B. mastomydis* in *M. erythroleucus* and *M. natalensis* rodents in Mali.

The identity percentages of *Bartonella* sp. Mali2 with the sequences available in GenBank were 94.57%, 92.49% and 97.10% for the genes *gltA*, *ftsz* and *rpoB* respectively, and those of *Bartonella* sp. Mali4 were 96.82%, 97.94% and 97.53% respectively for same gene. *Bartonella* sp Mali2 and Mali may be potential new species of *Bartonella* detected in rodents in Mali. The third and fifth groups were close to uncultivated *Bartonella* sp., previously detected in rodents and fleas. The sixth group was close to *B. elizabethae*, known to be a human pathogen commonly associated with rats of the genus *Rattus*.[42] *Bartonella elizabethae* causes infections in humans that have been associated with endocarditis and neuroretinitis.[43] *Bartonella elizabethae* has already been reported in small mammals and fleas in previous studies.[41, 42, 44, 45] For the first time, we report *B. elizabethae* in *R. norvegicus* from Mali. Sequences obtained from mite and flea specimens that were positive for *Bartonella* sp. by qPCR were not exploitable because they had a high double peak percentage. This may be due to the fact that these samples are co-infected by two different species of *Bartonella* sp. The presence of *Bartonella* sp. in other rodent mite species has already been reported in Africa[46], Asia[47, 48] and America.[49] Our study is the first to report the molecular presence of *Bartonella* sp. DNA in *L. echidnina* mites of small mammals in West Africa. The possible role of small

mammal mites in the transmission of bartonellosis should be considered by physicians and entomologists. Similarly, the presence of *Bartonella* sp. in fleas collected from small mammals has also been reported by previous studies.[50, 51] In Mali, *B. quintana* has already been found in head lice[52], although we report for the first time the presence of *Bartonella* sp. in fleas from Mali.

Coxiella burnetii is the agent of Q fever, which is a strict gram-negative intracellular bacterium that infects a large number of animals, from arthropods to humans. It is an airborne zoonosis transmitted to humans mainly by aerosols generated by exposure to infected placentas and the birth fluids of animals infected with Q fever. Ticks may also act as vectors. Typically asymptomatic in animals, *C. burnetii* infection may result in miscarriage, low offspring or infertility.[53] In humans, *C. burnetii* may be the cause of non-specific febrile illness, pneumonia, hepatitis, endocarditis and vascular infection.[53] We detected *C. burnetii* DNA in 49.23% (32/65) of ticks 2.30% of rodents (2 *M. erythroleucus* and 1 *P. daltoni*) and 1.06% of mites collected from rodents. In Mali, recent studies have reported the presence of *C. burnetii* in head lice and ticks[29, 52], but our study is the first to report the presence of *C. burnetii* in rodents and fleas collected from rodents, whose role in the epidemiology of Q fever is unknown. *Coxiella burnetii* has already been detected in fleas collected from rats, foxes and hares captured in different areas of Cyprus.[54]

Rickettsia africae and *E. ruminantium* transmitted primarily by ticks of the genus *Amblyomma* are the agent of African tick bite fever (ATBF) in humans and cowdriosis in cattle, respectively.[55, 56] We detected the DNA of *R. africae* and *E. ruminantium* in *Am. variegatum* nymphs. The presence of these bacteria has been reported in *Am. variegatum* ticks from Mali.[29, 57]

Bacteria of the genus *Wolbachia* are gram-negative endosymbiotic proteobacteria that appear to be intracellular endosymbionts located in the reproductive tissues of many arthropods and nematodes [58]. These bacteria are transmitted vertically from parents to their descendants and can alter the biology of the host by acting on feminisation, pathogenesis, killing of males and incompatibilities of spermatozoa.[58] The first *Wolbachia* species, named *W. pipientis,* was detected in *Culex pipiens* [59]. *Wolbachia* spp. have been detected in many arthropods including *Diptera*, *Lepidoptera*, *Hymenoptera*, *Coleoptera*, *Phthiraptera*, *Hemiptera*, *Thysanoptere*, *Blattaria*, *Isoptera*, *Orthoptera*, *Odonata*, *Oribatida*, *Prostigmata*, *Ixodida*, *Mesostigmata*, *Araneae*, *Scorpiones*, *Amphipoda*, and *Isopoda*.[60] In Mali, the presence of *Wolbachia* sp. has been reported in natural mosquito populations including malaria vectors such as *Anopheles gambiae* and *Anopheles coluzzii* mosquitoes.[61] In our study, 15.62% of the fleas were positive for *Wolbachia* sp. DNA. The presence of *Wolbachia* had already been reported in rodent fleas from Israel[62] and dog fleas from Spain.[63] We have reported, for the first time, the presence of *Wolbachia* sp. in small mammal fleas from Mali.

Conclusion

In summary, we have identified several small mammal species that can be considered as potential reservoirs of *Bartonella* spp. in Mali. These small mammals were infested by several species of arthropods, some of which are known to be disease vectors and others which could be studied extensively to understand their role in disease transmission. The results of our studies show that are likely to play a major role in the maintenance, circulation and transmission of *Bartonella* spp. or could simply be dead-end hosts that play little or no role in the transmission cycles. This data should draw the attention of doctors, veterinarians, biologists and public health authorities to the possibility of the existence of human bartonellosis in Mali and the establishment of diagnostic and monitoring strategies.

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Figure 1. Map of Mali showing small mammals trapping sites and percentages of animals captured by site.

Source: https://fr.wikipedia.org/wiki/Mali#/media/File:Mali_(orthographic_projection).svg; https://fr.wikipedia.org/wiki/Fichier:Mali_cercles.png

Figure 2: A consensus phylogenetic tree showing the relationships of the *Bartonella* species studied in this study based on a portion of *gltA* gene sequence comparison.

The GenBank accession numbers (or the only accession number of the genome) are indicated when the sequences come from Genbank at the beginning and the host names at the end. The sequences were aligned using ClustalW and phylogenetic inferences were obtained using Bayesian phylogenetic analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) within the integrated maximum likelihood application using the model K81uf I Γ. Nodes at nodes are percentages of bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree. Bootstrap values less than 80 have been removed from the final tree.

Figure 3: A consensus phylogenetic tree showing the relationships of the *Bartonella* species studied in this study based on a portion of *ftsZ* gene sequence comparison.

The GenBank accession numbers (or the only accession number of the genome) are indicated when the sequences come from Genbank at the beginning and the host names at the end. The sequences were aligned using ClustalW and phylogenetic inferences were obtained using Bayesian phylogenetic analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) within the integrated maximum likelihood application using the

model K81uf I Γ. Nodes at nodes are percentages of bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree. Bootstrap values less than 80 have been removed from the final tree.

Figure 4: A consensus phylogenetic tree showing the relationships of the *Bartonella* species studied in this study based on a portion of *rpoB* gene sequence comparison.

The GenBank accession numbers (or the only accession number of the genome) are indicated when the sequences come from Genbank at the beginning and the strain or the taxon number at the end. The sequences were aligned using ClustalW and phylogenetic inferences were obtained using analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) within the integrated maximum likelihood application using the model K81uf I Γ. Nodes at nodes are percentages of bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree. Bootstrap values less than 80 have been removed from the final tree.

Figure 5. Concatenated phylogenetic tree showing the relationships of the Bartonella species studied on the basis of a concatenated sequence of the *Bartonella gltA*, *ftsZ* and *rpoB* genes fragment.

. GenBank accession numbers are shown at the beginning. The concatenated sequences of *gltA*, *ftsz* and *rpoB* gene were aligned using Clustalw Multiple Alignment using Bioedit software and phylogenetic inferences obtained Bayesian model phylogenetic analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) using the substitution model K81uf + I + Γ.

660 Table 1: **Primers and probes used for real time PCRs and conventional PCRs in this**
661 **study**

Target	Gene name	Primers (5'-3') and probe (6FAM–TAMRA)	References
quantitative real-time PCRs(qPCRs)			
Rickettsia spp.	*gltA* ("RKND03")	F_GTGAATGAAAGATTACACTATTTAT	27
		R_GTATCTTAGCAATCATTCTAATAGC	
		6FAM-CTATTATGCTTGCGGCTGTCGGTTC-TAMRA	
Anaplasmataceae	23S rRNA	F_TGACAGCGTACCTTTTGCAT	31
		R_GTAACAGGTTCGGTCCTCCA	
		6FAM-CTTGGTTTCGGGTCTAATCC-TAMRA	
Borrelia spp.	*ITS4* ribosomal intergenic spacer	F_GGCTTCGGGTCTACCACATCTA	27
		R_CCGGGAGGGGAGTGAAATAG	
		6FAM-TGCAAAAGGCACGCCATCACC-TAMRA	
Bartonella spp.	*ITS 2* ribosomal intergenic spacer	F_GGGGCCGTAGCTCAGCTG	27
		R_TGAATATATCTTCTCTTCACAATTTC	
		6FAM- CGATCCCGTCCGGCTCCACCA-TAMRA	
Coxiella burnetii	*IS30a*	F_ CGCTGACCTACAGAAATATGTCC	27
	Intergenic spacer	R_ GGGGTAAGTAAATAATACCTTCTGG	
		6FAM-CATGAAGCGATTTATCAATACGTGTATGC-TAMRA	
Coxiella burnetii	*IS1111*	F_CAAGAAACGTAACGCTGTGGC	27
	Intergenic spacer	R_CACAGAGCCACCGTATGAATC	
		6FAM- CCGAGTTCGAAACAATGAGGGCTG-TAMRA	
Leishmania spp.	Leishk	F_CTTTCTGGTCCTCCGGGTAGG	28
		R_CCACCCGGCCCTATTTTACACCAA	

		FAM-TTTCGCAGAACGCCCCTACCCGC-TAMRA	
Leptospira spp.	lipL32	F_AGAGGTCTTTACAGAATTTCTTTCACTACCT	64
		R_TGGGAAAAGCAGACCAACAGA	
		6FAM-AAGTGAAAGGATCTTTCGTTGC-TAMRA	
Wolbachia spp.		F_CCAAAATTACAGCTAAGTGG	Unpublished
		R_AGTGAGCTGTTACGCTTTCT	
		6FAM-TACAGCTAGGAGGTTGGCTT-TAMRA	
Standard PCR			
Anaplasmataceae	23S rRNA gene	F_ATAAGCTGCGGGGAGTTGTC	31
		R_TGCAAAAGGTACGCTGTCAC	
	gltA	F_ACGTCGAAAAGAYAAAAATG	
		R_GTAATRCCAGAAATARAAATC	
	ftsZ	F_CCGTGAATAATATGATTAATGC	
		R_TTGAAATGGCTTTGTCACAAC	30
Bartonella spp.		1400F_CGCATTGGCTTACTTCGTATG	
		2028F_GGAAAATGATGATGCGAATCGTGC	
	ropB	1596R_GGACAAATACGACCATAATGCG	
		1873R_TCYTCCATMGCWGAMAGATAAA	
		2300R GTAGACTGATTAGAACGCTG	
Erlichia spp.	16S rRNA	F_GGTACCYACAGAAGAAGTCC	57
		R_TAGCACTCATCGTTTACAGC	
Fleas	*ITS2* ribosomal inter genic spacer	F_GGG TCG ATG AAG AAC GCA GC	32
		R_TTT AGG GGG TAG TCT CAC CTG	

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664 **Table 2:** The number of ectoparasites and organs collected from small mammals in the
665 different sites studied in Mali

		Faladjè	Bougouni	Bamako	Total
Ectoparasites	*Lealaps echidnina*	192	56	0	248
	Xenopsylla cheopis	22	42	0	64
	Haemaphysalis spp. (nymph)	53	9	0	62
	Amblyomma spp. (nymph)	0	3	0	3
Organs	Blood	65	44	0	109
	Liver	75	48	7	130
	Spleen	59	47	7	113
	Dropping	23	13	0	26
	Whatman paper with dried blood	75	48	0	123

666

667 **Table 3:** *Bartonella* spp. identified with the closest known species using three different genes in small mammals caught in Mali.

