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Epidémiologie et formes cliniques atypiques de la leishmaniose à *Leishmania infantum* Apport du génotypage parasitaire

Pour obtenir le grade de DOCTORAT d'AIX-MARSEILLE UNIVERSITÉ

SPÉCIALITÉ : Maladies Transmissibles et Pathologies Tropicales

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AVANT PROPOS

Le format de présentation de la thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe permettant un meilleur rangement des thèses traditionnelles.

Par ailleurs, la partie introduction et la bibliographie sont remplacées par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse.

Par ailleurs, la thèse est présentée sur article publié, accepté ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

SOMMAIRE

RESUME

Résumé :

La leishmaniose à *Leishmania infantum* est une zoonose transmise de mammifère à mammifère par la piqûre d'un insecte vecteur, le phlébotome femelle. S'il est classiquement décrit la leishmaniose viscérale avec la triade classique fièvre, pâleur et splénomégalie, de nombreuses formes cliniques peuvent être associées à ce parasite. Le portage asymptomatique est la forme la plus fréquente et la plus répandue dans l'Ancien Monde ou le Nouveau Monde. Entre la leishmaniose viscérale et le portage asymptomatique, plusieurs formes cliniques sont présentes. Ainsi certains sujets vont exprimer la leishmaniose sous forme d'adénopathies isolées. Ces formes sont intermédiaires entre une expression pauci symptomatique et une leishmaniose viscérale larvée. Alors que *L. infantum* n'est pas classiquement retrouvé dans des formes muqueuses, des cas ont été récemment décrits. Ces formes muqueuses isolées ne sont pas rares puisque sur les 3 CHU Marseille, Montpellier et Nice, entre 1997 et 2009, 10 cas de leishmaniose muqueuse à *L. infantum* ont été diagnostiquées principalement chez des sujets immunodéprimés. Il est important de faire le diagnostic de ces formes cliniques particulières puisqu'elles ont pour diagnostic différentiel des néoplasies (lymphome pour la première et néoplasie de la sphère ORL pour la seconde). Afin d'appréhender le rôle du parasite dans l'expression clinique, il a été réalisé le typage par les microsatellites de neuf souches isolées de sujets porteurs asymptomatiques. Il s'avère que ces neuf souches sont très peu polymorphes et que sept d'entre elles possèdent un génotype unique. De plus, elles sont très différentes des souches issues des sujets séropositifs pour le VIH. Si la génétique des souches semble avoir un rôle dans l'expression clinique de la maladie, l'environnement dans lequel vivent les sujets en zone d'endémie est associé à un sur-risque de développer une leishmaniose viscérale. En effet, tandis qu'à Marseille, les cas de leishmanioses viscérales

surviennent dans un environnement urbain, ceux de la région niçoise apparaissent dans un environnement rural, comme cela est plus classiquement décrit. Afin de déterminer si le parasite est impliqué dans les différences concernant le foyer marseillais et niçois, une étude des souches de *L. infantum* par les microsatellites est en cours dans ces deux foyers.

Abstract :

Leishmaniasis due to *Leishmania infantum* is a zoonotic disease transmitted from mammal to mammal through the bite of an insect vector the sandfly female. Beside the classical triad of visceral leishmaniasis symptoms: fever, pallor and splenomegaly, many clinical forms could be associated with this parasite infection. Asymptomatic carriage of *L. infantum* is the most common and the most widespread in the Old World and New World. Many other clinical forms are present and some subjects will develop only isolated lymphadenopathy. These forms are intermediate between pauci symptomatic and visceral leishmaniasis forms. Whereas *L. infantum* is not typically associated with mucosal forms, several cases have been described. Indeed, in the 3 academic hospitals of Marseille, Montpellier and Nice from 1997 to 2009, 10 cases were revealed mainly in immunocompromised patients. To understand the role of parasite in clinical expression, nine strains isolated from asymptomatic carriers were genotyped using microsatellite. The nine strains have few polymorphisms and seven of them are identical with a unique genotype. In addition, those strains are very different from strains of HIV-positive subjects. If the strains genetic appears to have a role in the clinical expression of the disease, the environment in which individuals live in endemic areas is associated with an excess of risk to develop visceral leishmaniasis. While in Marseille, cases of visceral leishmaniasis occur in an urban environment, they take place in Nice in a rural environment, as it is classically described. To investigate differences between parasite strains form Nice and Marseille studies with microsatellites are ongoing.

INTRODUCTION

Les leishmanioses sont des parasitoses dues à un protozoaire flagellé appartenant au genre *Leishmania*. Elles sont transmises de mammifère à mammifère par la piqûre d'un insecte vecteur, le phlébotome femelle. Il est décrit des leishmanioses zoonotiques et anthroponotiques (Desjeux, 2001). Dans les formes anthroponotiques, l'homme est considéré comme la seule source d'infection du vecteur alors que pour les formes zoonotiques, des animaux constituent le réservoir ce qui permet de maintenir et disséminer les parasites (Desjeux, 2001). Il existe plusieurs espèces ayant chacune chez l'homme un tropisme préférentiel entraînant des pathologies viscérales disséminées, des pathologies cutanées ou muqueuses (Tableau 1) (World Health Organization, 2010).

Tableau 1 :

Tropisme	Viscérotropique	Dermotropique		Mucotropique
Ancien Monde	*L. donovani* ***L. infantum***	*L. major* *L. tropica* *L. killicki*[a] *L. aethiopica* ***L. infantum***		
Nouveau Monde	***L. infantum***	***L. infantum*** *L. mexicana* *L. pifanoi*[a] *L. venezuelensis* *L. garnhami*[a] *L. amazonensis*	*L. braziliensis* *L. guyanensis* *L. panamensis* *L. shawi* *L. naïffi* *L. lainsoni* *L. lindenbergi* *L. peruviana* *L. colombiensis*[b]	*L. braziliensis* *L. panamensis*

[a] Le statut de ces espèces est soumis à discussion
[b] La position taxonomique est soumise à discussion

Dans le bassin méditerranéen, l'Asie et l'Amérique latine plusieurs espèces de *Leishmania* sont présentes mais une seule est commune à ces trois zones géographiques : *Leishmania infantum*. C'est une zoonose ayant le chien comme réservoir et principale victime (Aït-Oudhia et al., 2011). Du fait de la mise en évidence fréquente du parasite chez des enfants, Charles

Nicolle le baptise en 1908, *Leishmania infantum* ; agent du « kala-azar » infantile (Aït-Oudhia et al., 2011). Dans les années 1980, il est démontré qu'en plus des formes vicérales, *L. infantum* peut aussi donner des formes cutanées (Rioux et al., 1980). Depuis, la leishmaniose à *L. infantum* a été décrite chez les sujets immunodéprimés avec notamment une émergence importante de cas chez les sujets séropositifs pour VIH (Alvar et al., 2008). Actuellement, il est admis que *L. infantum* (syn. *Leishmania chagasi*) peut être responsable de plusieurs symptomatologies chez l'homme avec par ordre de fréquence : le portage asymptomatique, la leishmaniose viscérale, fatale si elle n'est pas traitée, et des formes cutanées. D'autres formes cliniques atypiques sont aussi décrites comme la leishmaniose ganglionnaire et la leishmaniose muqueuse. Afin de comprendre le rôle joué par le polymorphisme de *L. infantum* dans la variabilité des expressions cliniques chez l'hôte vertébré, diverses méthodes ont été développées. L'identification du zymodème des souches au moyen d'une étude izoenzymatique a longtemps constitué la base de l'étude du polymorphisme des *Leishmania* (Pratlong et al., 2004, 2009; Aït-Oudhia et al., 2011). Cependant, la majorité des souches du bassin méditerranéen, qu'il s'agisse de souches d'origine canine ou humaine, appartiennent au zymodème MON-1 (Pratlong et al., 2009; Aït-Oudhia et al., 2011). Si l'on considère plus précisément la région Provence Alpes Côtes d'Azur-Est (PACA-Est), le zymodème MON-1 est majoritaire tandis que le zymodème MON-24 est parfois retrouvé dans certains cas de leishmaniose cutanée chez l'immunocompétent et de leishmaniose viscérale chez des sujets séropositifs pour le VIH (Pratlong et al., 2004). L'étude izoenzymatique atteint donc rapidement ses limites lorsqu'il s'agit d'appréhender le rôle des souches et de leur virulence en pathologie humaine et vétérinaire. Afin de mieux étudier cet aspect, nous avons entrepris, au cours de cette thèse, l'étude des souches de *L. infantum* par l'analyse du polymorphisme de plusieurs microsatellites. Nous verrons que cet outil peut s'avérer discriminant pour différencier les souches issues de porteurs asymptomatiques de celles isolées chez les patients

présentant une forme clinique patente. Cependant, comme dans toutes les maladies parasitaires, l'expression clinique dépend aussi de l'hôte. Dans ce travail, le rôle de l'hôte ne sera pas directement abordé. Par contre, une étude de l'environnement associé aux cas de leishmaniose viscérale et en particulier des lieux de vie a été entreprise.

Ce travail a été réalisé dans le laboratoire d'accueil : Toxines microbiennes dans la relation hôte-pathogènes, INSERM 1065, Centre Méditerranéen de Médecine Moléculaire (C3M), Faculté de Médecine, Université de Nice Sophia Antipolis (Directeur Dr. Emmanuel LEMICHEZ).

L'objectif de cette thèse est de mieux caractériser les formes cliniques atypiques de la leishmaniose à *L. infantum*, d'étudier le polymorphisme des souches ainsi que l'environnement où circulent ces souches dans le sud de la France principalement.

Revue

IMPORTANCE OF WORLDWIDE ASYMPTOMATIC CARRIERS OF *LEISHMANIA INFANTUM* (*L. CHAGASI*) IN HUMAN

ACTA TROPICA

2011

LE PORTAGE ASYMPTOMATIQUE DE *L. INFANTUM*

Dans un premier, temps nous avons fait le point sur la forme clinique la plus fréquente qui est le portage asymptomatique humain de *Leishmania infantum*. Ce travail a donné lieu à une revue générale qui a été publiée dans Acta Tropica en 2011.