Gene	*gltA*						
	Small mammal species positive for *Bartonella*	**Closest GenBank match**	**% Similarity**	**Accession number**	**Hosts**	**% Similarity of Closest Known Bartonella**	**Accession number**
Bartonella sp. Mali1	*M. natalensis*, *M. erythroleucus*, *R. norvegicus*	*B. mastomydis*	98.64%	KY555066	*Mastomys erythroleucus*		
Bartonella sp. Mali2	*M. erythroleucus*, *P. daltoni*, *M. natalensis*	*Bartonella*s sp.	94.57%	KM233490	*Crocidura olivieri*	93.15% *B. acomydis*	AB444979
Bartonella sp. Mali 3	*M. erythroleucus*	Uncultivated *Bartonella* sp	99.09%	KC763960	*Rattus rattus*	97.74% *B. mastomydis*	KY555066
Bartonella sp. Mali4	*M. natalensis*	*Bartonella* sp.	96.82%	FJ492788	Rats	95.45% *B. coopersplainsensis*	HQ444160
Bartonella sp. Mali 5	*M. erythroleucus*	Uncultured *Bartonella* sp	100%	MF443365	*Xenopsylla cheopis*	94.97% *B. taylorii*	AB779517
Bartonella sp. Mali6	*Crocidura cf olivieri*	*Bartonella* sp.	99.10%	KM233490	*Crocidura olivieri*	97.48% *B. florencae*	HM622142

668

Gene	*ftsZ*						
	Small mammal species positive for *Bartonella*	**Closest GenBank match**	**% Similarity**	**Accession number**	**Hosts**	**% Similarity of Closest Known *Bartonella***	**Accession number**
Bartonella sp. Mali1	*M. natalensis, M. erythroleucus*,	*B. mastomydis*	100	KY555065	*Mastomys erythroleucus*		
Bartonella sp. Mali2	*M. erythroleucus*, *P. daltoni*, *M. natalensis*	*B. florencae*	92.49%	HM622141	*Crocidura russula*		
Bartonella sp. Mali3	*M. erythroleucus*	*B. mastomydis*	98.63%	KY555065	*Mastomys erythroleucus*		
Bartonella sp. Mali4	*M. natalensis*	*Bartonella* sp.	97.95%	KJ361691	Rodent	*B. japonica*	AB440633
*Bartonell*a sp. Mali5	*M. erythroleucus*	*B. mastomydis*	99.31%	KY555065	*Mastomys erythroleucus*		
Bartonella sp. Mali6	*R. norvegicus*	*B. elizabethae*	100%	AF467760	not available		

669

Gene	*rpoB*						
	Small mammal species positive for *Bartonella*	**Closest GenBank match**	**% Similarity**	**Accession number**	**Hosts**	**% Similarity of Closest Known *Bartonella***	**Accession number**
Bartonella sp. Mali1	*M. natalensis, M. erythroleucus*,	uncultivated *Bartonella* sp	99.65%	JQ425631	*Stenocephalemys albipes*	99.17% *B. mastomydis*	KY555068
Bartonella sp. Mali2	*M. erythroleucus*, *P. daltoni*, *M. natalensis*	*Bartonella* sp	97.10%	KT881102	*Mastomys* sp.	96.17% *B. mastomydis*	KY555068
Bartonella sp. Mali3	*M. erythroleucus*	uncultivated *Bartonella* sp	99.18%	JQ425631	*Stenocephalemys albipes*	98.59% *B. mastomydis*	KY555068
Bartonella sp. Mali4	*M. natalensis*	uncultivated *Bartonella* sp.	97.53%	JQ425631	*Stenocephalemys albipes*	97.29% *B. mastomydis*	KY555068
Bartonella sp Mali5	*M. erythroleucus*	uncultivated *Bartonella* sp.	99.66%	GU143503	*Rattus rattus brunneusculus*	99.06% *B. mastomydis*	KY555068
Bartonella sp. Mali6	*R. norvegicus*	*B. elizabethae*	100%	JX158367			

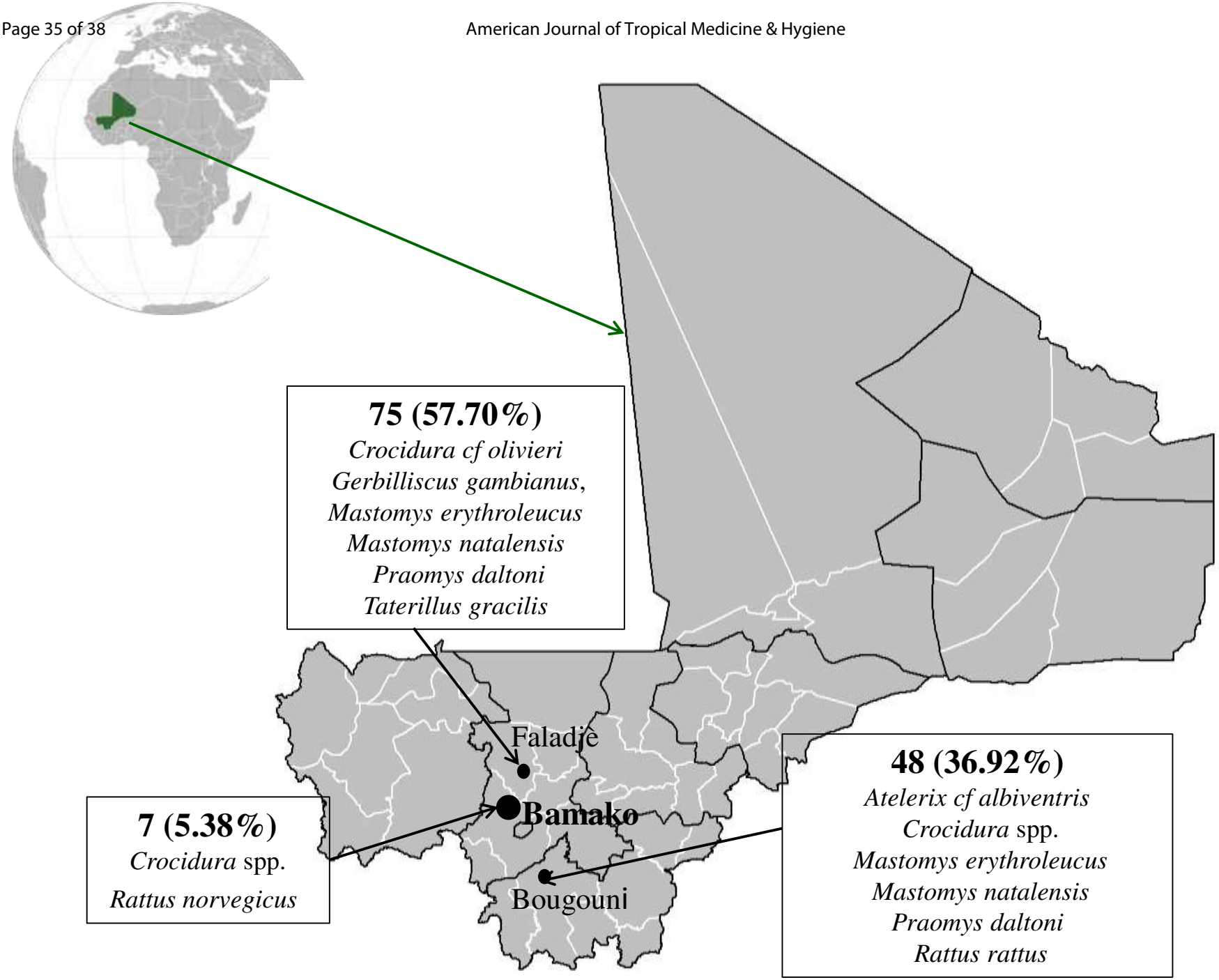
670

671 **S1 Table:** Description of the sequences deposited in the GenBank database with accession
672 numbers, genes, bacterial name and host.

Accession numbers	Genes	Bacterial names	Host
MK902921	*gltA*	*Bartonella* sp.	*Mastomys natalensis*
MK902922	*gltA*	Uncultured *Bartonella* sp.	*Mastomys erythroleucus*
MK902922	*gltA*	*Bartonella* sp.	*M. erythroleucus*
MK902924	*gltA*	*Bartonella* sp.	*M. natalensis*
MK892984	*ftsZ*	uncultured *Bartonella* sp.	*M. erythroleucus*
MK892985	*ftsZ*	uncultured *Bartonella* sp.	*M. erythroleucus*
MK892986	*ftsZ*	*Bartonella* sp.	*Paomys daltoni*
MK892986	*ftsZ*	*Bartonella mastomydis*	*M. natalensis*
MK913650	*rpoB*	uncultured *Bartonella* sp.	*M. erythroleucus*
MK913651	*rpoB*	uncultured *Bartonella* sp.	*M. erythroleucus*
MK9136552	*rpoB*	*Bartonella* sp.	*Paomys daltoni*
MK913653	*rpoB*	*Bartonella mastomydis*	*M. natalensis*
MK911751	23S Anaplasmataceae	*Wolbachia* sp.	*Xenopsylla cheopis*
MK911752	23S Anaplasmataceae	*Wolbachia* sp.	*X. cheopis*
MK911753	23S Anaplasmataceae	*Wolbachia* sp.	*X. cheopis*
MK911754	23S Anaplasmataceae	*Wolbachia* sp.	*X. cheopis*
MK911755	23S Anaplasmataceae	*Wolbachia* sp.	*X. cheopis*
MK911756	23S Anaplasmataceae	*Wolbachia* sp.	*X. cheopis*
MK911757	23S Anaplasmataceae	*Wolbachia* sp.	*X. cheopis*
MK920301	16S *Ehrlichia*	*Wolbachia* sp.	*X. cheopis*
MK920302	16S *Ehrlichia*	*Wolbachia* sp.	*X. cheopis*

MK920303	16S *Ehrlichia*	*Wolbachia* sp.	*X. cheopis*
MK920304	16S *Ehrlichia*	*Wolbachia* sp.	*X. cheopis*
MK920304	16S *Ehrlichia*	*Wolbachia* sp.	*X. cheopis*
MK920306	16S *Ehrlichia*	*Wolbachia* sp.	*X. cheopis*
MK920307	16S *Ehrlichia*	*Wolbachia* sp.	*X. cheopis*

673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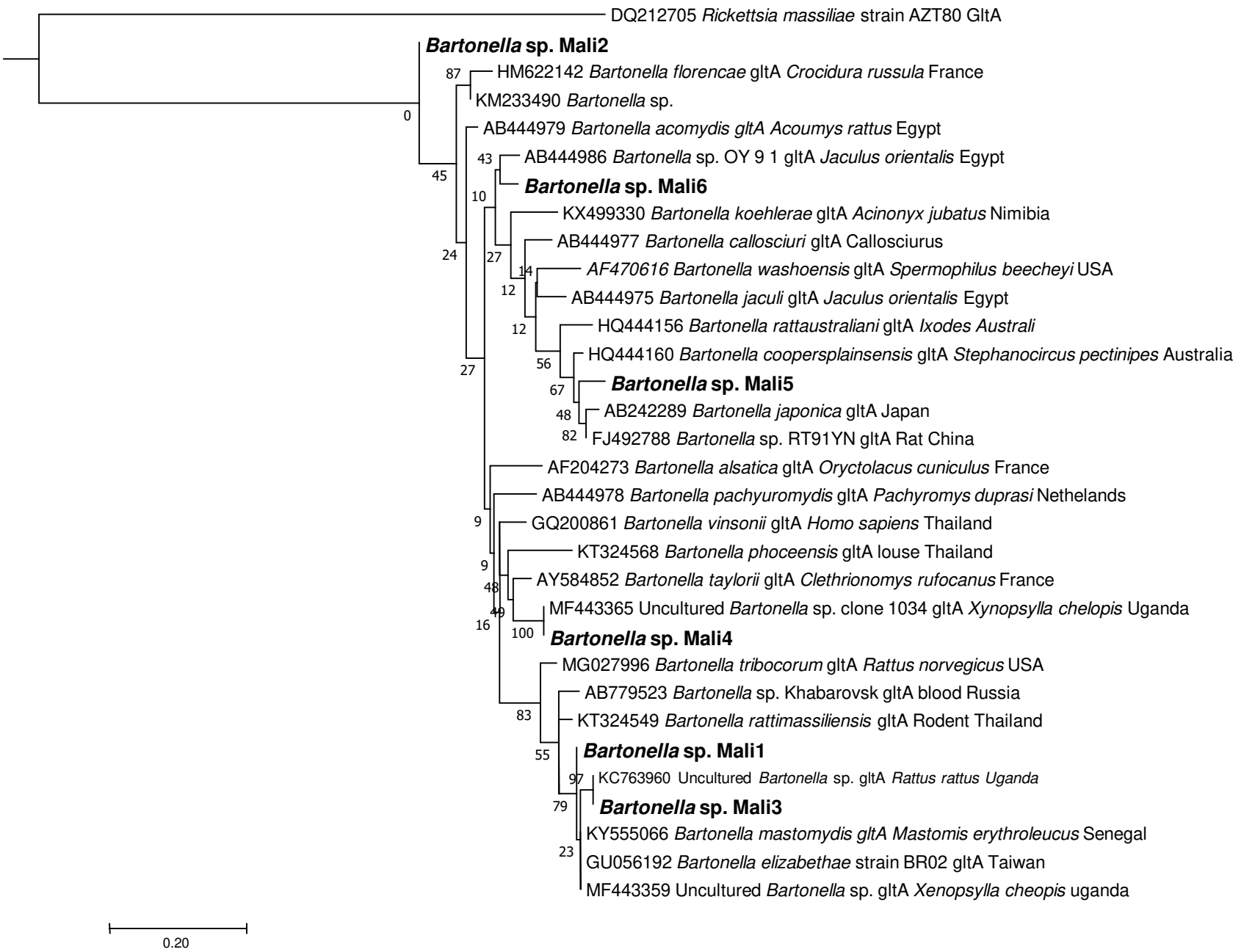
Crocidura cf olivieri
Gerbilliscus gambianus,
Mastomys erythroleucus
Mastomys natalensis
Praomys daltoni
Taterillus gracilis
Faladje
Bamako
Bougouni
7 (5.38%)
Crocidura spp.
Rattus norvegicus
48 (36.92%)
Atelerix cf albiventris
Crocidura spp.
Mastomys erythroleucus
Mastomys natalensis
Praomys daltoni
Rattus rattus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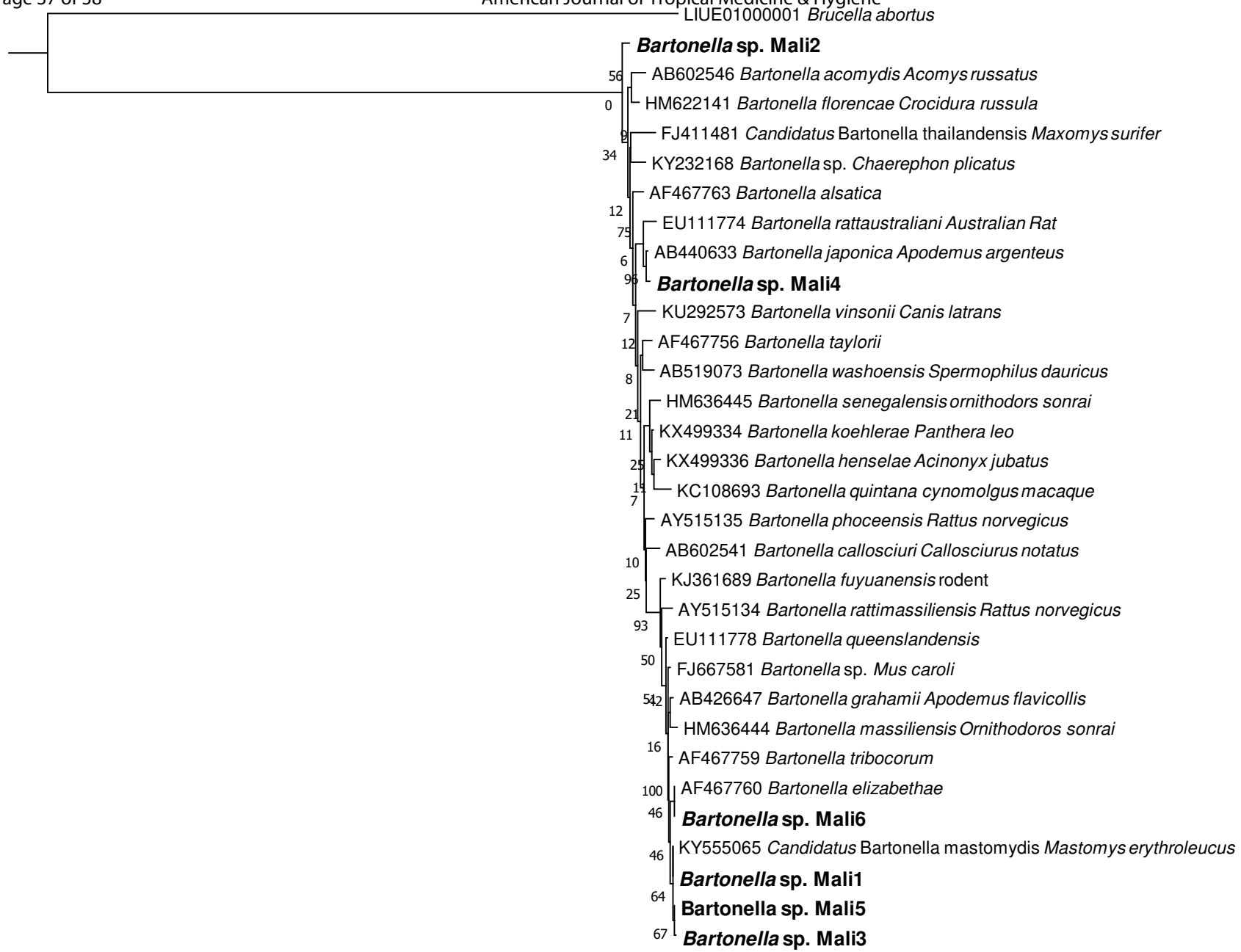
KM233490 *Bartonella* sp.
AB444979 *Bartonella acomydis gltA Acoumys rattus* Egypt
AB444986 *Bartonella* sp. OY 9 1 gltA *Jaculus orientalis* Egypt
***Bartonella* sp. Mali6**
KX499330 *Bartonella koehlerae* gltA *Acinonyx jubatus* Nimibia
AB444977 *Bartonella callosciuri* gltA Callosciurus
AF470616 Bartonella washoensis gltA *Spermophilus beecheyi* USA
AB444975 *Bartonella jaculi* gltA *Jaculus orientalis* Egypt
HQ444156 *Bartonella rattaustraliani* gltA *Ixodes Australi*
HQ444160 *Bartonella coopersplainsensis* gltA *Stephanocircus pectinipes* Australia
***Bartonella* sp. Mali5**
AB242289 *Bartonella japonica* gltA Japan
FJ492788 *Bartonella* sp. RT91YN gltA Rat China
AF204273 *Bartonella alsatica* gltA *Oryctolacus cuniculus* France
AB444978 *Bartonella pachyuromydis* gltA *Pachyromys duprasi* Nethelands
GQ200861 *Bartonella vinsonii* gltA *Homo sapiens* Thailand
KT324568 *Bartonella phoceensis* gltA louse Thailand
AY584852 *Bartonella taylorii* gltA *Clethrionomys rufocanus* France
MF443365 Uncultured *Bartonella* sp. clone 1034 gltA *Xynopsylla chelopis* Uganda
***Bartonella* sp. Mali4**
MG027996 *Bartonella tribocorum* gltA *Rattus norvegicus* USA
AB779523 *Bartonella* sp. Khabarovsk gltA blood Russia
KT324549 *Bartonella rattimassiliensis* gltA Rodent Thailand
***Bartonella* sp. Mali1**
KC763960 Uncultured *Bartonella* sp. gltA *Rattus rattus Uganda*
***Bartonella* sp. Mali3**
KY555066 *Bartonella mastomydis gltA Mastomis erythroleucus* Senegal
GU056192 *Bartonella elizabethae* strain BR02 gltA Taiwan
MF443359 Uncultured *Bartonella* sp. gltA *Xenopsylla cheopis* uganda
0.20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2
FJ411481 Candidatus Bartonella thailandensis Maxomys surifer
34
KY232168 Bartonella sp. Chaerephon plicatus
AF467763 Bartonella alsatica
12
75
EU111774 Bartonella rattaustraliani Australian Rat
6
AB440633 Bartonella japonica Apodemus argenteus
96
Bartonella sp. Mali4
7
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AF467756 Bartonella taylorii
8
AB519073 Bartonella washoensis Spermophilus dauricus
21
HM636445 Bartonella senegalensis ornithodors sonrai
11
KX499334 Bartonella koehlerae Panthera leo
25
KX499336 Bartonella henselae Acinonyx jubatus
19
7
KC108693 Bartonella quintana cynomolgus macaque
AY515135 Bartonella phoceensis Rattus norvegicus
AB602541 Bartonella callosciuri Callosciurus notatus
10
KJ361689 Bartonella fuyuanensis rodent
25
AY515134 Bartonella rattimassiliensis Rattus norvegicus
93
EU111778 Bartonella queenslandensis
50
FJ667581 Bartonella sp. Mus caroli
54
42
AB426647 Bartonella grahamii Apodemus flavicollis
HM636444 Bartonella massiliensis Ornithodoros sonrai
16
AF467759 Bartonella tribocorum
100
AF467760 Bartonella elizabethae
46
Bartonella sp. Mali6
46
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Bartonella sp. Mali1
64
Bartonella sp. Mali5
67
Bartonella sp. Mali3
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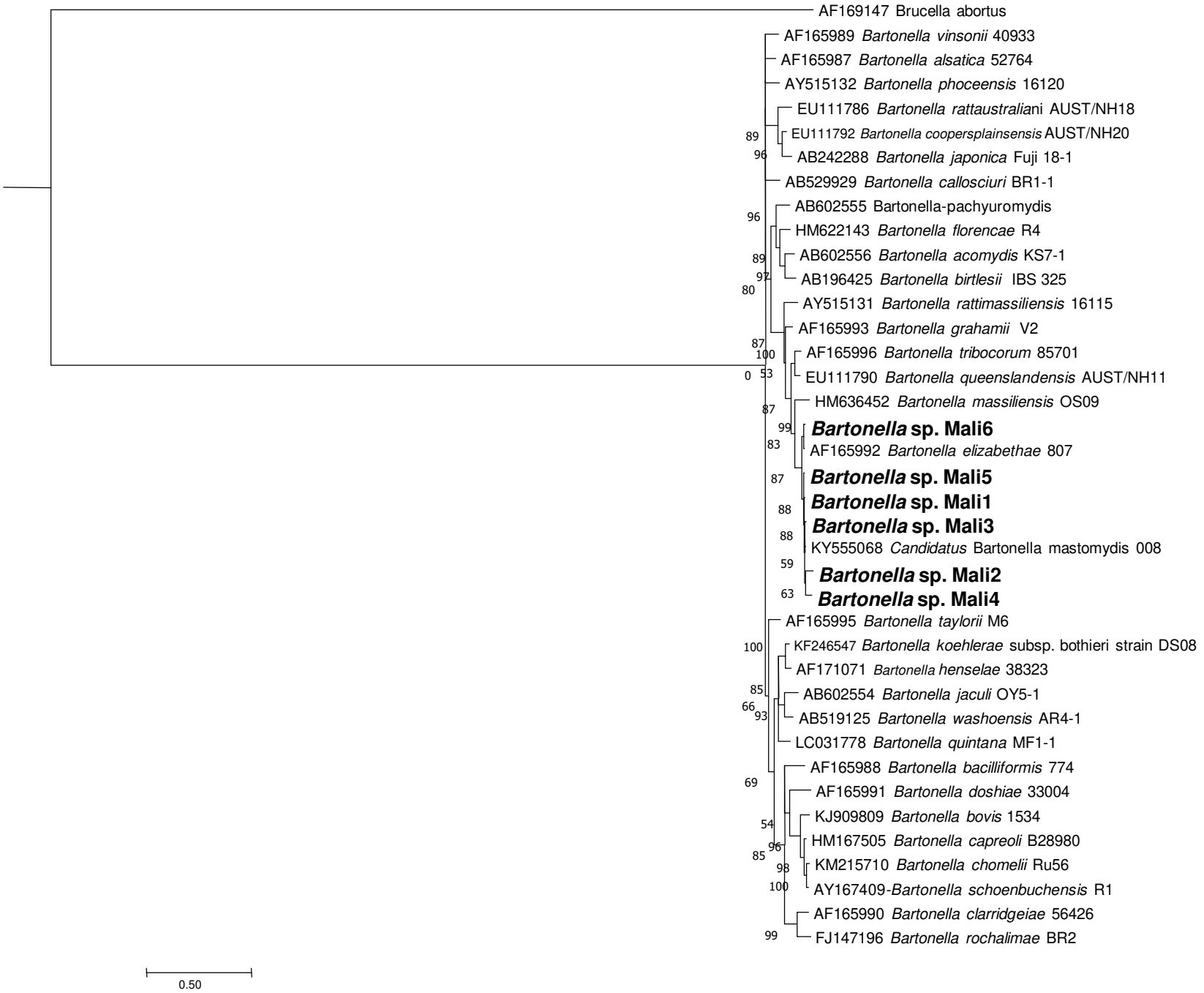
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EU111792 Bartonella coopersplainsensis AUST/NH20
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AB529929 Bartonella callosciuri BR1-1
AB602555 Bartonella-pachyuromydis
HM622143 Bartonella florencae R4
AB602556 Bartonella acomydis KS7-1
AB196425 Bartonella birtlesii IBS 325
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AF165993 Bartonella grahamii V2
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EU111790 Bartonella queenslandensis AUST/NH11
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Bartonella sp. Mali6
AF165992 Bartonella elizabethae 807
Bartonella sp. Mali5
Bartonella sp. Mali1
Bartonella sp. Mali3
KY555068 Candidatus Bartonella mastomydis 008
Bartonella sp. Mali2
Bartonella sp. Mali4
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AB602554 Bartonella jaculi OY5-1
AB519125 Bartonella washoensis AR4-1
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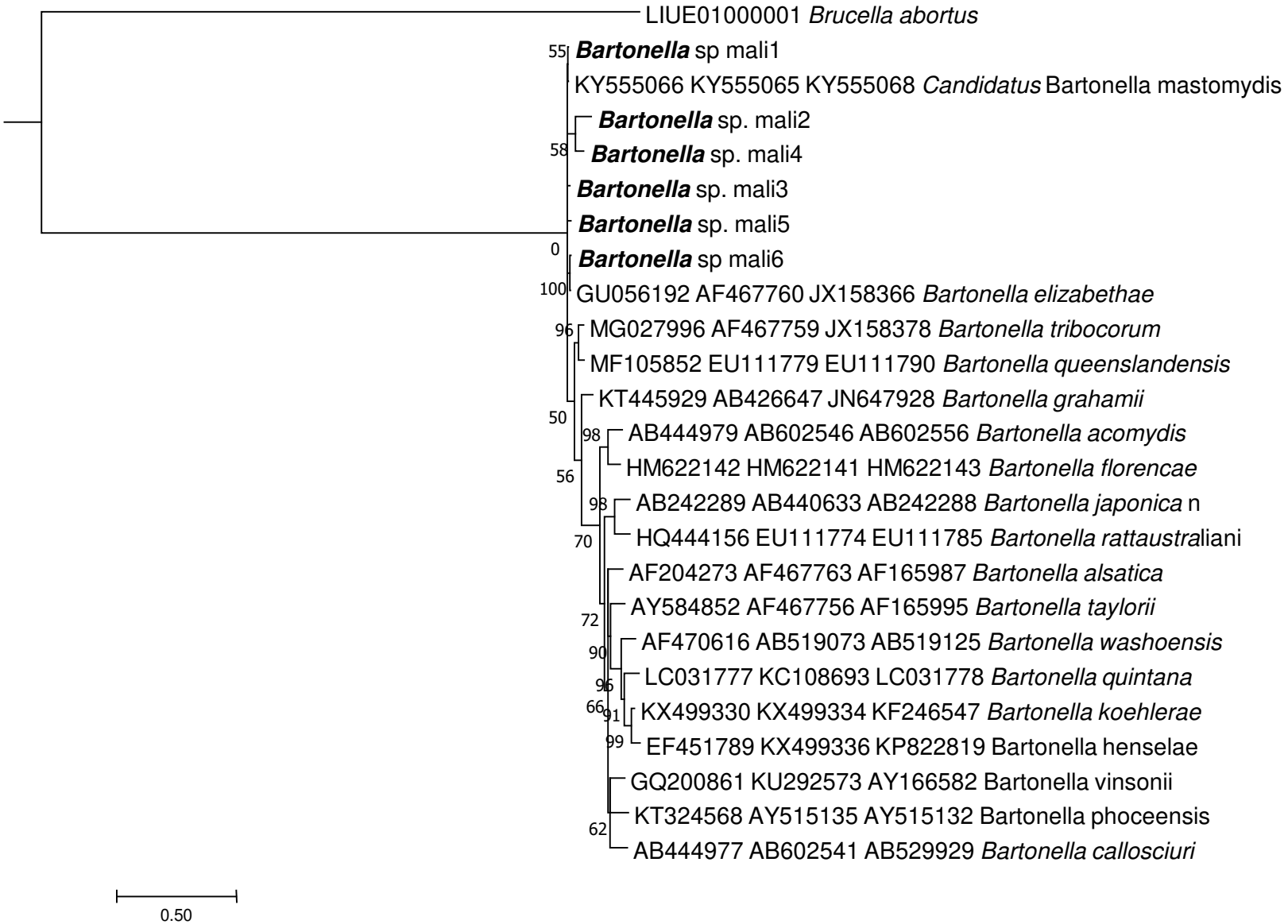
LIUE01000001 Brucella abortus
55
Bartonella sp mali1
KY555066 KY555065 KY555068 Candidatus Bartonella mastomydis
Bartonella sp. mali2
58
Bartonella sp. mali4
Bartonella sp. mali3
Bartonella sp. mali5
0
Bartonella sp mali6
100
GU056192 AF467760 JX158366 Bartonella elizabethae
96
MG027996 AF467759 JX158378 Bartonella tribocorum
MF105852 EU111779 EU111790 Bartonella queenslandensis
KT445929 AB426647 JN647928 Bartonella grahamii
50
98
AB444979 AB602546 AB602556 Bartonella acomydis
HM622142 HM622141 HM622143 Bartonella florencae
56
98
AB242289 AB440633 AB242288 Bartonella japonica n
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HQ444156 EU111774 EU111785 Bartonella rattaustraliani
AF204273 AF467763 AF165987 Bartonella alsatica
AY584852 AF467756 AF165995 Bartonella taylorii
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AF470616 AB519073 AB519125 Bartonella washoensis
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LC031777 KC108693 LC031778 Bartonella quintana
66
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KX499330 KX499334 KF246547 Bartonella koehlerae
99
EF451789 KX499336 KP822819 Bartonella henselae
GQ200861 KU292573 AY166582 Bartonella vinsonii
KT324568 AY515135 AY515132 Bartonella phoceensis
62
AB444977 AB602541 AB529929 Bartonella callosciuri
0.50