La revue bibliographique sur le portage asymptomatique de *Leishmania infantum* dans le monde permet de faire un bilan de ce qui a été réalisé sur cette entité clinique particulière. En effet, si la fréquence du portage asymptomatique à *L. infantum* chez le chien au niveau mondial a été largement documentée, il n'existait jusqu'à présent aucun travail sur la fréquence et les conséquences d'un portage asymptomatique à *L. infantum* chez l'homme au niveau mondial. Les données récoltées sont issues d'études effectuées principalement en Europe, au Brésil, en Chine et au Moyen Orient.

Le premier chapitre de l'article est consacré aux outils diagnostic. Ceux-ci apparaissent très variés en fonction des habitudes et des moyens de chaque investigateur et ils évoluent dans le temps. *A priori*, seuls les tests de diagnostic direct mettant en évidence la présence des parasites (culture, examen au microscope), peuvent traduire le portage asymptomatique. Cependant, les études montrant la concordance entre une PCR positive et un parasite viable indiquent que ce test peut être considéré comme un test direct. Néanmoins, la majorité des études publiées à ce jour ont été réalisées avec des tests indirects (Immunofluorescence, ELISA, Western blot, DAT et l'exploration de la réponse cellulaire à *Leishmania* par intradermoréaction). La concordance entre les tests directs et indirects mise en évidence dans certaines études, la répétition de la positivité chez certains individus au cours du temps, sont plus compatibles avec un portage asymptomatique qu'avec des séquences d'infections suivies d'élimination totale des parasites. Ainsi, les tests indirects peuvent être pris en compte comme

reflet du portage asymptomatique. Si les tests indirects ont été majoritairement utilisés et le sont encore, les dernières publications présentes dans cette revue incluent le diagnostic par biologie moléculaire qui sera probablement le plus réalisé dans les années à venir. En fonction des zones géographiques et selon les outils biologiques utilisés, il est obtenu des chiffres de prévalence du portage asymptomatique allant de 0,6 à 71,3 %.

Le portage asymptomatique étant bien plus fréquent que le développement de la maladie, des études comparant les profils immunologiques de ces deux populations ont été réalisées. Ces études montrent que les porteurs asymptomatiques expriment un profil mixte Th1 Th2 de cytokines produites par les lymphocytes T-helper (Th) tandis que les sujets malades expriment un profil Th2.

Les données actuelles sur la leishmaniose, montrent que la leishmaniose viscérale ne représente que le haut de l'iceberg et pose la question du rôle du portage asymptomatique en tant que réservoir, de la possibilité de la transmission du parasite par le don du sang et de l'évolution clinique de ce portage asymptomatique en cas d'immunodépression.

Revue

IMPORTANCE OF WORLDWIDE ASYMPTOMATIC CARRIERS OF *LEISHMANIA INFANTUM* (*L. CHAGASI*) IN HUMAN

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Importance of worldwide asymptomatic carriers of *Leishmania infantum* (*L. chagasi*) in human

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ABSTRACT

Leishmaniasis due to *Leishmania infantum* (syn. *L. chagasi*) infection is a zoonotic disease present mainly in Mediterranean basin, central Asia and Brazil. Besides a limited number of human cases of clinical visceral leishmaniasis, a great number of infections remains asymptomatic. In this review, the prevalence of asymptomatic carriers of *L. infantum* was evaluated worldwide using parasitological methods or indirect testing such as a skin test or serology. The consequences of the presence of asymptomatic carriers on parasite transmission by blood donation or the development of clinical visceral leishmaniasis in immunocompromised individuals and its possible role as reservoir are discussed.

Contents

1. Introduction

Human visceral leishmaniasis (VL) is a parasitic disease caused by obligate intracellular protozoans from the genus *Leishmania*. *Leishmania donovani* and *Leishmania infantum* in the Old World and *Leishmania chagasi* (*L. chagasi* and *L. infantum* are synonymous) in the New World, are the species that are implicated in disease. These protozoans are spread through the bite of infected female phlebotomine sandfly. For *L. infantum* (*L. i*), dogs represent the main reservoir and victims. In dogs, the number of asymptomatic carriers (AC) is well documented (Miro et al., 2008) and ranges from almost 25% to more than 80% depending on the geographic area and detection technique used (Berrahal et al., 1996; Papadopoulou et al., 2005; Queiroz et al., 2009). In humans, patent VL due to *L. i* is characterized by a classical clinical triad of irregular fever, pallor, and splenomegaly. Nevertheless, the outcome of

Abbreviations: VL, visceral leishmaniasis; *L. i*, *Leishmania infantum*; AC, asymptomatic carriers.

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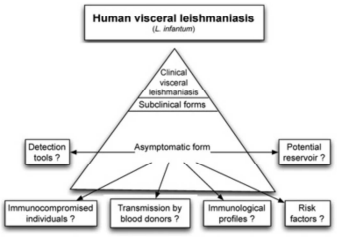

Fig. 1. Asymptomatic carriers of *Leishmania infantum*, importance and question marks.

infection can be variable ranging from an asymptomatic form to obvious disease with subclinical forms also termed intermediate or oligosymptomatic forms. Asymptomatic forms characterize individuals infected by *Leishmania* parasite and in apparently healthy condition. Subclinical forms are characterized by at least one clinical manifestation such as lymphadenopathy or mild symptoms (fever, cough, diarrhea, malaise, mild hepatomegaly and eventually splenomegaly) associated with at least one positive biological test currently used in VL diagnosis (Badaro et al., 1986). Diagnosis of leishmaniasis caused by *L. i* is established upon the demonstration of parasite by microscopic examination, culture, or PCR, performed from tissue samples. In addition, detection of specific anti *L. i* antibodies is sometimes concomitantly performed as an aid for the diagnosis. The estimated annual incidence of VL due to *L. i* is around 50 000 new cases. However the full-blown VL cases represent only the tip of the iceberg. World Health Organization (WHO) reports that "Infections due to *Leishmania infantum* are apparently often asymptomatic. Most individuals who have evidence of exposure to *Leishmania*, with a positive leishmanin skin test or positive serology [. . .] do not recall having a clinical illness". Actually, the majority of infected persons do not progress to patent VL (Badaro et al., 1986; WHO, 1991; Pearson and Sousa, 1996). The aim of this review is to draw an overview of AC in *L. i* infection worldwide in humans including the available biological tools for diagnosis and their limitations. Additionally, we discuss the prevalence of AC in immunocompetent and immunocompromised individuals and the factors associated with the susceptibility to asymptomatic infection. We also present data on the immunological status of the AC. Finally, the possible role of AC as reservoir and particularly their possible involvement in transmission by blood transfusion are discussed (Fig. 1).

2. Detection of asymptomatic carriers

The first test able to detect AC was the leishmanin skin test (LST). In 1959, Manson-Bahr, using the Montenegro LST, found positive subjects without symptoms of patent VL (Pampiglione et al., 1975). The term "asymptomatic infection" was used for the first time in 1974 by Pampiglione et al. in a study on *L. i* using the LST in Italy and confirmed by Badaro et al. in 1981 to 1984 in Brazil (Pampiglione et al., 1974; Badaro et al., 1986). Since that time, AC demonstration has benefited from the direct and indirect methods used for the diagnosis of patent VL due to *L. i.*

Direct methods are based on the demonstration of parasite by microscopic examination, culture, or PCR. Indirect methods include LST and antibody searches by various techniques including Immunofluorescence antibodies test (IFAT), direct agglutination test (DAT), ELISA with single (rk39) or crude *Leishmania* antigens, Western blot, or immunochromatographic test (rk39). AC have been assessed using antigen recall experiments *in vitro*, where immune memory response against *L. i* is assessed following challenge with *L. i* antigens generally through cytokine production (de Gouvea Viana et al., 2008). From a strict point of view, only the direct methods which demonstrated *L. i* parasites in humans could be used for the diagnosis of AC. According to this criterion AC has only been evidence in three studies (Table 2) (le Fichoux et al., 1999; Riera et al., 2004, 2008). Importantly, PCR testing can be considered as a true direct method for detecting the parasite presence, as recent studies showed that *Leishmania* nucleic acids detected by PCR came from living parasites and were rapidly degraded following parasite death (de La Llave et al., 2011). On the contrary, the reliability of indirect tests for establishing an asymptomatic infection can be questioned. Indeed, a positive LST or serology at a given time may not reflect a chronic infection but more simply a recent contact with parasite followed by sterile cure. However, the sporadic but repetitive positive serology or LST observed overtime in some individuals living in endemic areas in some cases are better explained by an asymptomatic infection rather than cycles of infection following by total parasite clearing (le Fichoux et al., 1999; Martin-Sanchez et al., 2004; de Gouvea Viana et al., 2008; Moreno et al., 2009; Silveira et al., 2009, 2010b; Biglino et al., 2010). In this case, parasites occurring in safe targets cells (cells that are not able to exert antiparasite activities) could occasionally, and for non-elucidated reasons, proliferate leading to repetitive antigenic stimulation of the innate and adaptative immune system (Bogdan, 2008). This in turn would confer, at least in immunocompetent individuals, resistance to the development of clinical VL or to reinfection. This concept is further supported by the appearance of clinical VL in some AC following severe immunosuppression and parasite reactivation. However, the concept does not rule out that in AC further parasite contact can contribute to maintain or increase antibody or T cell responses. For these reasons, as in nearly all previous studies, we shall consider that even indirect tests are likely to reflect an asymptomatic infection. All the techniques used for measuring the frequency of AC exhibit variable sensitivity and specificity for detecting the expected low parasite burden occurring in safe targets and the immune response associated with asymptomatic infection (Bogdan, 2008). For example, PCRs generally detects more AC than LST, the ELISA or IFAT tests being the least sensitive (Mary et al., 2006). ELISA tests using crude antigens are generally more sensitive but less specific than those developed with single *Leishmania* protein (D'Oliveira Junior et al., 1997). Detection of antibodies against 14–18 kDa *L. i* antigens by Western blot analysis is generally more sensitive and specific than ELISA with crude antigens (Mary et al., 1992; Marty et al., 1994; Biglino et al., 2010). It is noteworthy that the weak overlap observed in some studies between direct and indirect testing and even between two serological tests indicates that the use of more than one diagnostic method increases the proportion of AC detected (Alborzi et al., 2008; de Gouvea Viana et al., 2008). Therefore, the use of a single serological method for assessing the frequency of AC may represent a risk of case underestimation.

3. Asymptomatic carriers in endemic areas

Data on the prevalence of *L. i* AC worldwide is summarized in Table 1. As DAT or ELISA Kmp11 was used only once, studies conducted with these tests are not taken into account in the following analysis. Most epidemiological studies were conducted in Brazil and in the south of Europe. Strikingly the prevalence of AC, detected by single test, was highly variable (0.6–71.3%), depending on the test used for detection. The most frequently used technique was LST, followed by ELISA, with crude *Leishmania* antigens, and in the most recent studies PCR. As already mentioned, the use of more than one technique increases the number of detected cases and this was exemplified by the increase rate of positive detection found in one study using five different tests (de Gouvea Viana et al., 2008).

Table 1
Surveys of asymptomatic infection in endemic areas.

Country/Place	Techniques	Number of tested inhabitants	Results (%)	References
South of Europe				
Sicily, Italy	LST	n/a	16.6	Pampiglione et al. (1975)
Abruzzi, Italy	LST	n/a	27.5	Cited in Marty et al. (1992)
Tuscany, Italy	LST	n/a	15.3	Bettini et al. (1983)
Pyrénées Orientales, France	LST	n/a	15	Cited in Marty et al. (1992)
Sardina, Italy	LST	640	9.7	Gramiccia et al. (1990)
Alpes Maritimes, France	LST	237	30	Marty et al. (1992)
Alpes Maritimes, France	LST/Western blot	50	32/38	Marty et al. (1994)
Alpes Maritimes, France	LST/Western blot	47	46.8/42.5	Marty et al. (1995)
Granada and Malaga province, Spain	LST	1286	44	Acedo Sanchez et al. (1996)
Granada and Malaga province, Spain	LST	1258	42.40	Morillas et al. (1996)
Alacantí region, Spain	LST	184	52.8 of adults and 11.5 of children	Moral et al. (2002)
population of Castilla-Leon, Spain	ELISA (crude antigen)	4825	4.90	Garrote et al. (2004)
Seville, southern Spain	IFAT	95	24.00	Martin-Sanchez et al. (2004)
Epirus region, Greece	IFAT	1200	0.50	Papadopoulou et al. (2005)
Macedonia and Thrace regions, Greece	IFAT and ELISA (crude antigen)	1525	2.80	Diza et al. (2008)
Area of Marseilles	PCR	81	58%	Mary et al. (2006)
Central Piedmont, Italy	WB/PCR in WB+/PCR in WB-	526	7.41/56.4/4.2	Biglino et al. (2010)
South America				
State of Bahia, Brazil	LST/ELISA (crude antigen)	135	45/27	D'Oliveira Junior et al. (1997)
Brotas, Brazil	ELISA (crude antigen)	920	10.30	Jeronimo et al. (2000)
Rio Grande do Norte, Brazil	ELISA (rK39)/ELISA (crude antigen)	168	2.9/8.3	Braz et al. (2002)
Piauí, Brazil	LST/ELISA (crude antigen)	167/168	50/13.9	Werneck et al. (2002)
Piauí, Brazil	PCR/LST	101/87	7.9/71	Costa et al. (2002)
Rio Grande do Norte, Brazil	ELISA (KMP11 antigen)	27	0.05	Passos et al. (2005)
Maranhão, Brazil	LST/ELISA (rK39)/ELISA (crude antigen)	1520	61.1/19.4/19.7	Nascimento Mdo et al. (2005)
General Carneiro district, Brazil	IFAT/ELISA (crude Antigen)/rK39 immunochromatographic	1604	2.4/3.4/5.6	Moreno et al. (2006)
Maranhão, Brazil	Western blot	1100	0.60	Mendes et al. (2007)
Araçatuba, Brazil	rK39 dipstick	A1: 125 A2: 125	Two area: 20 in A1 and 4.8 in A2	Barao et al. (2007)
Minas Gerais, Brazil	LST/IFAT/ELISA (crude antigen)/ELISA (rK39)/PCR	138	2.9/5.1/9.4/5.1/18.1	de Gouvea Viana et al. (2008)
Belém, Brazil	LST/IFAT	946	11.2/3.4	Crescente et al. (2009)
Belém, Brazil	LST/IFAT	946	20.8/6.5	Silveira et al. (2009)
Belo Horizonte, Brazil	PCR/ELISA (crude antigen Amazonensis)/ELISA (crude antigen *Infantum*)/ELISA (rK39)	136	71.3/60.2/40/28.7	Moreno et al. (2009)
Cametá municipality, Brazil	LST and/or IFAT	174	15.8	Silveira et al. (2009)
Minas Gerais, Brazil	ELISA (crude antigen)/ELISA rK39/ELISA rK26/IFAT	1017	26.6/14.6/12.7/8.5	Romero et al. (2009)
Minas Gerais, Brazil	ELISA (crude antigen)/ELISA rK39/ELISA rK26/IFAT	224	19.2/37.5/21.4/11.2	Romero et al. (2009)
Belém, Brazil	LST and/or IFAT	120	73.4	Silveira et al. (2010a)
China				
Endemic area in China	PCR	n/a	33	Gao et al. (2006)
Gansu Province, China	PCR/ELISA (crude antigen)/rK39-dipstick	n/a	30.9/24.2/0	Wang et al. (2007)
Middle East				
Denizli province, Turkey	Western blot	81	7.41	Sakru et al. (2007)
Eskisehir, Bilecik, Kutahya and Afyon provinces, Turkey	ELISA (commercial ELISA)/ELISA (crude antigen)/ELISA (rK39)/IFAT	572	5.2/4.7/3.6/3.8	Dogan et al. (2008)
Fars province, Iran	DAT/PCR	802	North-western: 1.9/16; South-east: 1.5/8.5	Fakhar et al. (2008)
Ghir Karzin and Sar Mashhad in Iran	LST/IFAT/PCR	388	34/54.4/24.5	Alborzi et al. (2008)

4. Immunological profiles of human *L. i* asymptomatic carriers

Several studies have demonstrated that clinical forms of VL are under the control of the host's innate and adaptative immune response. In particular, accumulating data have suggested that innate immune responses play a pivotal role during host resistance, both in controlling parasite growth during the early stages of infection and in driving the cytokine milieu in which T cells are primed. Data derived from *in vitro* challenge experiments of human peripheral blood mononuclear cells (PBMC) or T cell clones with *Leishmania* antigens have been used to classify the immune responses into type 1 and type 2 according to the associated cytokine profiles (Carvalho et al., 1989; Kharazmi et al., 1999). As in mouse models, a type 1 response is characterized by IFN-γ, IL-12 and TNF-α and is associated with resistance to infection, whereas predominant type 2 response is accompanied by IL-4, IL-5 and IL-10 production and is linked to disease progression. However, contrary to what has been reported in the BALB/c mouse model infected with *L. major*, the immune response to *L. i* infection observed in BALB/c mouse model (Ferrua et al., 2006) or in patients with clinical VL to *L. i*, is not polarized (de Gouvea Viana et al., 2008). Experiments done on *L. chagasi* infection in human showed that patent VL is accompanied by circulating mixed type1/type2 cytokine pattern including IL-8, IFN-γ, TNF-α, IL-6 and IL-10 (Peruhype-Magalhaes et al., 2006). *Ex vivo* short-term culture of

Table 2
Surveys of asymptomatic infection among blood donors.

Country/Place	Techniques	Number of tested inhabitants	Results (%)	References
Rome and Sicily, Italy	Indirect hemagglutination	374/217	2.7/4.1	Federico et al. (1991)
South of France	WB/Culture	463	13.2/1.9	Kubar et al. (1997)
Rio Grande do Norte, Brazil	Fucose–mannose ligang (FML) ELISA	1194	9	Luz et al. (1997)
South of France	WB/PCR in WB+/Culture in WB+	566/73/73	13.4/12/12	le Fichoux et al. (1999)
Crete, Greece	WB/PCR/Flow cytometry	2000	15.2/1.7/1.65	Kyriakou et al. (2003)
Balearic Island, Spain	ELISA/WB/Nested-PCR/Culture	656/656/122/67	2.4/3.5/22.1/4.5	Riera et al. (2004)
Sicily, Italy	ELISA/IFAT	500/298	0/0	Colomba et al. (2005)
Balearic Island, Spain	WB/DTH/Nested-PCR/Culture	1437/73/304/304	3.1/11/5.9/0.6	Riera et al. (2008)
Western Sicily, Italy	IFAT/PCR and dot blot hybridization in IFAT positive	1449/11	0.76/36.4	Scarlata et al. (2008)

PBMC showed a similar pattern but the frequency of monocytes producing TNF-α was reduced as compared to controls or AC. This decreased frequency with IL-10 production was associated with the development of clinical disease. A similar study showed that following antigen challenge *in vitro* PBMC of VL patients exhibited a predominant type 2 response whereas cells of AC produced a mixed profile (Peruhype-Magalhaes et al., 2005). This mixed response would ensure both effective control of parasite proliferation and preservation of immune homeostasis by blocking an excessive cellular response (Machado et al., 2009). A mixed cytokine pattern (IFN-γ produced by $CD8^+$ cells and $CD4^+$ cells and IL-5 produced by $CD4^+$) was also found to be associated to the AC of *L. i* (Mary et al., 1999).