CONCLUSION GENERALE ET PERSPECTIVES

Au cours de cette thèse, nous avons développé et évalué la capacité du MALDI-TOF MS à identifier les arthropodes collectés sur le terrain dans les pays du sud. Dans un premier temps nous avons mis au point un protocole rapide, simple permettant d'identifier les tiques collectées au Mali et conservées dans de l'alcool et nous avons montré la nécessité de créer une base de données avec les spécimens conservés en alcool. Ce protocole est actuellement utilisé pour le traitement des tiques conservées en alcool dans notre laboratoire. Nous avons également enrichit la base de données de référence du laboratoire par l'ajout des espèces nouvelles. Dans le second travail qui était une première à utiliser le MALDI-TOF MS pour l'identification des moustiques et leur repas de sang conservés avec des spécimens conservés dans des tubes avec du silicagel collecté au Tchad. Nous avons montré que cet outil était efficace pour l'identification des moustiques mais moins pour leur repas de sang. Ainsi nous pensions que cette méthode de conservation avec du silicagel simple, moins couteuse et facile pour le transfert des échantillons de moustiques du terrain vers les laboratoires pourrait être une alternative à la méthode de conservation par congélation qui est fastidieuse et très couteuse. Le MALDI-TOF MS nous a permis aussi d'identifier de nouvelles espèces de moustiques (*Culex perexiguus*, *Culex rima* et *Culex watti*) qui n'étaient pas dans notre base de données.

Nos travaux sur l'étude des répertoires des micro-organismes, nous ont permis de détecter *Rickettsia* spp., *Coxiella burnetii*, *Anaplasmataceae* et *Borrelia* spp. dans les tiques collectées sur les bovins et chiens au Mali. Nous avons identifié l'ADN de *R. massiliae*, *R. aeschlimannii*, *A. platys*, *A. marginale*, *A. ovis Theileria equi*, *T. annulata*, *T. ovis T. buffeli*, *B. bovis*, «*Candidatus* Ehrlichia urmitei», «*Candidatus* Rickettsia barbariae» et *Borrelia* spp. chez les animaux et les tiques dans le Nord-est de l'Algerie. Nous avons également trouvé *B. mastomydis*, *B. elizabethae*, *Bartonella* spp., *Coxiella burnetii*, *R. africae* et *E. ruminantium* les petits mammifères et leurs ectoparasites au Mali. Nous avons contribué à l'amélioration

des connaissances du répertoire de micro-organismes associés avec les tiques en Algérie et au Mali.

Nous avons rédigé une revue sur les tiques et maladies transmises par les tiques aux humains et animaux en Afrique de l'Ouest. Les données disponibles nous ont permis de constater que les maladies transmises par les tiques sont très peu étudiées dans cette zone bien que des études signalent la présence de ces pathogènes chez les tiques et les hôtes animaux. Les personnels de santé ainsi que les autorités sanitaires devraient penser à ces pathogènes comme pouvant être les causes des certaines fièvres inconnues non palustres.

Mon expérience sur l'utilisation des outils innovants au sein du laboratoire VITROME de l'IHU m'a permis de collaborer actuellement avec un étudiant en Master 2 de la République Démocratique du Congo pour mener une étude sur utilisation du MALDI-TOF MS pour l'identification des tiques collectées sur les animaux domestiques et sauvages conservées en alcool. Ce travail est en cours.

Il a été montré au Sénégal que l'installation d'un MALDI TOF pour la microbiologie clinique permet son utilisation en entomologie médical sans coût additionnel. A plus long terme, je souhaite dans ma carrière d'entomologiste participer au transfert de cette technologie et de mes compétence au Mali et dans les autres pays du sud par des formations et ateliers scientifiques mais aussi de réaliser une étude future à grande échelle sur les tiques collectées à des endroits divers et variés géographiquement en fin d'avoir le maximum d'espèces pour enrichir et consolider la base de donnée MALDI-TOF MS arthropode de notre groupe.

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ANNEXES

Article 6

***Using MALDI-TOF MS to identify mosquitoes collected in Mali and their blood meals*.**

Fatalmoudou Tandina, Sirama Niaré, Maureen Laroche, Abdoulaye K Koné, Adama Zan Diarra, Abdoulaye Ongoiba, Jean-Michel Berenger, Ogobara Doumbo, Didier Raoult, Philippe Parola

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Résumé

La spectrométrie de masse à temps de vol par de désorption/ ionisation laser assistée par matrice (MALDI-TOF MS) a récemment été décrite comme un outil innovant et efficace pour l'identification des arthropodes et des sources de repas de sang de moustiques. Pour tester cette approche dans le cadre d'une enquête entomologique sur le terrain, des moustiques ont été collectés dans cinq zones écologiquement distinctes du Mali. Nous avons analysé avec succès les repas de sang provenant de 651 abdomens de moustiques écrasés sur du papier filtre Whatman (WFP) sur le terrain avec MALDI-TOF MS. Les pattes de 826 moustiques ont ensuite été soumises à une analyse MALDI-TOF MS afin d'identifier les différentes espèces de moustiques. Huit espèces de moustiques ont été identifiées, dont *Anopheles gambiae* Giles, *Anopheles coluzzii*, *Anopheles arabiensis*, *Culex quinquefasciatus*, *Culex neavei*, *Culex perexiguus*, *Aedes aegypti* et *Aedes fowleri* au Mali. Les moustiques sur le terrain pour lesquels la MALDI-TOF MS n'a pas fourni l'identification réussie n'étaient pas disponibles auparavant dans notre base de données. Ces spécimens ont ensuite été identifiés par la biologie moléculaire. Les sources de repas du sang trouvées dans cette étude étaient appariées au sang humain (n = 619), au sang de poulet (n = 9), au sang de vache (n = 9), au sang d'ânesse (n = 6), au sang de chien (n = 5) et le sang de mouton (n = 3). Cette étude confirme le fait que MALDI-TOF MS est un outil prometteur pour les enquêtes entomologiques.

Parasitology

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Using MALDI-TOF MS to identify mosquitoes collected in Mali and their blood meals

Fatalmoudou Tandina[1,2], Sirama Niaré[1,2], Maureen Laroche[1], Abdoulaye K Koné[2], Adama Z Diarra[1,2], Abdoulaye Ongoiba[2], Jean Michel Berenger[1], Ogobara K Doumbo[2], Didier Raoult[1] and Philippe Parola[1]

[1]Aix Marseille Univ, IRD, AP-HM, IHU-Méditerranée Infection, SSA, VITROME, Marseille, France and [2]Department of Epidemiology of Parasitic Diseases, Malaria Research and Training Center, University of Science, Techniques and Technologies of Bamako, Mali

Research Article

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Author for correspondence:
Philippe Parola, Email: philippe.parola@univ-amu.fr

Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been recently described as an innovative and effective tool for identifying arthropods and mosquito blood meal sources. To test this approach in the context of an entomological survey in the field, mosquitoes were collected from five ecologically distinct areas of Mali. We successfully analysed the blood meals from 651 mosquito abdomens crushed on Whatman filter paper (WFPs) in the field using MALDI-TOF MS. The legs of 826 mosquitoes were then submitted for MALDI-TOF MS analysis in order to identify the different mosquito species. Eight mosquito species were identified, including *Anopheles gambiae* Giles, *Anopheles coluzzii*, *Anopheles arabiensis*, *Culex quinquefasciatus*, *Culex neavei*, *Culex perexiguus*, *Aedes aegypti* and *Aedes fowleri* in Mali. The field mosquitoes for which MALDI-TOF MS did not provide successful identification were not previously available in our database. These specimens were subsequently molecularly identified. The WFP blood meal sources found in this study were matched against human blood ($n = 619$), chicken blood ($n = 9$), cow blood ($n = 9$), donkey blood ($n = 6$), dog blood ($n = 5$) and sheep blood ($n = 3$). This study reinforces the fact that MALDI-TOF MS is a promising tool for entomological surveys.

Introduction

Mosquito-borne infectious diseases are a public health concern in tropical countries, and an emerging problem in temperate areas (Becker *et al.* 2010). The main mosquito vectors, which may transmit pathogens during their blood meals, belong to three main genera, namely *Aedes*, *Culex* and *Anopheles* (Becker *et al.* 2010). *Aedes* spp. mosquitoes are vectors for several arboviruses including the Yellow Fever, Dengue, Chikungunya and Zika viruses, which have come to the world's attention in recent years (Gardner and Ryman, 2010; Vasilakis *et al.* 2011; Caglioti *et al.* 2013). *Culex* mosquitoes are responsible for West Nile Virus (WNV) and Japanese encephalitis virus transmission around the world (Komar, 2003; Anosike *et al.* 2005; de Wispelaere *et al.* 2017). *Anopheles* mosquitoes are the primary vectors of malaria. Female *Anopheles* mosquitoes are able to transmit six species of *Plasmodium* to humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale wallikeri*, *P. ovale curtisi*, *P. knowlesi* and *P. simium* (WHO, 2016; Brasil *et al.* 2017). Malarial transmission remains high in Africa, with 117 886 deaths in 2015 (WHO, 2016). In Mali, West Africa, 1544 deaths were recorded as being attributable to malaria in 2015 (WHO, 2016). It is reasonable to assume that the number of malaria-associated deaths remains underestimated.

The precise identification of mosquito fauna is essential in entomological surveys, and in order to plan control measures and monitor their impact (Bass *et al.* 2007). Furthermore, the identification of mosquito blood meal sources is essential to understanding the biting behaviour of mosquito vectors (anthropophilic or zoophilic) (Muturi *et al.* 2013).

Mosquitoes are most frequently identified at the genus and species levels by morphological characteristics and using molecular tools. Morphological identification requires well-trained entomologists using dichotomous identification keys (Gillies MT 1987). Morphological identification continues to be the standard approach for arthropod studies. However, it presents some limits in terms of discriminating cryptic or sibling species. In recent years, molecular tools have emerged and can identify mosquitoes by amplifying different target genes. The target gene, such as the cytochrome *c* oxidase (COI), internal transcribed spacer 2, IGS regions of rDNA, has been used to satisfactorily identify mosquitoes up to sibling species with great specificity and sensitivity (Folmer *et al.* 1994).

Several approaches have been developed to identify the host vertebrate blood source of mosquito meals. The main tools include a serological approach which involves precipitin tests and enzyme-linked immunosorbent assays (ELISA) (Fyodorova *et al.* 2006; Gomes *et al.* 2013). However, these techniques present several limitations, including the availability of specific antisera against a broad diversity of host species and the cross-reactivity of antibodies for close species. To this end, molecular methods have also been developed to identify

mosquito blood meal sources, such as mammalian blood and avian blood from *Culex pipiens* complex (Gomes *et al.* 2013). However, molecular methods also present several constraints, such as their cost, the time they take and the need for bulky equipment.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently been used as an alternative tool for rapid arthropod identification. The mass spectrum from a new sample, generated using MALDI-TOF MS, is compared with a library of spectra from a reference database. In our laboratory, the MALDI-TOF MS approach has been routinely used to identify arthropods such as ticks (using their legs) (Yssouf *et al.* 2013, 2015; Kumsa *et al.* 2016), fleas (bodies of fleas without the abdomen) (Yssouf *et al.* 2014*b*), sand flies (using their thoraces, wings and legs) (Lafri *et al.* 2016), adult mosquitoes (using their legs) (Yssouf *et al.* 2013, 2014*a*) and mosquito larvae (using whole mosquitoes) (Dieme *et al.* 2014). Preliminary studies have also reported that MALDI-TOF MS may be used for mosquito blood meal identification. When the MS spectra obtained from the abdomen of mosquitoes which had been experimentally engorged on different blood meals source were tested, the MS protein profiles were clearly distinct according to the origin of the mosquito blood meals, up to 24 h post-feeding (Niare *et al.* 2016). During entomological surveys, it may be difficult to preserve samples, and entomologists frequently use Whatman filter papers (WFPs) to preserve mosquito blood meals in the field by crushing the engorged abdomens onto WFPs.