5. Asymptomatic carriers among blood donors

There has been a questioning of the risk of *Leishmania* transmission by blood donors that are AC among the healthy population. Moreover, recent changes in travel, immigration patterns and disease migration owing to climate changes have altered the rates of disease in non-endemic areas (Cardo, 2006a; Biglino et al., 2010). In the 1990s, WHO raised the possibility of transmission of *Leishmania* via blood transfusion (WHO, 1991). While some cases of *Leishmania* contamination by transfusion have been reported, transmission by blood transfusion is not necessarily followed by clinical VL (Colomba et al., 2005; Riera et al., 2008). Most studies on frequency of AC in blood donors have been performed in southern Europe (Table 2). In this population, AC proportion ranged from 0% to 36.4% depending on the test used and the number of individuals studied. However, the lack of gold standard and reliable methods for detecting AC has made it difficult to determine the extent of the cryptic infection, and up to now there is no routine testing done in blood donors to detect *Leishmania* infection (Reesink, 2005; Cardo, 2006b). The existence of AC exhibiting alternative positivity and negativity render a negative test difficult to interpret (Moreno et al., 2009). The transmission of *Leishmania* by transfusion requires that amastigotes are present in the blood, either within monocytes, or free in the plasma, survive processing and storage. To reduce the risk of transmission, several methods were tested including riboflavin and ultraviolet light for inactivation of *L. i* in plasma and platelet concentrates (Cardo, 2006b), photo inactivation of *L. i* by thiopyrylium in red cell suspension (Wagner et al., 2006) and pre-storage leucodepletion (Kyriakou et al., 2003; Riera et al., 2008). The two first techniques resulted in 5 or 7 log reduction in parasite load. Following leukodepletion, which is routinely used in most of developed countries for preventing various pathogens transmission, *Leishmania* DNA was undetectable in red blood cells. Therefore the current use of leukodepletion filter is effective in markedly reducing the risk of *Leishmania* transmission by transfusion (Cardo, 2006b; Riera et al., 2008). Except for Ireland, most other European countries do not question their donors for risk of *Leishmania*. Only Ireland and the USA defer donors for 12 months when they had visited Iraq (Reesink, 2005).

6. Asymptomatic carriers and immunosuppression

AC of *L. i* has been repeatedly demonstrated in immunocompromised people (HIV-positive individuals and patients under immunosuppressive therapy) and results from controlled primo-infection before, during or after immunosuppression (Kubar et al., 1998; Desjeux and Alvar, 2003; Mary et al., 2004; Basset et al., 2005; Pittalis et al., 2006; Alvar et al., 2008; Scarlata et al., 2008; Colomba et al., 2009; Bourgeois et al., 2010; Molto et al., 2010). In all cases, AC can evolve to clinical VL.

Concerning the HIV seropositive population, patent VL generally occurs in the advanced stages of the HIV disease, characterized by $CD4^+$ lymphocyte counts below 200×10^6/l (Kubar et al., 1998). The correlation between HIV viral load and parasitemia underlines a synergism between *Leishmania* and HIV (Colomba et al., 2009). HIV increases the risk of developing VL by 100- to 2320-fold and VL promotes the clinical progression of HIV and development of AIDS (Alvar et al., 2008). The implementation of highly active antiretroviral therapy (HAART) has led to a reduction in the incidence of symptomatic VL in HIV-infected patients but successful treatment of the viral infection is insufficient to prevent the relapse of the clinical disease (Alvar et al., 2008). Moreover, recent studies show that the patients coinfected with HIV and *Leishmania* never cleared the parasite, presenting episodes of clinical, oligosymptomatic VL as well as asymptomatic periods (Mary et al., 2004; Bourgeois et al., 2010).

Transplant recipients, and more generally, patients receiving immunosuppressive chemotherapy are also at risk of developing patent VL. Of note, unusual forms of leishmaniasis could occur in these cases leading to diagnostic delay and sometimes partial response to therapy (Basset et al., 2005; Antinori et al., 2008; Machado et al., 2009). In transplant recipients, VL clinical cases could result by transmission via the transplanted tissue, reactivation of a quiescent infection, or *de novo* natural infection (Basset et al., 2005). Some authors recommend that donors should be tested for asymptomatic infection. Indeed, the geographical origin of the donor is not always a valid criterion, as travel is common, and asymptomatic infection can occur even during a short stay in an endemic area. Additionally, it has been proposed that transplant patients living or travelling in endemic areas should be tested regularly for leishmaniasis. Currently there is no consensus recommendation to detect AC in endemic area for the donors, recipients or before patients receive immunosuppressive therapy (Pittalis et al., 2006; Antinori et al., 2008; Machado et al., 2009). In light of the risk presented above, screening asymptomatic infection prior to immunosuppressive chemotherapy may be an effective method to prevent the impact of a potential VL (Basset et al., 2005).

7. Asymptomatic carriers and associated risk factors

Schematically, AC result from both parasite inoculation by vector, and control of the parasite spreading by the host immune system. Only in a very few cases, parasite growth is not controlled

by the immune system resulting in clinical VL (Bogdan et al., 1993). Therefore, factors associated with asymptomatic infection are grossly on the one hand, those favouring parasite transmission in endemic areas and in the other hand those related to the host. For example, AC are more frequent in rural areas, where sandfly vector is abundant, than in the cities (Moral et al., 2002; Garrote et al., 2004; Biglino et al., 2010). In the same way, the non-randomly distributed positivity of the LST was associated with variation in bioclimatic zone probably through influencing vector presence (Acedo Sanchez et al., 1996). Long-lasting and uninterrupted contact with both the reservoir and the vector may also increase the asymptomatic infection and thus a close relationship has been reported between canine seroprevalence and the proportion of LST positive individuals (Acedo Sanchez et al., 1996; Biglino et al., 2010). In addition to environmental conditions, host factors can directly or indirectly influence the frequency of AC. For example, in one study adult males were more frequently found to be AC than females, and generally the proportion of AC increased with age reflecting cumulative transmission over multiple years (Marty et al., 1992; Acedo Sanchez et al., 1996; Morillas et al., 1996; Moral et al., 2002; Werneck et al., 2002; Crescente et al., 2009; Silveira et al., 2009, 2010a; Biglino et al., 2010). In addition, variations in the ratio of patent VL cases versus AC cases observed in some studies has been associated to host differences including socioeconomic status, nutritional aspects and genetic background, rather than to parasite transmission (Alvar et al., 2006). For example, the ratio between symptomatic and asymptomatic infection was higher for people living in shanty town than for those with a good socioeconomic status (Barao et al., 2007). As well, high level of vitamin A (Maciel et al., 2008), high birth weight, high albumin concentration and high red meat consumption have been suggested to favour asymptomatic infection in Brazil (Barao et al., 2007). Furthermore, despite the limited size of populations studied, variations in the outcome of *L. i* infection have been suggested to be strongly influenced by genetic factors. For example, certain tribes or family are less susceptible than others to develop clinical VL, and infection outcome was reported to be controlled by several genes including the innate resistance gene SLC11A1, which regulates macrophage activation and markers on chromosome 22 or on TNF-α and IL-4 locus (Wilson et al., 2005; Ettinger et al., 2009).

8. Human *L. i* asymptomatic carriers as potential reservoir

As already described (WHO, 2010), the incrimination of a host reservoir should fulfill the following criteria. First, a reservoir host must be abundant and long lived. Second, repetitive contacts between host and sandfly are necessary to increase the chance of transmitting the parasite. Third, the proportion of host individuals must exceed 20%. Fourth, the infection should be long enough and non-pathogenic to permit a long time survival of the parasites. Finally, sufficient parasite loads should be present in the skin and blood. Humans living in endemic zones of *L. i* and particularly those living in South America, generally fulfill the four first criteria. Contrary to the high parasite loads observed in blood from immunocompetent or immunocompromised patients with active VL (ranging from 32 to 188,700 parasites/ml) very low numbers of *L. i* parasites (0–0.2 parasite/ml blood by quantitative PCR) were detected in the blood of AC or immunocompromised infected patients, apparently cleared without relapse after Ambisome® therapy (Mary et al., 2004). This strongly suggests that contrary to active VL, AC in immunocompetent subjects are generally accompanied by very low to undetectable parasitemia. Only one study, which compared patients with active or cured VL, asymptomatic individuals with positive LST, or individuals living close to a patient with VL has been devoted to evaluate the competence of the human host as a reservoir for *L. chagasi* (Costa et al., 2000). In this case, only the group with active VL was capable of infecting the sandfly vector *Leishmania longipalpis*. However the reservoir competence of individual persons with asymptomatic infections cannot be excluded because they represent a large segment of the population of several Brazilian cities (Costa et al., 2000). Therefore, while AC are markedly less infective than individuals with active VL, their huge numbers would constitute a formidable reservoir of infection (Costa et al., 2002). Consequently, their contribution as a reservoir for parasite transmission needs further evaluation (perhaps by xenodiagnosis). Finally, because of the direct character of parasite transmission, asymptomatic pregnant woman and intravenous drug users exchanging syringes may constitute a human reservoir (Meinecke et al., 1999; Alvar et al., 2008).

9. Conclusion

Epidemiological studies conducted worldwide in endemic areas of VL to *L. i* strongly suggest that AC represent a rather large proportion of individuals, depending on the screening test used and the size of the population studied. Most studies were performed using indirect testing and in the absence of parasite demonstration the use of two or more indirect tests has been highly recommended for improving sensitivity detection of AC. Recent studies using direct tests such as PCR confirm the high proportion of AC in endemic zones. While a few cases of *L. i* transmission by blood donation have been reported, prestorage leukodepletion is greatly effective in reducing the risk of possible parasite transmission by blood donors. Immunocompromised AC such as HIV-coinfected patients or patients receiving immunosuppressive chemotherapy are at high risk of developing clinical VL and therefore screening of this population for parasite presence is highly recommended. Various factors linked to environmental conditions or to the host have been suggested to control prevalence of AC as well as the AC to clinical VL ratio. While AC exhibited, at least in immunocompetent individuals, very low parasitemia level, their potential role as parasite reservoir need further studies.

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LEISHMANIOSES HUMAINES ET POLYMORPHISME CLINIQUE

Si la précédente revue est centrée sur le portage asymptomatique de *Leishmania infantum*, le travail suivant publié porte sur le polymorphisme clinique des principales leishmanioses humaines. Dans cette revue, toutes les formes cliniques de toutes les leishmanioses sont prises en compte. Les mécanismes immunologiques de la réponse de l'hôte sont abordés, confirmant non seulement l'implication de la réponse immune Th1 et Th2 dans l'expression clinique, mais aussi celle des cellules régulatrices T et des cellules Th17 qui jouent un rôle dans la susceptibilité et la résistance de l'hôte. La compréhension de la pathogénicité des leishmanioses doit tenir compte à la fois du fond génétique de l'hôte lui permettant de lutter contre le parasite, et des signaux moléculaires utilisés par le parasite pour échapper à la réponse immune et survivre dans l'hôte. Enfin, il faut prendre en compte le vecteur qui lui aussi est impliqué dans la virulence notamment par les composés de la salive du phlébotome et par les interactions entre le parasite et le phlébotome. Récemment, de l'ARN viral a été retrouvé dans des formes métastatiques de leishmaniose à *L. guyanensis*. Cet ARN pourrait accroître la virulence du parasite. Si chaque espèce de *Leishmanie* est associée à un tropisme et une expression clinique préférentielle, ce phénomène se retrouve aussi à un niveau infra-spécifique. Finalement, pour comprendre l'expression de la maladie, l'étude des porteurs asymptomatiques s'avère indispensable. Ils pourraient constituer un réservoir de parasites même si, pour l'instant, cela n'a pas été clairement mis en évidence pour la leishmaniose à *L. infantum*. L'état de santé du sujet, des facteurs génétiques, ethniques ou nutritionnels influent sur le fait de développer ou pas la maladie. Si le portage asymptomatique a surtout donné lieu à des études sur l'hôte (asymptomatique *versus* malade), le parasite lui-même pourrait, selon les souches, être plus ou moins virulent. Cet aspect est beaucoup moins étudié du fait de la rareté des souches isolées de porteurs asymptomatiques.

Revue

CLINICAL PLEIMORPHISM IN HUMAN LEISHMANIASES, WITH SPECIAL MENTION OF ASYMPTOMATIC INFECTION

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Clinical pleiomorphism in human leishmaniases, with special mention of asymptomatic infection

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Abstract

This review gives an update of current knowledge on the clinical pleiomorphism of *Leishmania*, with a special emphasis on the case of asymptomatic carriage. The first part describes the numerous unusual expressions of the disease that occur besides the classic (visceral, cutaneous, and mucocutaneous) forms of leishmaniases. The second part deals with progress in the understanding of disease outcome in humans, and the possible future approaches to improve our knowledge in the field. The third part highlights the role of the too often neglected asymptomatic carrier compartment. This group could be key to understanding infraspecific differences in virulence and pathogenicity of the parasite, as well as identifying the genetic determinants involved in the expression of the disease.

Keywords: Asymptomatic carriage, clinical diversity, host susceptibility, immune response, *Leishmania*, parasite genetic diversity, unusual clinical forms

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Introduction

Infectious diseases remain one of the major challenges in public health, particularly in developing countries. Nevertheless, the causes of the variety of clinical expression in humans are largely unknown. This is to a great extent because of the complexity of infectious systems within which various parameters act and interact: microorganisms, hosts, vectors (for vector-borne diseases), and environment. Such an understanding requires study of the diversity, dynamics and ecology of infectious agents and endemic foci.

For a large number of pathogens, we have only a faint idea of the biological processes explaining why, in some cases, there is no disease (asymptomatic carriage) and in others there is a variety of forms of clinical expression, from benign to critical forms. This is especially the case for leishmaniases. *Leishmania* species, of which there are about 30, can produce a large spectrum of clinical manifestations in humans, from asymptomatic to fatal visceral disease, through benign or conversely severe cutaneous forms; for this reason, the term is used in the plural. Leishmaniases are widespread in the world, and still constitute a major public health problem, causing considerable morbidity and mortality (for further epidemiological information, see [1–7]).

The objectives of this review are to give an update of the knowledge on the clinical pleiomorphism of leishmaniases. We will also address the question of asymptomatic carriers (ACs). Indeed, in view of the many aspects of the parasite cycle, epidemiology and clinical expression of the disease that

remain unclear, the study of ACs could be a valuable source of information and understanding. This area, too often neglected, could constitute a key element in understanding why some strains are more or less virulent at the infraspecific level, as well as for identifying the genetic determinants involved in the expression of the disease. It will also be essential to better characterize the role that ACs play in transmission and the consequences of this for control strategies.

Clinical Diversity and Disease Complications

Human leishmaniases are classically subdivided into three main clinical forms: cutaneous, mucocutaneous, and visceral. Within these three categories, *Leishmania* infection is able to produce a large variety of atypical and rare variants. Since 1980, more than 150 papers have been referenced in PubMed reporting unusual clinical presentations, both for the Old World and the New World.

Cutaneous leishmaniasis (CL) can be the most complex form to diagnose, as an incredible variety of lesions exists. CL is classically associated with several species in the Old World and New World, e.g. *Leishmania major*, *Leishmania tropica*, *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania braziliensis*, *Leishmania peruviana*, and *Leishmania guyanensis* (see [5,7–9] for reviews and Table 1). *Leishmania infantum*, a classically visceralizing species, also frequently causes benign cutaneous lesions at the biting site of the sandfly [10,11], at a ratio of at least one CL case for one visceral leishmaniasis (VL) case in France, which is probably an underestimate (L. Lachaud and P. Bastien, unpublished data from the national notification system); the same has been described in Central America for its synonym *Leishmania chagasi* [11] and, to a much lesser extent, for *Leishmania donovani* in Sri Lanka [12] and in Yemen [13]. *L. donovani* also produces a particular cutaneous disease termed post-kala azar dermal leishmaniasis (PKDL) (see below). CL occurs in humans as a localized or diffuse form, depending on the infecting species of *Leishmania* [8,14,15] (Table 1) and human host factors (reviewed in [16]). The typical and most common CL lesion is a torpid self-healing round ulcer, leaving an ineradicable and unattractive scar with sometimes dramatic social consequences. However, lesions can be highly polymorphic, and, apart from the 'classic' ulcer, frequently appear as single crusted papules ('uta'), verrucous or papulonodular. Necrosis of the external ear caused by *L. mexicana* ('chiclero' ulcer) and sporotrichoid lesions caused by *L. guyanensis* ('pian-bois') have also been well described (reviewed in

TABLE 1. Main *Leishmania* species associated with different clinical outcomes in leishmaniases

	Cutaneous leishmaniasis	Mucocutaneous leishmaniasis	Visceral leishmaniasis
Main clinical form	*L. major* *L. tropica* *L. killicki* *L. aethiopica* *L. mexicana* *L. amazonensis* *L. braziliensis* *L. peruviana* *L. guyanensis* *L. infantum*[a] *L. shawi*[a] *L. naiffi*[a] *L. lainsoni*[a] *L. donovani*[b]	*L. braziliensis* *L. panamensis*[a] *L. amazonensis*[a] *L. guyanensis*[b]	*L. donovani* *L. infantum/chagasi* *L. tropica*[b]
Particular forms of cutaneous disease	*L. donovani*: PKDL *L. mexicana*: 'chiclero ulcer' *L. peruviana*: 'uta' *L. guyanensis*: 'pian-bois' *L. aethiopica*[a], *L. mexicana*[a], *L. amazonensis*[a]: DCL *L. tropica*[a], *L. aethiopica*[a], *L. braziliensis*[b], *L. panamensis*[b], *L. guyanensis*[b], *L. amazonensis*[b]: recidivans and disseminated leishmaniasis		
Immunosuppressed patients (mainly HIV-positive patients)	Most species	*L. donovani*[a] *L. infantum*[b,c] *L. major*[b,d] *L. guyanensis*[b] *L. amazonensis*[b]	*L. infantum*[c] *L. braziliensis*[b] *L. mexicana*[b] *L. amazonensis*[b]

DCL, disseminated cutaneous leishmaniasis; HIV, human immunodeficiency virus; PKDL, post kala-azar dermal leishmaniasis.
[a]Rarely. [b]Exceptionally. [c]Including 'dermotropic' zymodemes. [d]Local extension to the mucosa.
Bibliographic references can be found in the text.

[8,17]). Besides these well-known presentations, numerous unusual variants associated with many different species have been reported, described as 'eczematoid', 'infiltrative plaques', 'paronychial', 'chancriform', 'erysipeloid', 'zosteriform', 'lupoid', 'whitlow', 'sprototrichoid', etc. [9,18–25].

Classically, in most cases, cutaneous lesions appear at the site of the sandfly bite. Thus, all exposed parts of the body are susceptible to cutaneous lesions. Nevertheless, unusual locations, such as genital organs and palms, have been observed [26–30]. Furthermore, multiple lesions are frequently observed, possibly owing to multiple inoculations [31–33]. With respect to disease evolution, CL may reappear as satellite lesions along the route of lymphatic drainage or, in rare cases, progress around the site of the original lesion as recidivans leishmaniasis [25,34,35], or, even worse, evolve towards disseminated CL; all of these are clinically and epidemiologically well-described forms [17,36,37]. Disseminated CL is essentially caused by *Leishmania aethiopica* in East Africa and *L. amazonensis* and *L. mexicana* in South America (Table 1); it produces diffuse, non-ulcerated nodular lesions that fail to heal spontaneously, relapse after treatment, and may generalize to the whole body, leaving highly unsightly scars and sometimes causing death [9]. This form has been also associated with human immunodeficiency virus (HIV) co-infection and with the immune reconstitution inflammatory syndrome (IRIS), but is caused by unusual *Leishmania* species [28,38–40]. PKDL is a particular evolution of VL caused by *L. donovani* that develops in spite of a successful primary response to therapy, usually 1–2 years after apparent clinical cure [36,41]. It presents as hypopigmented macules, papules, nodules, or facial erythema. PKDL has been reported in 50–60% and 5–10% of VL patients in Sudan and the Indian subcontinent, respectively [41].