In this study, the goal was to use the proteomic MALDI-TOF MS approach to identify mosquitoes collected in Mali and determine the sources of their blood meals. For this purpose, mosquitoes were collected in different ecological areas of Mali and tested by MALDI-TOF MS in Marseille, France. The abdomens of engorged female mosquitoes were crushed onto WFP to determine the blood meal sources using MALDI-TOF MS.

Materials and methods

Ethics statement

Consent was obtained from the heads of families where the mosquitoes were collected. Ethical approval for the collection of mosquito was granted by authorities from the National Malaria Control Program (NMCP) and approved by the Faculty of Medicine Ethical Committee, Bamako, Mali (N°2016/113/CE/FMPOS). The mosquito samples were processed and stored in line with the World Health Organization (WHO) Good Laboratory Practices guidance and documents on mosquito sampling handling procedures.

Collection sites

This study was conducted in three different localities in Mali, namely Donéguébougou, Bougoula-hameau and Bamako. In Bamako, the collection was performed in the three semi-urban areas of Sotuba, Yirimadio and Missabougou. The geo-positions of each collection site are as follows: Bougoula-hameau (−5° 66′13.1″, 11°30′95.2″E); Donéguébougou (−7°98′39.8″N, 12° 80′44.9″E) and the semi-urban areas of Bamako, Sotuba (−9° 18′65.7′N, 8°23′07.4′E), Yirimadio (−9°18′56.5 ′N, 6°23′01.8″E) and Missabougou (−9°18′77.5″N, 8°23′03.9″E).

Mosquito collection

Mosquitoes were collected from the various sites during the middle of the rainy season between July and August 2016 (WHO, 1992). The peak densities and consequentially of anopheline mosquitoes in Mali occur in August (Sogoba *et al.* 2007). Mosquitoes were collected over three consecutive days per week. On each day, mosquitoes were aspirated from 10 houses using a mouth aspirator (Model 612, John W Hock, Gainesville, Florida, USA). All mosquitoes were collected indoors in the morning between 8 am and noon. The mosquito specimens were identified using morphological criteria (Gillies MT 1987). After being collected, mosquito specimens were kept at room temperature (RT) between 2 and 4 h during the female abdomens crushed process and then were stored at −20 °C. Each mosquito specimen was then individually transferred to a 1.5 mL Eppendorf tube labelled with a reference number, the gender of the specimen, the date and site of collection.

Mosquito abdomens with visible blood meals were crushed on WFPs (Whatman International Ltd., Maidstone, England, approved by BSI). Following the entomological stage, all samples were transported to Aix-Marseille University for mosquito and blood meal identification using MALDI-TOF MS in September and October 2016.

Preparation of samples for MALDI-TOF MS analysis

Mosquito identification

The legs of the specimens were cleaned in 70% (v/v) ethanol for between one to two minutes, then rinsed in high performance liquid chromatography (HPLC) grade water. The legs from each mosquito were individually placed in 1.5 mL Eppendorf tubes with glass powder (Sigma, Lyon, France), 15 μL of 70% (v/v) formic acid (Sigma, Lyon, France), and 15 μL of 50% (v/v) acetonitrile (Fluka, Buchs, Switzerland). The samples were crushed using a TissueLyser device (Qiagen, Hilden, Germany) over three cycles of 30 m s^{-1} for 60 s (Nebbak *et al.* 2016). The samples were centrifuged at 200 ***g*** for one minute, and 1.5 μL of supernatant of each homogenate was deposited on the MALDI-TOF target plate in quadruplicate (Bruker Daltonics, Wissembourg, France) and covered with 1.5 μL of CHCA matrix solution composed of saturated α-cyano-4-hydroxycynnamic acid (Sigma, Lyon, France), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Aldrich, Dorset, UK), and HPLC grade water (Yssouf *et al.* 2013; Nebbak *et al.* 2016). The target plate was dried for several minutes at RT and placed in the Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Wissembourg, France) for analysis (Yssouf *et al.* 2013, 2016; Nebbak *et al.* 2016).

Bloody Whatman filter papers (BWFPs)

A piece of the WFPs (i.e. about 1 mm^2) containing crushed abdomens from engorged mosquitoes was individually cut using a sterile scalpel and transferred to a new 1.5 mL Eppendorf tube (Niare *et al.* 2017). For each piece of WFPs, 20 μL of formic acid (70%, v/v) plus 20 μL of acetonitrile (50% v/v) (Fluka, Buchs, Switzerland) was added and incubated for 10 min at RT. After a fast spin (i.e. 10 000 rpm for 20 s), 1 μL of the supernatant of each sample was loaded onto the MALDI-TOF target plate in quadruplicate and covered with 1 μL of CHCA matrix (Niare *et al.* 2016). After drying for several minutes at RT, the MALDI-TOF target plate was placed in the Microflex LT MALDI-TOF Mass Spectrometer (Bruker Daltonics, Bremen, Germany) for analysis. To control loading on mass spectra steel, matrix quality and MALDI-TOF apparatus performance, the matrix solution was loaded in duplicate onto each MALDI-TOF plate with or without a bacterial test standard (Bruker protein Calibration Standard I) (Niare *et al.* 2016).

Spectral analysis

Protein mass profiles were acquired using a Microflex LT MALDI-TOF Mass Spectrometer, with detection in the linear positive-ion mode at a laser frequency of 50 Hz within a mass range of 2-20 kDa. The acceleration voltage was 20 kV, and the extraction delay time was 200 ns. Each spectrum corresponded to ions obtained from 240 laser shots performed in six regions of the same spot and automatically acquired using the AutoXecute of the Flex Control v.2.4 software (Bruker Daltonics, Bremen, Germany). The spectrum profiles obtained from mosquito legs and bloody WFPs were visualized with Flex analysis v.3.3 software and were exported to ClinProTools version v.2.2 (Bruker Daltonics, Bremen, Germany) and MALDI-Biotyper v.3.0. (Bruker Daltonics, Bremen, Germany) for data processing (smoothing, baseline subtraction, peak picking) and evaluated using cluster analysis. Spectra of low quality were excluded from the study.

MALDI-TOF identification of mosquitoes

We used our in-lab arthropod MALDI-TOF database, which includes spectra obtained from various arthropods listed in Table 1. The database was upgraded with the spectra of three *Culex quinquefasciatus* mosquitoes and one *Culex neavei* mosquito collected and molecularly identified during this study. A comparison of the spectrum of each specimen of mosquito legs from Mali was evaluated against the home-made MS reference spectra database using the MALDI-Biotyper software v3.0. tool (Bruker Daltonics, Bremen, Germany). The level of significance was determined using the log score values (LSVs) provided by the MALDI-Biotyper software v.3.3. corresponding to a matched degree of signal intensities of mass spectra of the query and the reference spectra. LSVs ranged from zero to three. To determine the origin of blood meals, MALDI-TOF MS spectra from the abdominal proteins of engorged mosquitoes crushed on WFPs were also blindly queried against the database. A sample was considered to be correctly and significantly identified at the species level when the queried spectrum had a log score value (LSV) ⩾1.8 (Niare *et al.* 2016).

Cluster analysis

Cluster analysis on MSP (MSP, Main Spectrum Profile) spectra was performed and the comparison of the main spectra given by the MALDI-Biotyper software was clustered according to protein mass profile (i.e. their mass signals and intensities). We performed hierarchical clustering of the mass spectra of two specimens per mosquito species using the MSP dendrogram function. The clustering analyses were performed to visualize the homogeneity level of MS spectra from specimens belonging to the same species level. The resulting MSP dendrogram shows how samples are related to one another.

Molecular identification

A molecular tool was used to confirm MALDI-TOF MS identification in randomly selected mosquitoes. Molecular identification was also conducted for specimens whose spectra did not match with any mosquito spectrum in our database. When it was demonstrated that a high quality spectrum had been obtained from a mosquito species missing from our database, this new spectrum was added to the database. DNA extractions from individual mosquito heads and thorax samples were performed using the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. A set of primers specifically amplifying a fragment of 710 bp of the mosquito's cytochrome *c* oxidase I gene (mCOI) was used (LCO1490 (forward): 5'-GGTCAAC

Table 1. List of the arthropod species present in our home-made MALDI-TOF MS[a] database.

Mosquitoes	*Imago*: *Aedes albopictus*, *Ae. excrucians*, *Ae. vexans*, *Ae. rusticus*, *Ae. dufouri*, *Ae. cinereus*, *Ae. fowleri*, *Ae. aegypti*, *Ae. caspius*, *Anopheles gambiae* Giles, *An. coluzzii*, *An. funestus*, *An. ziemanni*, *An. arabiensis*, *An. wellcomei*, *An. rufipes*, *An. pharoensis*, *An. coustani*, *An. claviger*, *An. hyrcanus*, *An. maculipennis*, *Culex quinquefasciatus*, *Cx. pipiens*, *Cx. modestus*, *Cx. insignis*, *Cx. neavei*, *Mansonia uniformis*, *Culiseta longiareolata*, *Orthopodomyia reunionensis*, *Coquillettidia richiardii* and *Lutzia tigripes*. *Larvae*: *Aedes aegypti*, *Ae. albopictus*, *Anopheles gambiae* Giles, *An. coluzzii*, *Cx. pipiens*, *Cx. molestus*, *Culiseta* sp.
Sand flies	*Phlebotomus papatasi*, *P. (Larrousius) longicuspis*, *P. (Larrousius) perfiliewi*, *P. (Larrousius) perniciosus*, *P. (Paraphlebotomus) sergenti* and *Sergentomyia minuta*
Triatomines	*Triatoma infestans*, *Rhodnius prolixus*, *Rh. pictipes*, *Rh. robustus*, *Eratyrus mucronatus* and *Panstrongylus geniculatus*
Ticks	*Legs: Amblyomma cohaerens*, *Am. gemma*, *Am. variegatum*, *Dermacentor marginatus*, *D. reticulatus*, *Haemaphysalis leachi*, *Hae. concinna*, *Hae. spinulosa*, *Hyalomma marginatum rufipes*, *H. truncatum*, *H. detritum*, *Rhipicephalus decoloratus*, *Ixodes hexagonus*, *I. ricinus*, *Rh. bergeoni*, *Rh. e. evertsi*, *Rh. praetextatus*, *Rh. pulchellus*, *Rh. sanguineus*, *Rh. sulcatus*, *Rh. microplus*, *Rh. annulatus*, *Rh. turanicus* and *Rh. bursa*. *Hemolymph: Am. variegatum*, *D. marginatus*, *H. marginatum rufipes*, *Rh. bursa* and *Rh. sanguineus*.
Mites	*Leptotrombidium chiangraiensis*, *L. imphalum* and *L. deliense*
Bedbugs	*Cimex lectularius*
Lice	*Pediculus humanus*, *Damalinia bovis*, *D. caprae*, *D. ovis*, *Haematopinus eurysternus*, *Linognatus vituli* and *L. africanus*
Fleas	*Ctenocephalides felis*, *Ct. canis*, *Archaeopsylla erinacei*, *Xenopsylla cheopis* and *Stenoponia tripectinata*
Abdomen of mosquitoes engorged	*Anopheles gambiae* Giles fed on: *Homo sapiens*, *Equus caballus*, *Ovis aries*, rabbit, Balb/C mouse, *Rattus norvegicus*, *Canis familiaris*, *Bos taurus*, *Capra hircus*, *Gallus gallus*, *Equus asinus*, *Tapirus indicus*, *Tapirus terrestris*, *Carollia perspicillata*, *Thraupis episcopus*, *Erythrocebus patas* and *Callithrix pygmaea* blood *Aedes albopictus* fed on: *Homo sapiens* blood
Anopheles gambiae Giles blood meals from Whatman Filter paper	*Ovis aries* and *Homo sapiens* blood

[a]MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

AAATCATAAGATATTGG-3'; HC02198 (reverse): 5'-TAAAC TTCAGGGTGACCAAAAAATCA-3' (Folmer *et al.* 1994). We used gene Acetylcholinesterase-2 to amplify a fragment of 610 bp of *Culex pipiens* and a fragment of 274 bp of *Cx. quinquefasciatus*. The primers set were ACEquin (forward): 5′-CCTT CTTGAATGG CTGTGGCA-3′, ACEpip (forward): 5′-GGAAA CAACGACGTATGTACT-3′, B1246s (reverse): 5′-TGGAGCC TCCTCTTCACGGC-3′ (Smith and Fonseca, 2004).

A set of primers specifically amplifying a fragment of 310 bp of the *Anopheles gambiae* mosquito complex Acomplex_28S_MBF 5′-AGCKCGTCTTGGTCTGGGG-3′ and Acomplex_28S_MBR 5′-GCCGACAAGCTCAYTAGTGT-3′ was designed in our laboratory based on the work by Fanello *et al.* and PCR reactions were processed as described (Fanello *et al.* 2002). Molecular identification of the blood was carried out on the bloody WFPs from 41 specimens randomly selected from the Malian samples, as previously described (Niare *et al.* 2016). Positive PCR products were then purified and sequenced using the same primers with the BigDye version 1-1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA) and an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). The sequences were assembled and analyzed using the ChromasPro software (version 1.34) (Technelysium Pty. Ltd., Tewantin, Australia) and the NCBI BLAST website (http://blast.ncbi.nlm.nih.gov).