With this high number of non-specific and atypical presentations, CL often mimics other infectious diseases and cancers (see [28,42,43] for examples), leading to misdiagnosis and delay in starting treatment.

Mucocutaneous leishmaniasis (MCL), the other prominent clinical manifestation of New World tegumentary leishmaniasis, is also known as 'espundia', and is essentially associated with *L. braziliensis*; rare forms have been observed with *Leishmania panamensis*, and exceptionally with *L. guyanensis* and *L. amazonensis* (reviewed in [5,8,44–46]). It causes extensive destruction of oronasal and pharyngeal cavities, with hideously disfiguring lesions, mutilation of the face, and great life-long distress for the patient. Although the nose mucosa is the preferred site for MCL (90% of mucosal lesions), the lips, palate, mouth, pharynx and larynx can also be affected, as well as the middle ear [47–50]. Mucosal involvement is usually metastatic, i.e. secondary, several years after cutaneous lesions [37], although cases have been reported where the mucosa was the primary site [48]. Dissemination from cutaneous to mucosal lesions appears to take place through the lymphatic system or blood vessels, but rarely (if ever) by direct contact between the mucosa and the cutaneous lesions [48]. In the Old World, MCL has rarely been observed in Sudan and North Africa, where leishmaniases result from either direct inoculation of the parasites in the mucosa or from local extension of the cutaneous lesions to the mucosa [9]. Finally, there are rare reports of mucosal involvement with *L. infantum* or dermotropic species in immunosuppressed patients [37,51] (Table 1).

VL, also called kala-azar in the Indian subcontinent, is the most severe form of leishmaniasis, as it is fatal if left untreated. It is estimated that VL causes over 50 000 deaths annually, a rate surpassed among parasitic diseases only by malaria [1]. It is characterized by undulating fever, loss of weight, hepatosplenomegaly and/or lymphadenopathies, and pancytopenia, particularly anaemia. This form is generally caused by *L. donovani* senso lato, including *L. infantum*/*L. chagasi* (Table 1). Whereas *L. infantum* is essentially a parasite causing zoonotic disease, mainly in dogs, and is responsible for low endemicity with regard to humans, *L. donovani* is human-specific and may be responsible for large-scale epidemics with a high fatality rate [37,52–55] (see also [7], this issue). Some 'unorthodox' expressions of the disease result from a peculiar presentation of classically visceralizing species (the *L. donovani* complex). For example, as described above, *L. infantum*, which is classically responsible for VL and localized CL, has been described as causing purely mucocutaneous leishmaniasis (see examples in [51,56]). In contrast, visceralization of *Leishmania* species considered to be dermotropic has been observed [46,57,58] (Table 1).

The number of atypical presentations reported with 'visceral' species has been growing with the increasing frequency of immunosuppression, showing the essential role of immune control in the expression of the disease. For example, a case of pseudotumoral-like recurrence located in the nasal mucous membrane 4 years after a classic VL episode caused by *L. infantum* was observed in a 7-year-old girl treated with anti-tumour necrosis factor and steroids [59].

The clinical manifestations are particularly diverse during *Leishmania*–HIV co-infection, where VL has emerged as an important opportunistic infection. In AIDS patients, VL usually has atypical presentations, often severe but also frequently attenuated, both clinically and biologically, making the diagnosis difficult for practitioners (reviewed in [60,61]). In particular, numerous unusual locations of the parasite have been described (reviewed in [28]). Most importantly,

it follows a relapsing course that may last for years, the relapse risk depending on the $CD4^+$ T-cell levels [28,61–63]. Thus, a novel nosological entity termed 'active chronic visceral leishmaniasis' has been proposed for immunocompromised patients presenting with continuous blood circulation of the parasite associated with numerous secondary VL episodes despite both preventive and curative treatment [64].

Although *Leishmania*–HIV co-infection concerns mainly VL, several cases of CL have been reported from different countries (reviewed in [28]). *L. braziliensis*, *L. mexicana*, and *L. amazonensis*, as well as dermotropic isoenzymatic variants of *L. infantum*, have been reported to cause visceral disease in HIV-positive patients [28] (Table 1). Cutaneous involvement has been reported to occur in 8–12% of patients with *Leishmania*–HIV co-infection [28]. It is worth noting that these patients may present highly polymorphic lesions (papulonodular, ulcerative, infiltrative, lepromatous and diffuse, psoriasis-like, cheloid, histioid, or Kaposi's sarcoma-like) at the same time. Some patterns of presentation share similarities with other specific infections, such as disseminated or extrapulmonary coccidioidomycosis or histoplasmosis [28].

Finally, the asymptomatic forms of leishmaniasis probably constitute a further and extreme example of clinical diversity, as will be examined in the third part of this review.

Our present knowledge of *Leishmania* tropism in humans is largely influenced by the fact that clinical diagnosis is based on the sampling of defined body tissues, which are primarily microscopically examined (usually, bone marrow, lymph nodes and spleen for VL, and cutaneous lesions for CL). The introduction of PCR in the 1990s has modified our views regarding tropism, particularly if an 'ultrasensitive' PCR assay is used [65]. For example, except for rare reports [66–68], blood circulation of *L. infantum* in humans was largely unsuspected before the application of PCR in 1995 [69]. Similarly, delayed and/or 'non-classic' clinical forms suggest that the parasites' distribution in the body extends beyond the apparently affected tissues, e.g. to the healthy skin, from where they can be more easily detected in case of immunodeficiency, as was demonstrated experimentally in a murine model [70]. Also, the common view that *L. infantum* is viscerotropic may be contradicted by the fact that the parasite massively invades the skin in the dog reservoir [65]. Thus, the issue of pathogen tropism in leishmaniases remains a topical debate [71], as it depends upon both parasite features [72,73] and immunogenetics of the host (see below), probably in infinitely variable proportions (see Supporting Information, Fig. S1).

Scientific Progress on the Clinical Pleiomorphism of Leishmaniases: Experimental, Mechanistic and Genetic Aspects

Disease manifestations of infections in humans depend on multifactorial parameters, such as host response or susceptibility, parasite genetic background, and, potentially, factors depending on the vector (see Supporting Information, Fig. S1). Indeed, the different clinical forms are closely related to the adaptive immune response of the host. The nature of the pathogen, notably the species but also at the infraspecific level, seems to be a strong factor as well (Table 1). How *Leishmania* species cause human diseases and why the clinical symptoms are so variable remain unclear.

Concerning the human host response, different patterns of immunological response are observed, according to the clinical manifestation and exposure to different *Leishmania* species. Resistance and susceptibility to *Leishmania* infection have been associated with the generation of T-helper (Th)1 and Th2 responses, respectively. This infectious disease model was later used to identify and assess the role of key factors, such as interleukin-12 and interleukin-4, in Th1 and Th2 maturation [74]. Nylen and Gautam [75] recently reviewed this issue, and showed that, although still valid in several respects, the Th1/Th2 paradigm is an oversimplification. Numerous studies have demonstrated that a number of other T-cell subsets, including regulatory T-cells and Th17 cells, alo have important roles in susceptibility and resistance to both experimental and human leishmanial disease.

Although there is certainly much more to explore regarding purely immune response mechanisms, in our view, the main research field to develop in this scientific area comprises the interactions between *Leishmania* and its insect and mammalian hosts. One promising approach is the study of immune evasion mechanisms that allow parasites to avoid the development of sterilizing immunity and secure their transmission to a new host (reviewed in [74]). The importance of these processes has been known for a long time for many pathogens [76], and also for *Leishmania* [77]. This suggests that it is crucial to identify the diverse mechanisms by which these agents modulate or evade their hosts' defences, creating a dynamic interplay between the human immune system and the parasite population. For example, a recent study using *L. amazonensis* and *L. braziliensis* (species that are usually able to produce infection or only transient self-healing cutaneous lesions in BALB/c mice) clearly showed that it is the involvement of parasite molecules, the immune

response and the host genetic background together that determines the disease outcome [78]. The same close interplay between host, parasite and vector-dependent factors was shown for human VL in Sudan [79]. Nevertheless, studies focusing on specific factors are necessary to target the main processes involved in pathogenesis. For example, the analysis of leishmaniasis symptomatology in mice by monitoring skin lesions, hepatosplenomegaly and seven immunological parameters allowed the detection and mapping of 17 *L. major* response (*Lmr*) gene loci that control the symptoms of infection [80]. To directly identify the potential genes involved in human diseases, several studies genetically compared populations presenting different evolution patterns of leishmaniasis. Some studies identified significant associations between human gene polymorphisms and expression of the disease, such as the regulatory *CCL2* (coding for proinflammatory monocyte chemoattractant protein 1) promoter polymorphism for MCL/CL differentiation [81], and other genetic polymorphisms for VL or CL involvement [79,82–88].

With respect to the role of *Leishmania* parasite diversity on disease manifestations, it is evident that each *Leishmania* species has its own tropism and specific clinical forms (for example, *L. tropica* is well known to produce more severe lesions that heal less rapidly than those caused by *L. major*). Even at the infraspecific level, numerous studies have shown the role played by the parasitic strains in the outcome of the disease (reviewed in [5]). As an additional example, Alimohammadian *et al.* [89] reported that different genetic strains of *L. major* isolated from distinct endemic areas in Iran were able to cause major differences in parasite load and immunogenicity of susceptible BALB/c mice. Nevertheless, more work is required to relate the immune responses in mice to those in human and canine infections. On the genetic side, several studies have linked genetic polymorphisms to the tropism observed in humans or to immune evasion [90–94]. Besides these specific studies, sequencing of the whole genomes of *L. major*, *L. braziliensis* and *L. infantum* will provide interesting insights into disease phenotype at the global level. Surprisingly, the *Leishmania* genome is highly conserved, and has <1% of species-specific genes [95]. These genes mainly encode proteins involved in host–pathogen interactions and parasite survival in the macrophage. Thus, they might be good candidates for functional analysis to determine their roles in the establishment of infection or specific disease manifestations (see Supporting Information, Fig. S2) [96]. The low level of differentiation between the genomes and gene expression suggests that studies of expressed proteins in the amastigote (vertebrate) life-cycle stage of the parasite are crucial [97]. Because there seems to be no regulation of gene expression at the transcriptional level in these parasites [98], these proteins are likely to be regulated at the level of translation control or protein stability; hence, studies need to also focus on protein modifications and how they affect disease pathogenicity. Finally, epigenetic regulation is not well known in *Leishmania*, but certainly adds more complexity to the role played by the parasite [99].

In addition, extrinsic factors may influence the course of infection. For example, the nature of the sandfly bite itself has become a tremendously important parameter of infection. Multiple cutaneous lesions are, in part, caused by the inability of the vector to feed when its 'oesophagus' is blocked by an accumulation of parasites fixed upon the stomodeal valve [100]. The exacerbation of the establishment and development of CL by sandfly saliva, owing to its immunomodulatory properties, has long been documented for several *Leishmania* species [101]. However, this is complicated by the discovery that previous exposure to salivary components can have a protective effect against *Leismania* infection [102]. More recently, another natural component regurgitated by the fly, consisting of a proteophosphoglycan-rich gel secreted by the parasite inside the midgut, was also shown to benefit the establishment of VL caused by *L. infantum* [103]. Finally, another recent study has opened new and amazing research perspectives: Ives *et al.* [104] showed that *Leishmania* RNA viruses could control the severity of MCL. More specifically, recognition of the virus within metastasizing *L. guyanensis* parasites by the host promoted inflammation and subverted the immune response to promote parasite persistence.

All of these data show that we are still far from understanding all of the mechanisms underlying *Leishmania* infection outcome (see Supporting Information, Fig. S1). In our view, asymptomatic carriage may also be a fundamental aspect to study in order to achieve progress in our knowledge of disease expression.

The Asymptomatic Compartment: a Key Element in the Understanding of Pathogenesis and Epidemiology?

AC is used in this review to define mammalian hosts infected with *Leishmania* parasites but that do not show any sign of disease. Identification of an AC is not easy, as one may consider that only conventional methods, such as microscopic observation and parasite cultivation, can provide a definite diagnosis, as immunological or molecular techniques, such as PCR, do not demonstrate the presence of live parasitic cells. Nevertheless, recent studies showed that *Leishmania* nucleic acids detected by PCR came from live parasites and were rapidly degraded following parasite death [105,106].

For many infectious diseases, ACs constitute a key point in transmission, and also in the evolution from the asymptomatic to the patent stage. ACs may never develop the disease, or the asymptomatic carriage may be a temporary stage before the development of clinical signs [107,108]. There are numerous examples of ACs with bacteria, viruses, or parasites [109–112]. Numerous reports (see below) support the existence of asymptomatic leishmaniasis in animals as well as in humans. Failures of certain control programmes suggest that the elimination of the seropositive or clinically ill reservoir is not enough to stop transmission. For example, the elimination of such patently infected dogs in Brazil has failed to stop or prevent epidemics of urban VL caused by *L. infantum*, suggesting that other secondary (asymptomatic) reservoirs may be important in propagating the infection [113–116]. Moreover, various authors have shown that asymptomatic dogs can be infective to the sandfly vectors, just like symptomatic dogs [117–119]. Thus, as the natural reservoir hosts of *Leishmania* often remain asymptomatic [120], it is now essential to investigate the potential role of ACs in the maintenance of transmission in leishmaniasis foci. Epidemiological studies using different tools have demonstrated that variable percentages of human ACs of *L. infantum* exist in the Mediterranean area, e.g. 4% in northwestern Italy [121], 3% in Monaco [122], 22% in the Balearic Islands [123], 24% in Iran [124], and 58% in southern France [125] and, among HIV-infected subjects, 30% in Spain [126], but 0.03% [127] in western Sicily and 16% [128] in Palermo area, Sicily. In the highly endemic state of Bihar, India, a recent study estimated the number of asymptomatic seropositive subjects in the population without a history of VL to be almost 13%, suggesting a high rate of ACs also with *L. donovani* [129]. Asymptomatic *Leishmania*-infected dogs have become the subject of sustained interest, with a median ten-fold increase in the number of yearly publications on the subject since the report of the first PCR diagnostic field studies [65,130,131]. Indeed, 65% of asymptomatic dogs in southern France, 60% in Spain [131] and between 22% and 80% in southern Italy [65,107,130,132] have been found to harbour blood-circulating parasites by PCR. The variable prevalence rates between studies in countries of similar epidemiology are probably attributable to the highly variable performances of the diagnostic methods used, particularly for serology and PCR [125,133–137]. Although PCR methods, particularly the 'ultrasensitive' ones used in most of these studies, may always be suspected of yielding a proportion of false-positive results, the body of evidence is sufficient to believe that the number of ACs of visceralizing *Leishmania* is larger than that of hosts presenting with patent disease.

The relatively recent discovery of the importance of ACs in VL poses a number of interesting questions. The first concerns the issue of disease risk resulting from blood transfusion from asymptomatic donors. The possibility of leishmaniasis transmission from an asymptomatic blood donor exists [138–142], and the risks to public health deserve to be assessed [143]. In France, there is no blood screening for *Leishmania*, even in the south, where *L. infantum* is endemic; but the French Institute for Public Health Surveillance estimates that the leukodepletion almost completely rules out the risk of transmission [143], and this was confirmed by the work of Riera *et al.* [144] in the Balearic Islands. Also, classic pathogen inactivation of blood concentrates proved to be efficient against *Leishmania* parasites [145]. Nevertheless, the risk might be critical for immunocompromised patients, who are more likely to develop the disease. In southern Europe, a considerable proportion of HIV-infected patients are ACs of *L. infantum* [28,123,126]. VL is also frequently observed in other immunosuppressed patients, such as graft recipients, those with blood cancers, and those undergoing long-term steroid treatment [146–148]. It therefore appears essential to define worldwide common recommendations to screen for ACs in VL-endemic areas.

Another interesting question is the possible cause of asymptomatic carriage. We know very little of the relative contributions of the host, the parasite or any other factor in the evolution of *Leishmania* infection towards an asymptomatic outcome. Considering the host, there are numerous studies showing that host factors are crucial for infection evolution. Nevertheless, the factors determining the host resistant/susceptible status appear to be complex, and are mostly unknown. In endemic areas, it is striking to observe, for a given parasite species, a wide range of inter-individual variability in susceptibility/resistance to disease (reviewed in [16]). In contrast with ACs, some subjects are unable to control parasite dissemination and/or multiplication, and develop clinical symptoms of diverse severity. The general state of health and physiological condition of the host, in particular subtle immunity variations, do influence disease progression, as shown, for example, by the classically higher incidence of *L. infantum* VL in infants and elderly people [9,149,150]. Nutritional state is also an essential factor, as shown by the devastating VL epidemics that took place in refugee populations in Sudan in the years 1984–1994, 1997, and 2009 [53,151,152]. Genetic predisposition also undoubtedly plays a major role in determining disease outcomes [16]. Profound ethnic differences in the ratio of asymptomatic to symptomatic infections have been observed [86,153], and, as described above, certain genes have been incriminated in the susceptibility to visceral disease. All in all, it is most likely that asymptomatic carriage is strongly mediated by nutritional, physiological and immunogenetic host factors (see Supporting Information, Fig. S1).

The parasite itself might provide another clue to the absence of symptoms. Specific clinical features are grossly associated with particular *Leishmania* species, but we do not know whether the parasite or the host determines the different clinical forms encountered for the same species (see Supporting Information, Fig. S1; Table 1). Similarly, particular strains might be less virulent than others, i.e. less able to cause patent disease. Very little is known about the *Leishmania* strains associated with asymptomatic carriage, in great part because of the difficulty in isolating them *in vitro*. We recently demonstrated that *Leishmania* isolates obtained from ACs in France are genetically different from those obtained from patients with VL or CL (M. Hide, E. Marion, P. Marty, R. Fisa, A.L. Bañuls, in preparation). This observation opens a large study area, suggesting the possibility of identifying genes or genotypes involved in asymptomatic vs. symptomatic disease outcomes. To better understand the mechanisms involved in leishmaniasis (whether VL, CL, or MCL) pathogenesis and immunity, it now appears essential to include strains from ACs with the classic symptomatic strains in all studies of host immune response, pathogenicity, gene expression, etc. However, isolating a representative sample number of these strains remains a major problem.

In summary, besides the three classic categories of leishmaniasis (CL, VL, and MCL), the clinical expression of the disease is highly polymorphic, depending on both the host and the parasite, and, for the latter, at the species, infraspecific and probably 'individual' (strain) levels. Leaving aside the difficulties in patient care caused by such pleiomorphism, the role of ACs needs to be explored to obtain a better understanding of the molecular cellular and immune mechanisms underlying pathogenicity and progression of the infection outcome. The exploration of the subtle variations in hosts and parasites that determine these differences poses challenges similar to those of 'personalized medicine', and promises to be an exciting venture for the coming years.

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Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Factors acting on the clinical pleiomorphism of leishmaniases in humans. *Leishmania* infection is able to produce a large variety of clinical expressions in humans. This is, to a large extent, because of the complexity of infectious systems within which various factors act and interact: microorganisms, hosts, vectors, and environment.

Fig. S2. Species-specific genes in *Leishmania*. The Venn diagram shows how many of the 8187 protein-coding genes are species-specific or shared between two of the three sequenced *Leishmania* species. These genes are subdivided into those that have a predicted function (mostly through sequence identity) and those that are of unknown function at the time of publication ('hypotheticals').