Results

Identification of the mosquitoes by MALDI-TOF MS

A total of 865 mosquitoes were captured by aspiration in Mali from various collection sites, including 257 in Bougoula-hameau, 168 in Donéguébougou, 230 in Sotuba, 125 in Missabougou and 85 in the Yirimadio semi-urban zones of Bamako (Fig. 1). All specimens collected were morphologically identified to genus level as *Anopheles* spp. (287/865; 33.18%), *Culex* spp. (573/865; 66.24%) and *Aedes* spp. (5/865; 0.58%).

For MALDI-TOF analysis, MS spectra of good quality were obtained from 272 legs of *Anopheles* spp. Of these 272 *Anopheles* spp. tested against the arthropod MS database, 97% ($n = 264/272$) were identified with a log score value (LSV) ranging between 1.70 and 2.575. These 264 *Anopheles* specimens were identified as *Anopheles gambiae* Giles (95.80%, $n = 253/264$), *Anopheles coluzzii* (3.40%, $n = 9/264$) and *Anopheles arabiensis* (0.80%, $n = 2/264$) (Fig. 2) by MALDI TOF MS. The remaining eight *Anopheles* spp. were subjected to molecular identification.

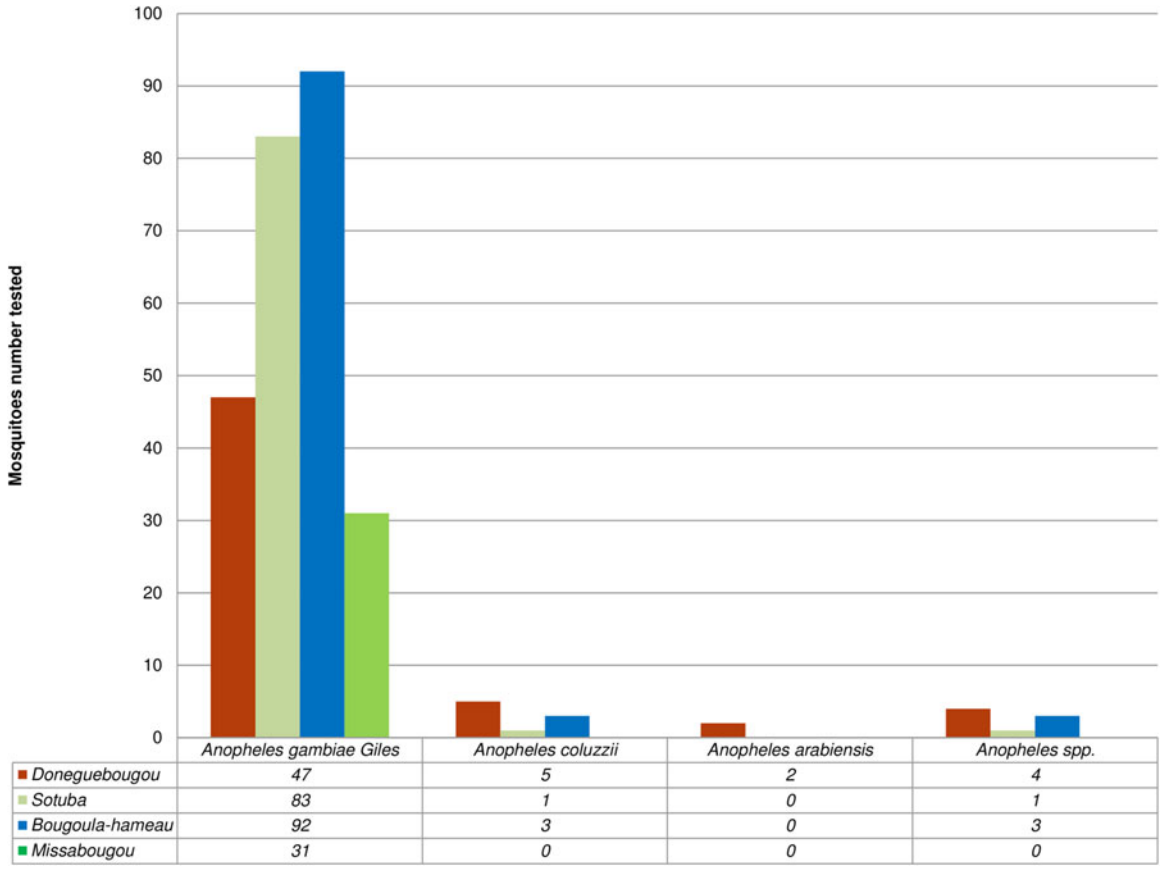
We tested the MS spectra from the legs of 549 *Culex* spp. against our arthropod database.

Of these 549 *Culex* spp. high-quality spectra, 98% ($n = 537/549$) were identified as species contained in our database. The 537 *Culex* specimens were identified by MALDI-TOF MS as *Cx. quinquefasciatus* (98%, $n = 527/537$) and *Cx. neavei* (2%, $n = 10/537$) from Mali (Fig. 3). These 537 *Culex* obtained LSVs ranging from 1.713 to 2.611. The remaining twelve *Culex* spp. were subjected to molecular identification.

The five *Aedes* specimens were identified by MALDI-TOF MS as *Aedes fowleri* ($n = 4$) and *Aedes aegypti* ($n = 1$), with log score values ranging between 2.128 and 2.418.

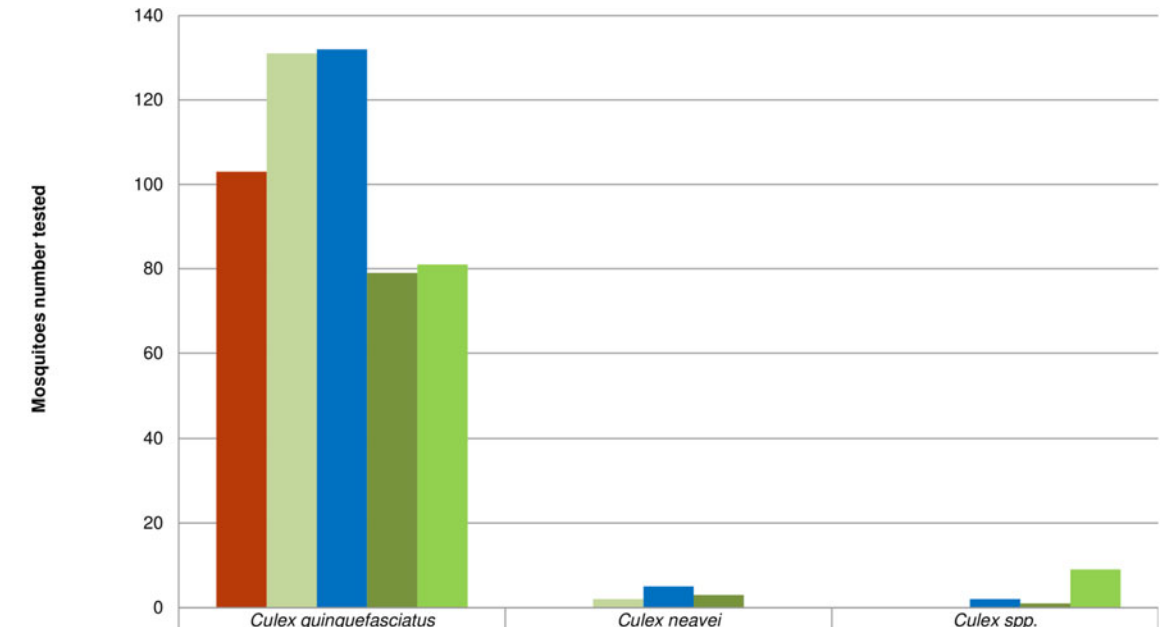
The MS spectra comparison from different mosquito species with Flex analysis software revealed an intra-species reproducibility and an inter-species specificity (Fig. 4). Visually, the signals and intensity of mosquito species' protein profiles (Fig. 4) were consistent for MALDI-TOF identification and revealed eight

Fig. 1. Ecological patterns and geographic distribution of mosquito collection in Mali. Sikasso: Bougoula-hameau (rural area), Bamako: Sotuba (peri-urban area), Missabougou, Yirimadio (urban areas) and Kati: Doneguebougou (rural area).



	Anopheles gambiae Giles	Anopheles coluzzii	Anopheles arabiensis	Anopheles spp.
Doneguebougou	47	5	2	4
Sotuba	83	1	0	1
Bougoula-hameau	92	3	0	3
Missabougou	31	0	0	0

Fig. 2. MALDI-TOF MS Identification of 272 *Anopheles* spp. collected in Mali.



	Culex quinquefasciatus	Culex neavei	Culex spp.
Doneguebougou	103	0	0
Sotuba	131	2	0
Bougoula-hameau	132	5	2
Yirimadio	79	3	1
Missabougou	81	0	9

Fig. 3. MALDI-TOF MS Identification of 549 *Culex* spp. captured in Mali.

different species, namely *Anopheles gambiae* Giles, *An. coluzzii*, *An. arabiensis*, *Cx. quinquefasciatus*, *Cx. neavei*, *Culex perexiguus*, *Ae. fowleri* and *Ae. aegypti*. Clustering analysis of MSP spectra from two specimens per mosquito species was used to generate a dendrogram. Clustering analysis revealed a gathering on distinct branches, following the eight species which were loaded

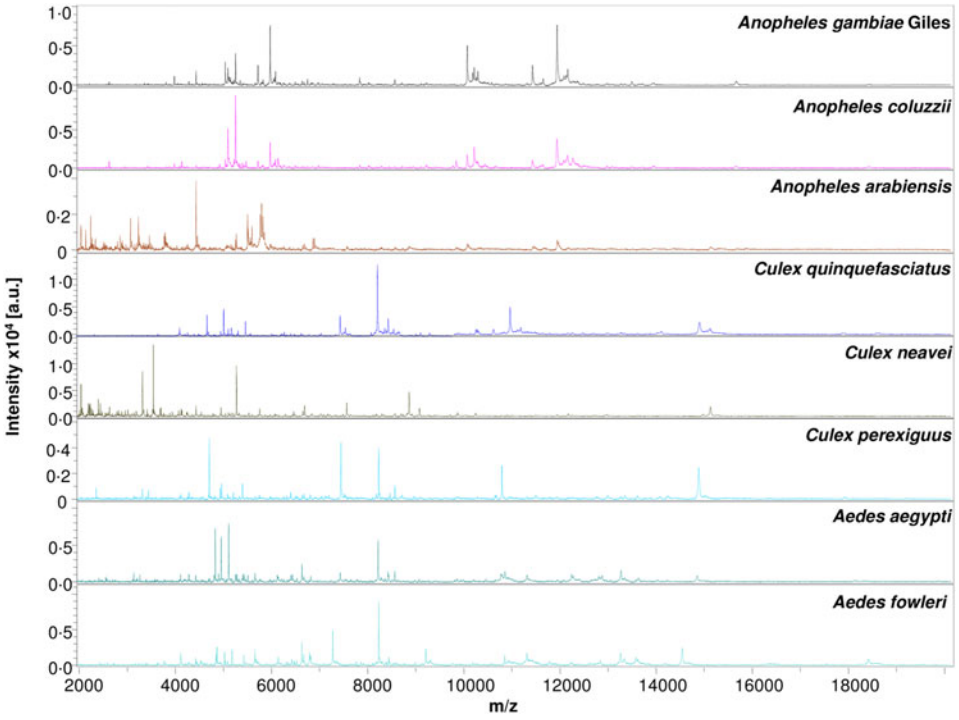


Fig. 4. Comparison of MALDI-TOF MS profiles of eight mosquito species collected in Mali. Spectra analysis was performed using Flex analysis 3.3 software. Abbreviations: a.u., arbitrary units; *m/z*, mass-to-charge ratio.

(*Anopheles gambiae* Giles, *An. coluzzii*, *An. arabiensis*, *Cx. quinquefasciatus*, *Cx. neavei*, *Cx. perexiguus*, *Ae. fowleri* and *Ae. aegypti*) (Fig. 5). The clusters formed were consistent with the intra-species reproducibility and inter-species specificity visually observed on protein profiles.

Molecular identification of mosquitoes collected in Mali

Molecular biology was performed to confirm the mosquito identification resulting from the MALDI-TOF MS analyses. For this purpose, we randomly selected 20/253 *An. gambiae* Giles, 2/9 *An. coluzzii*, 15/527 *Cx. quinquefasciatus*, 1/10 *Cx. neavei* for sequencing. The *28S* gene sequencing of *Anopheles* corroborated the MALDI-TOF MS identification in all cases, with between 97.51 and 99.27% identity with Genbank sequences (Table 2).

The acetylcholinesterase-2 and *COI* genes were used to identify the *Culex* species. Sixteen specimens of *Cx. quinquefasciatus* (n = 15) and *Cx. neavei* (n = 1) were randomly selected for sequencing. The molecular results were found to be highly consistent with the MALDI-TOF MS identification. Sequences obtained from *Cx. quinquefasciatus* and *Cx. neavei* were shown to share between 98.90 and 100% identity with Genbank (Table 2).

Molecular biology was also carried out on the mosquitoes that were not identified by MALDI-TOF MS (low scores), including the eight *Anopheles* spp. and 12 *Culex* spp. Sequencing of the *28S* gene was performed to identify the eight *Anopheles* spp. (3%, n = 8/264). The matching sequences corresponded to *Anopheles gambiae* Giles (n = 4) and *An. coluzzii* (n = 4), which were shown to share between 98.52 and 100% identity with Genbank (Table 2).

The acetylcholinesterase-2 and *COI* genes were amplified to identify the 12 *Culex* spp. which were misidentified (2%, n = 12/549) by MALDI-TOF MS. The sequences obtained correspond to the *Cx. quinquefasciatus* (n = 11) which were shown to share between 98.90 and 100% identity with Genbank and 100% identity with *Cx. perexiguus* (n = 1) (Table 2).

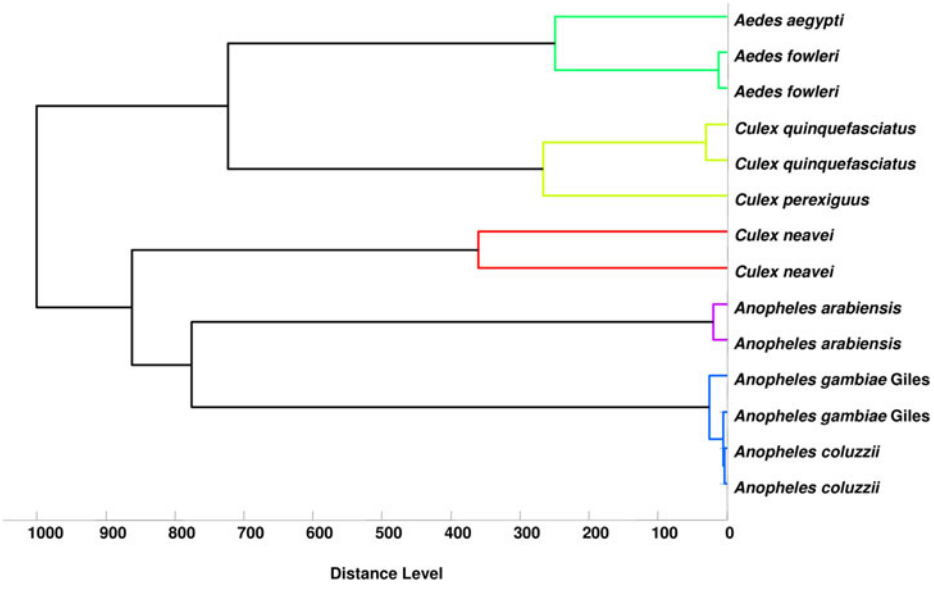


Fig. 5. MSP (Main Spectrum Profile) dendrograms of MALDI-TOF MS spectra of eight mosquito species collected in Mali. Clustering analysis was performed using MALDI Biotyper software. Distance unit corresponds to the relative similarity calculated from the distance matrix.

Table 2. Molecular identification of mosquitoes collected in Mali

Collection sites	Morphological identification	MALDI-TOF MS identification	Log score value	Genes	Molecular identification	% Identities Genbank	Accession number
Sotuba	*Anopheles* spp.	Human	[1.754]	28S	*Anopheles coluzzii*	100	AF470112.1
Donéguébougou	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.470]	28S	*Anopheles gambiae* Giles	99.25	AF470116.1
Donéguébougou	*Anopheles* spp.	Human	[1.854]	28S	*Anopheles coluzzii*	98.51	AF470113.1
Donéguébougou	*Anopheles* spp.	Less relevant	[1.553]	28S	*Anopheles coluzzii*	98.51	AF470113.1
Donéguébougou	*Anopheles* spp.	Less relevant	[1.610]	28S	*Anopheles coluzzii*	99.25	KT284724.1
Donéguébougou	*Anopheles* spp.	Human	[2.008]	28S	*Anopheles gambiae* Giles	98.14	AF470116.1
Donéguébougou	*Anopheles* spp.	*Anopheles coluzzii*	[1.864]	28S	*Anopheles coluzzii*	98.51	AF470113.1
Bougoula -hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.383]	28S	*Anopheles gambiae* Giles	98.88	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.389]	28S	*Anopheles gambiae* Giles	99.25	AF470115.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.404]	28S	*Anopheles gambiae* Giles	99.25	AF470116.1
Bougoula-hameau	*Anopheles* spp.	Less relevant	[1.581]	28S	*Anopheles gambiae* Giles	98.51	AF470115.1
Bougoula-hameau	*Anopheles* spp.	Human	[2.286]	28S	*Anopheles gambiae* Giles	98.88	AF470115.1
Bougoula-hameau	*Anopheles* spp.	Human	[1.961]	28S	*Anopheles gambiae* Giles	98.88	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles coluzzii*	[1.964]	28S	*Anopheles coluzzii*	98.51	AF470113.1
Bougoula -hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.237]	28S	*Anopheles gambiae* Giles	99.27	AF470116.1
Bougoula -hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.406]	28S	*Anopheles gambiae* Giles	99.27	AF470116.1
Bougoula -hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.390]	28S	*Anopheles gambiae* Giles	98.59	AF470116.1
Missabougou	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.099]	28S	*Anopheles gambiae* Giles	99.27	AF470116.1
Missabougou	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.401]	28S	*Anopheles gambiae* Giles	98.90	AF470116.1
Missabougou	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.283]	28S	*Anopheles gambiae* Giles	98.54	AF470116.1
Missabougou	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.258]	28S	*Anopheles gambiae* Giles	98.90	AF470116.1
Missabougou	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.368]	28S	*Anopheles gambiae* Giles	98.91	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.293]	28S	*Anopheles gambiae* Giles	98.16	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.293]	28S	*Anopheles gambiae* Giles	98.13	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.049]	28S	*Anopheles gambiae* Giles	99.26	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.311]	28S	*Anopheles gambiae* Giles	97.51	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[1.704]	28S	*Anopheles gambiae* Giles	97.84	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.110]	28S	*Anopheles gambiae* Giles	98.50	AF470116.1
Bougoula-hameau	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.233]	28S	*Anopheles gambiae* Giles	97.70	AF470116.1
Donéguébougou	*Anopheles* spp.	*Anopheles gambiae* Giles	[2.204]	28S	*Anopheles gambiae* Giles	98.13	AF470116.1
Bougoula-hameau	*Culex* spp.	*Culex pipiens*	[2.022]	Ace2	*Culex quinquefasciatus*	99.26	FJ416029.1

(*Continued*)

Table 2. (*Continued.*)

Collection sites	Morphological identification	MALDI-TOF MS identification	Log score value	Genes	Molecular identification	% Identities Genbank	Accession number
Bougoula-hameau	*Culex* spp.	*Culex pipiens*	[2.229]	Ace2	*Culex quinquefasciatus*	98.90	FJ416029.1
Donéguébougou	*Culex* spp.	*Culex quinquefasciatus*	[1.791]	Ace2	*Culex quinquefasciatus*	99.26	FJ416029.1
Missabougou	*Culex* spp.	Human	[2.182]	COI	*Culex quinquefasciatus*	99.38	KU920694.1
Missabougou	*Culex* spp.	Less relevant	[1.610]	COI	*Culex perexiguus*	100	KU380476.1
Missabougou	*Culex* spp.	*Culex pipiens*	[1.761]	Ace2	*Culex quinquefasciatus*	99.63	FJ416025.1
Missabougou	*Culex* spp.	Human	[2.042]	COI	*Culex quinquefasciatus*	99.08	KU920694.1
Missabougou	*Culex* spp.	*Culex pipiens*	[2.139]	Ace2	*Culex quinquefasciatus*	98.90	FJ416025.1
Missabougou	*Culex* spp.	*Culex pipiens*	[2.231]	Ace2	*Culex quinquefasciatus*	98.90	FJ416025.1
Missabougou	*Culex* spp.	Human	[2.514]	COI	*Culex quinquefasciatus*	99.84	KU920694.1
Missabougou	*Culex* spp.	*Culex pipiens*	[2.088]	Ace2	*Culex quinquefasciatus*	98.90	FJ416029.1
Missabougou	*Culex* spp.	Human	[2.294]	COI	*Culex quinquefasciatus*	99.69	KU920694.1
Yirimadio	*Culex* spp.	*Culex pipiens*	[2.032]	Ace2	*Culex quinquefasciatus*	98.90	FJ416029.1
Donéguébougou	*Culex* spp.	*Culex quinquefasciatus*	[2.000]	Ace2	*Culex quinquefasciatus*	99.26	FJ416029.1
Donéguébougou	*Culex* spp.	*Culex quinquefasciatus*	[2.009]	Ace2	*Culex quinquefasciatus*	99.27	FJ416025.1
Donéguébougou	*Culex* spp.	*Culex quinquefasciatus*	[2.207]	Ace2	*Culex quinquefasciatus*	98.90	FJ416025.1
Bougoula-hameau	*Culex* spp.	*Culex quinquefasciatus*	[2.406]	Ace2	*Culex quinquefasciatus*	98.90	FJ416019.1
Bougoula-hameau	*Culex* spp	*Culex neavei*	[2.839]	COI	*Culex neavei*	99.16	KU380473.1
Bougoula-hameau	*Culex* spp	*Culex quinquefasciatus*	[2.037]	Ace2	*Culex quinquefasciatus*	99.11	FJ416029.1
Bougoula-hameau	*Culex* spp	*Culex quinquefasciatus*	[2.197]	Ace2	*Culex quinquefasciatus*	99.55	FJ416029.1
Bougoula-hameau	*Culex* spp	*Culex quinquefasciatus*	[2.146]	Ace2	*Culex quinquefasciatus*	99.56	FJ416029.1
Bougoula-hameau	*Culex* spp	*Culex quinquefasciatus*	[2.383]	Ace2	*Culex quinquefasciatus*	99.56	FJ416029.1
Bougoula-hameau	*Culex* spp	*Culex quinquefasciatus*	[2.322]	Ace2	*Culex quinquefasciatus*	99.13	FJ416029.1
Donéguébougou	*Culex* spp.	*Culex quinquefasciatus*	[2.445]	Ace2	*Culex quinquefasciatus*	99.57	FJ416029.1
Donéguébougou	*Culex* spp.	*Culex quinquefasciatus*	[2.067]	Ace2	*Culex quinquefasciatus*	98.71	FJ416029.1
Donéguébougou	*Culex* spp.	*Culex quinquefasciatus*	[2.151]	Ace2	*Culex quinquefasciatus*	100	FJ416029.1
Missabougou	*Culex* spp.	*Culex quinquefasciatus*	[2.244]	Ace2	*Culex quinquefasciatus*	98.68	FJ416029.1
Missabougou	*Culex* spp.	*Culex quinquefasciatus*	[2.159]	Ace2	*Culex quinquefasciatus*	99.56	FJ416029.1

MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Ace 2: acetylcholinesterase-2; COI: the cytochrome *c* oxidase; %, per cent.

Table 3. Identification of the blood meals of mosquitoes collected in distinct ecological areas in Mali

			Blood meals identified by MALDI-TOF MS							
Sites	Morphological ID	Mosquito identified by MALDI-TOF MS	Human	Chicken	Cow	Donkey	Dog	Sheep	Not identified	Total
Bougoula-hameau	*Anopheles*	*Anopheles gambiae* Giles	87		3	5			3	98
	Anopheles	*Anopheles coluzzii*	3							3
	Culex	*Culex quinquefasciatus*	86	5			1			92
	Culex	*Culex neavei*	3							3
Donéguébougou	*Anopheles*	*Anopheles gambiae* Giles	46		6	1		2	4	59
	Anopheles	*Anopheles coluzzii*	5							5
	Anopheles	*Anopheles arabiensis*	2							2
	Culex	*Culex quinquefasciatus*	93	1			1		5	100
Missabougou	*Anopheles*	*Anopheles gambiae* Giles	13						2	15
	Culex	*Culex quinquefasciatus*	69	1					12	82
Sotuba	*Anopheles*	*Anopheles gambiae* Giles	51				3	1	6	61
	Culex	*Culex quinquefasciatus*	97						2	99
	Culex	*Culex neavei*	1							1
Yirimadio	*Aedes*	*Aedes aegypti*	1							1
	Culex	*Culex quinquefasciatus*	61	2					16	79
	Culex	*Culex neavei*	1							1
Total			619	9	9	6	5	3	50	701

ID, Identification; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Identification of the bloody WFPs sources by MALDI-TOF MS

A total 701 abdomens of engorged mosquitoes were crushed in WFPs in the field in Mali. The 701 bloody BWFPs were submitted for MALDI-TOF MS analysis in Marseille one month after sampling. Of the 701 BWFPs, 651 (93%) high-quality spectra were obtained. The 651 BWFPs MS high-quality spectra were queried against our blood source MALDI-TOF MS database for identification. They matched with spectra from our database, including those of mosquito abdomens engorged with human blood ($n = 619$), chicken blood ($n = 9$), cow blood ($n = 9$), donkey blood ($n = 6$), dog blood ($n = 5$) and sheep blood ($n = 3$) (Table 3). These blood meals were identified using MALDI-TOF MS with log score values (LSVs) ranging from 1.707 to 2.731. The MS spectra comparison of different host blood revealed an intra-species reproducibility and an inter-species specificity by Flex analysis (Fig. 6).

Molecular identification of the bloody mosquito WFPs

A total of 41 bloody WFPs identified by MALDI-TOF MS as mosquito abdomens engorged with human blood ($n = 21$), donkey blood ($n = 5$), chicken blood ($n = 4$), cow blood ($n = 5$), dog blood ($n = 4$) and sheep blood ($n = 2$) were randomly selected for sequencing by *COI* gene amplification. Thirty-three bloody WFPs sequences were obtained which confirmed the accuracy of the MS identification. However, for eight bloody WFPs, no quality sequences could be obtained. The results of the PCR based on bloody WFP sequencing highly correlated with the results of MALDI TOF MS identification (Table 4). The sequences obtained from seventeen bloody WFPs had identities between 98.52 and 100% against Genbank NCBI (Table 4).

Discussion

The goal of this work was not to provide precise data on the presence and abundance of various mosquito species in specific areas in Mali. Indeed, these data vary according to the type of climate and the seasons. However, we did want to test the usefulness of MALDI-TOF MS using mosquitoes collected in the field, as most preliminary studies have used laboratory specimens.

The use of MALDI-TOF MS has recently emerged in medical entomology, including for the identification of arthropods, their blood meals and the detection of potential microorganisms (Schaffner *et al.* 2014; Yssouf *et al.* 2016). The choice of the arthropod body part is critical for specimen identification by MALDI-TOF MS (Yssouf *et al.* 2016). For example, the legs from adult mosquitoes have been shown to be sufficient for identification, whereas whole specimens have been used for aquatic stages (larvae) (Nebbak *et al.* 2017).

Here, the MS spectra from mosquito legs collected in Mali, including 264 *Anopheles*, 549 *Culex* and five *Aedes*, permitted MALDI-TOF MS identification. The MS spectrum analyses from the mosquito legs revealed an intra-species reproducibility and inter-species specificity consistent with molecular validation (Fig. 5). Accurate identification of mosquitoes queried against the home-made MS database corresponded to 100% concordance with molecular identification results (Table 2). The consistent

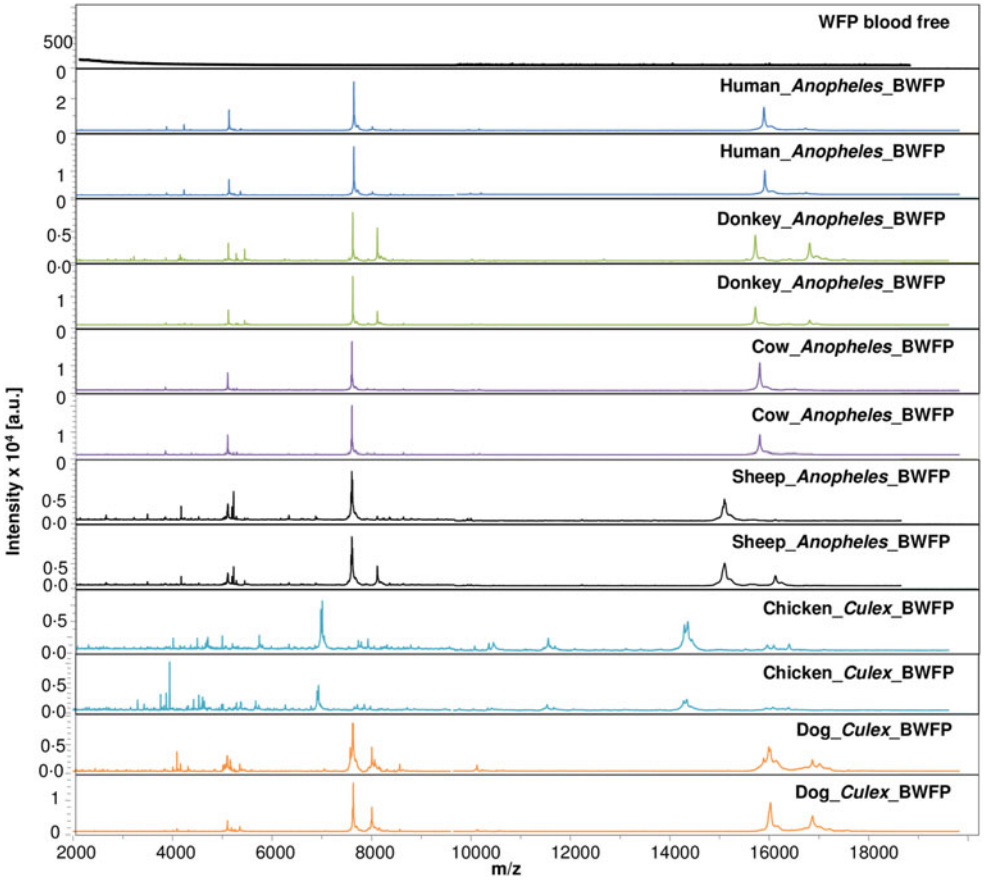


Fig. 6. The MS spectrum alignment from mosquito abdomen engorged on vertebrate host bloods and then crushed on Whatman filters. All bloody WFPs (BWFPs) were obtained from the field mosquitoes collected in Mali and crushed on WFPs. The MS spectrum alignment was performed by Flex analysis 3.3 software. The WFP blood free corresponds to the MS profiles of WFPs where no mosquito blood meal was released. The representative MS spectra from abdominal protein corresponds to *Anopheles gambiae* Giles abdomens BWFPs feed on human, donkey, cow and sheep blood, and *Culex quinquefasciatus* abdomens feed on chicken and dog blood. a.u. arbitrary units; *m*/*z* mass-to-charge ratio.

Table 4. Molecular identification of the blood from mosquito's abdomens crushed on Whatman filter papers

Mosquito blood meals sources identification by MALDI-TOF MS	Mosquito blood meals sources identification by COI gene amplification	% Identities Genbank	Accession number Genbank
Human	*Homo sapiens*	100	KM102136.1
Human	*Homo sapiens*	99.84	KY595668.1
Human	*Homo sapiens*	99.64	HM185231.1
Human	*Homo sapiens*	98.52	KM102136.1
Human	*Homo sapiens*	98.86	KY595669.1
Human	*Homo sapiens*	98.72	MF058292.1
Human	*Homo sapiens*	99.68	KF161694.1
Human	*Homo sapiens*	99.68	MF058210.1
Human	*Homo sapiens*	99.34	MF058210.1
Human	*Homo sapiens*	100	MF058210.1
Human	*Homo sapiens*	99.37	MF058210.1
Human	*Homo sapiens*	98.68	MF058210.1
Human	*Homo sapiens*	99.67	MF058210.1
Human	*Homo sapiens*	99.18	AY275535.2
Human	*Homo sapiens*	99.52	MF588867.1
Human	*Homo sapiens*	99.36	MF057217.1
Human	*Homo sapiens*	99.18	MF588867.1
Human	*Homo sapiens*	99.05	AY922271.1
Human	*Homo sapiens*	99.52	MF588867.1
Human	*Homo sapiens*	99.35	KM101695.1
Human	*Homo sapiens*	99.36	KF163046.1
Donkey	*Equus asinus*	99.37	KX683425.1
Chicken	*Gallus gallus*	99.68	KX781318.1
Chicken	*Gallus gallus*	99.22	KX781318.1
Chicken	*Gallus gallus*	99.21	KX781318.1
Cow	*Bos taurus*	99	KY650678.1
Donkey	*Equus asinus*	98.91	KX683425.1
Cow	Failed	–	–
Cow	*Bos taurus*	99	KY650678.1
Cow	Failed	–	–
Cow	Failed	–	–
Donkey	Failed	–	–
Chicken	*Gallus gallus*	98.52	KX781318.1
Dog	Failed	–	–
Dog	Failed	–	–
Dog	Failed	–	–
Donkey	*Equus asinus*	99.52	KX683425.1
Donkey	*Equus asinus*	100	KX683425.1
Sheep	*Ovis aries*	99.84	KP998473.1
Sheep	*Ovis aries*	99.21	KR868678.1
Dog	Failed	–	–

MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; COI, the cytochrome *c* oxidase; %, per cent.

identification between molecular biology and MALDI-TOF MS was validated by the choice of the *28S* gene for *Anopheles* species identification, and the acetylcholinesterase-2 and *COI* genes for *Culex* species identification. As shown in previous studies, the choice of these genes was highly relevant to discriminate and assess the phylogenetic relation between different mosquito species (Folmer *et al.* 1994; Fanello *et al.* 2002; Smith and Fonseca, 2004). Here, the quality of spectra was a very important element for identification, as more than 98% of the good quality spectra were identified with LSVs >1.8. The MALDI-TOF MS reference database has been updated with other mosquito species. It is necessary to create a reference database, which could subsequently be shared, and open access could be provided for routine arthropod identification. In this study, *Aedes* mosquitoes collected in Mali were correctly identified as *Ae. fowleri* using a database containing reference spectra of this species collected from La Reunion Island only, which is located in the Pacific Ocean. Therefore MALDI-TOF MS appears as an efficient tool for the identification of arthropods collected from distant geographical areas.

For Raharimalala *et al.* (2017), the usefulness and accuracy of MALDI-TOF MS as a tool to identify vector mosquito species requires the creation of an international database (Raharimalala *et al.* 2017). In this study, 2.40% of inconsistent MS leg results were attributed to low-quality MS spectra for identification. The MS spectra of some legs ($n = 9$) (Table 2) that matched with reference spectra of mosquito abdomens engorged with human blood were attributed to traces of blood on the legs during the abdomen crushing process onto WFPs. This phenomenon of low-quality spectra, leading to lower identification rates have been reported in arthropod identification such as at the aquatic mosquito stage (Dieme *et al.* 2014). According to the reproducibility of MS spectra, the hierarchical clustering showed that all specimens from the same species were grouped in the same branch. These results are similar to previous studies supporting interspecies reproducibility for mosquito identification (Yssouf *et al.* 2013). Additionally, we stress that MS cannot yet be considered a reliable tool for the phylogenetic study of mosquito species (Yssouf *et al.* 2013).

Our results showed that 95% of the collected mosquitoes had fed on human blood. This result is not surprising because all mosquitoes were collected inside homes. The advantage of our MALDI-TOF approach is its rapidity, effectiveness and reliability in determining bloody WFPs, since more than 100 bloody WFPs specimens were processed per day. Previously, the authors had demonstrated that the profiles of abdominal spectra of mosquito females engorged on human blood are the same, regardless of whether they were crushed or not crushed on WFPs (Niare *et al.* 2017). Indeed, the home-made database contains filter papers with *Anopheles gambiae* engorged blood such as human blood and sheep blood. These authors tested WFP either with the crushed abdomen of a non-engorged mosquito or simply as a control (Niare *et al.* 2017). These results suggest that MALDI-TOF MS is not time-consuming in comparison with molecular tools and serological techniques. The eight bloody WFPs which failed molecular biology identification may be attributed to blood meal digestion. As previously reported, the time of the host blood digestion in the mosquito has an impact upon blood meal identification by MALDI-TOF MS and molecular biology (Niare *et al.* 2016). Moreover, the molecular biology results of the seventeen BWFPs sequences obtained by *COI* gene amplification corroborated the MALDI-TOF MS identification (Table 4).

Interestingly, as we have recently found that MALDI-TOF may also recognize mixed blood meals (unpublished), we did not find any mixed blood meals either by molecular tools nor by MALDI-TOF. The authors experimentally engorged *An. gambiae* Giles mosquitoes with a mixture of blood from distinct vertebrate

hosts, such as human, sheep and dogs. Their results demonstrate that mixed mosquito blood meals can be successfully identified, depending on the concentration ratio (unpublished). Recently, some authors have also used the proteomic approach to identify the sources of tick mixed blood meals (Onder *et al.* 2013).

Of the mosquitoes identified by MALDI-TOF MS, *A. gambiae* Giles and *Cx. quinquefasciatus* were widely distributed across all collection sites. Our work enabled *Cx. neavei* and *Cx. perexiguus* to be detected for the first time in Mali. Currently, few studies have been carried out on the *Culex* species in Mali, particularly on their abundance, ecology and the infectious pathogens transmitted by these vectors. *Culex* species are widely distributed in West Africa and are found in any type of breeding sites (clear and polluted water), whereas the *Anopheles* species colonizes sunny, fresh water (Becker *et al.* 2010). There is an abundant literature on these mosquitoes, the well-known distribution of *Cx. neavei* and *Cx. perexiguus* in sub-Saharan Africa and their implication in the transmission of many arboviruses (Jupp *et al.* 1986; Fyodorova *et al.* 2006; Nikolay *et al.* 2012; Fall *et al.* 2014; Gould *et al.* 2017). The presence of these potential vectors in Mali might be of epidemiological importance.

Our study is the first to use MALDI-TOF MS as a tool for monitoring field mosquitoes in Africa, particularly in Mali, an endemic malarial area. Moreover, when the MALDI-TOF MS device is bought for clinical microbiology purposes, it can also be used for medical entomology at no additional cost. For example, at the Dakar hospital in Senegal, the MALDI-TOF MS equipment that was initially bought for clinical microbiology has been used for field entomology surveys and has successfully identified *Culicoides* (Sambou *et al.* 2015). In Senegal, the acquisition of MALDI-TOF MS equipment has revolutionized bacteriology laboratories and clinical microbiology domains, suggesting that this technique can be used as a front-line tool in tropical countries (Lo *et al.* 2015).

Although the time period for blood meal source determination by MALDI-TOF MS was shorter than that of molecular biology or ELISA, the rapidity and low cost of the reagents made this proteomic method a financial and reliable competitive strategy. However, the relatively high cost of the machine could be an impediment to implementation of this innovative tool in laboratories. The cost of purchasing the MALDI-TOF MS equipment in under-developed countries such as Mali (sub-Saharan Africa) could be a limitation to estimating the local vector-borne risk. However, when the device is bought by a microbiology lab it can be used in medical entomology at no additional cost.

Concluding remarks

The present study successfully identified field mosquitoes and the sources of their blood meals using MALDI-TOF MS. The mosquitoes collected in Mali were correctly identified based on reproducibility and specificity from the protein profiles of leg extracts. The innovative MALDI-TOF MS tool enabled the rapid identification of eight mosquito species in Mali during entomological surveys. The challenge is to maintain and develop collaboration between north and south to facilitate the acquisition of the MALDI-TOF MS equipment.

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Author contributions. PP, TF and NS designed and developed the protocol. TF and NS performed the protocol. PP, TF, NS and ML analysed the data. KKA, DZA, OA, BMJ, OD and RD contributed reagents/materials/analysis tools. PP, TF and NS wrote the paper. OD and RD contributed to editing the paper. All authors agreed to publication.

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