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FORME CLINIQUE ATYPIQUE : ADENOPATHIES ISOLEES ET LEISHMANIOSE A *L. INFANTUM*

Parfois, la différence entre le sujet malade et le sujet porteur asymptomatique peut s'avérer minime. En effet, certains sujets, sans toutefois être totalement asymptomatiques, n'expriment pas complètement la maladie. C'est ainsi qu'au lieu de présenter la triade classique de la forme viscérale : fièvre, anémie et splénomégalie, des patients vont exprimer une symptomatologie larvée ou atypique. Plusieurs cas de leishmaniose à *L. infantum* ont ainsi été diagnostiqués sur une diarrhée chronique inexpliquée ou une dysphagie liée à une atteinte duodénale (Alvarez-Nebreda et al., 2005; Hicks et al., 2009; Jawhar, 2011). Parfois même, la présentation clinique se résume à des adénopathies isolées. Il est important dans ce cas de faire le diagnostic de leishmaniose non seulement pour éliminer un lymphome, principal diagnostic différentiel à envisager, mais aussi parce que ces formes qui n'évoluent pas toujours vers la guérison spontanée peuvent donner lieu au développement d'une leishmaniose viscérale. Pour l'instant, il n'existe pas de facteurs permettant de prédire l'évolution préférentielle vers l'une de ces expressions cliniques. Les deux articles qui suivent portent sur cette forme clinique particulière de l'infection à *L. infantum* constituée par des adénopathies isolées.

ISOLATED LYMPHADENOPATHY IN *LEISHMANIA INFANTUM* INFECTION: THREE CASE REPORTS

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LOCALIZED LEISHMANIAL LYNPHADENITIS: AU UNSUAL MANIFESTATION OF THE DISEASE IN AN IMMUNOCOMPETENT PATIENT

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SHORT COMMUNICATION

Isolated lymphadenopathy in *Leishmania infantum* infection: three case reports

Human leishmaniasis caused by *Leishmania infantum* is a zoonotic parasitic disease that is endemic in southern Europe. Most locally acquired infections cause visceral leishmaniasis (VL) that is typically associated with fever, anaemia and splenomegaly — the three 'cardinal signs' (Peachey *et al.*, 1994; Dujardin *et al.*, 2008). The results of studies conducted over the past few decades have, however, demonstrated that the clinical presentation and course of *L. infantum* infection in humans depend on complex interactions between host and parasite, ranging from atypical, disseminated and life-threatening VL in immunocompromised patients to frequently asymptomatic infection or carriage among the general population (Gramiccia and Gradoni, 2005; Alborzi *et al.*, 2008). Although isolated lymphadenopathy has been reported in *Leishmania* infection, it is rare and its clinical significance is currently unknown (Peachey *et al.*, 1994; Harms *et al.*, 2001; Aoun *et al.*, 2002). Three patients who recently presented with isolated lymphadenopathy and *L. infantum* infection are described below.

CASE REPORTS

Case 1

A 63-year-old woman was admitted to San Remo hospital, in the Italian region of Liguria, in January 2007, for investigation of cervical lymphadenopathy. The patient was taking alprazolam, for mild depression, and reported having pulmonary tuberculosis 30 years earlier. During clinical examination, a firm, non-tender and mobile lymphadenopathy (measuring approximately 1 cm in diameter) was detected in the left anterior cervical area. An ultrasound examination revealed intraparotid lymphadenopathy but no spleen or liver enlargement. A tuberculin skin test gave a positive result. The patient's blood-cell count and liver enzymes appeared normal and the results of serological tests for Epstein–Barr virus (EBV), cytomegalovirus (CMV), toxoplasmosis and *Bartonella henselae* were either negative or not indicative of recent infection. Histopathological examination revealed a necrotizing granulomatous reaction, with classical *Leishmania* amastigote forms. Although *Leishmania*-specific IFAT and ELISA gave negative results, the 14-, 18-, 23- and 31-kDa bands seen in an *L. infantum*-specific western blot were indicative of patent leishmaniasis (Marty *et al.*, 1995). Promastigotes multiplied in cultures of lymph-node samples, both in Schneider's *Drosophila* medium with 20% heat-inactivated foetal calf serum and on solid Novy–MacNeal–Nicolle (NNN) medium. Iso-enzyme analysis indicated that the parasite involved was *L. infantum* of the MON-1 zymodeme. A sample of blood collected pretreatment was tested for *Leishmania* by culture and for leishmanial DNA by PCR but was found negative by both methods. The patient was given liposomal amphotericin B (3 mg/kg.day on days 1–5, 14 and 21) and this treatment appeared to clear the lymphadenopathy without any adverse effects.

Case 2

In July 2008, a 38-year-old man from Vallauris, in the Alpes-Maritimes province

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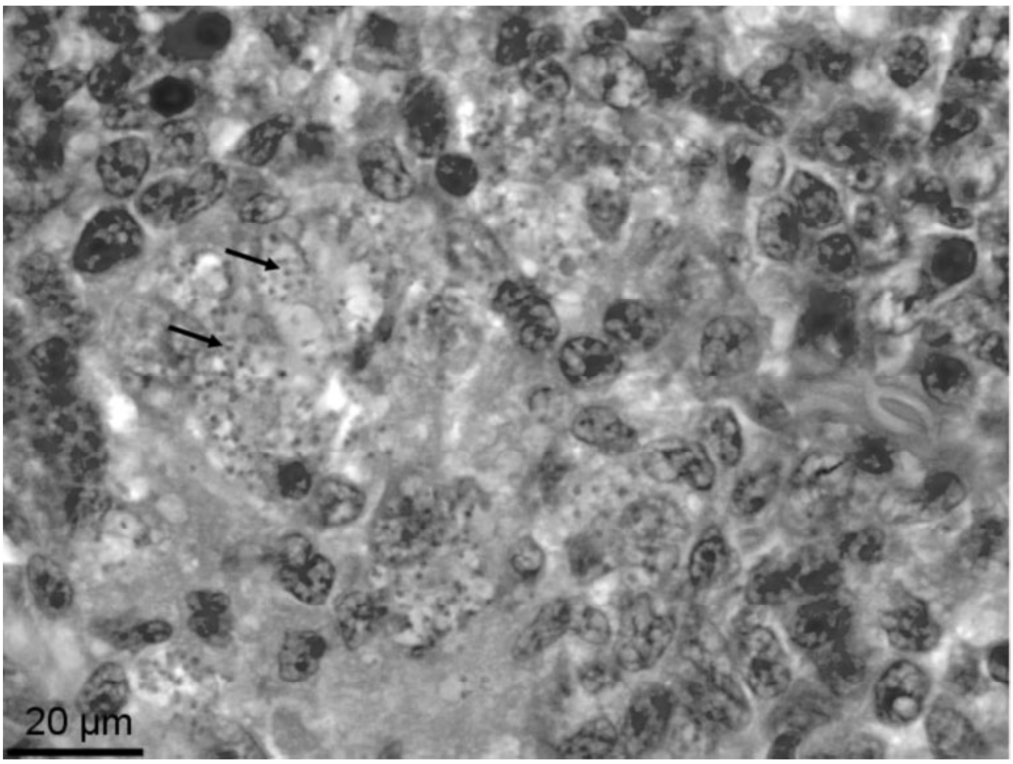

FIG. Part of a histological section of a lymph-node biopsy from Case 2, showing amastigotes within some of the macrophages (arrows). The section was stained with haematoxylin–eosin–saffron.

of south–eastern France, presented with left supra-clavicular lymphadenopathy associated with asthenia, as his sole symptom. A computed-tomography scan of his chest and abdomen confirmed the presence of a left supra-clavicular lymphadenopathy (2.3 cm in diameter), associated with mediastinal (1 cm) and axillary (1.3 cm) lymph nodes. The patient's blood-cell count, liver enzymes and renal function appeared normal. The results of serological tests for EBV, HIV, CMV, hepatitis B virus, hepatitis C virus and toxoplasmosis were either negative or not indicative of recent infection. An ultrasound-guided cytopuncture of the left supra-clavicular lymph node ruled out metastatic cells. Histological examination of the same lymph node, following surgical biopsy, revealed amastigotes within some macrophages (see Figure). Recent or current leishmaniasis was confirmed by the detection of antibodies against *L. infantum* in ELISA and IFAT, and patent leishmaniasis by a western blot showing 14-, 18-, 21-, 23- and 31-kDa bands (Marty *et al.*, 1995) and by the demonstration, using real-time PCR (Mary *et al.*, 2004), of leishmanial DNA in a sample of the patient's peripheral blood. The PCR result indicated that the patient had a low parasitaemia, of less than one parasite/ml blood. As there were no signs or symptoms indicative of systemic disease, no further invasive investigations, such as bone-marrow aspiration, were performed. After the patient received liposomal amphotericin B (10 mg/kg/day on two consecutive days), he showed a transient increase in serum creatinine (Syriopoulou *et al.*, 2003). Blood samples collected on days 14 and 36 post-treatment were PCR-negative for leishmanial DNA.

Case 3

In February 2009, a 73-year-old woman attended the San Remo hospital complaining of a right anterior cervical

lymphadenopathy that had appeared 4 weeks before, associated with low-grade fever. She was taking furosemide, amlodipine, irbesartan and atorvastatin, for hypertension and hyperlipidaemia. The results of a clinical examination were normal apart from a palpable and tender right submandibular lymph node (3×3 cm). There was no lymphangitic streaking or cellulitis and the overlying skin was normal. The results of a full blood count, C-reactive-protein and liver-enzyme evaluations and protein electrophoresis were all normal. A tuberculin skin test and serological tests for anti-*Bartonella* IgG and IgM gave negative results but the results of serological tests for *Toxoplasma gondii*, CMV and EBV indicated current or recent infection with all three pathogens. An ultrasound examination confirmed the clinical findings and excluded spleen and liver enlargement. Histopathological examination of a biopsy of the affected lymph node revealed a necrotizing granulomatous reaction with *Leishmania* amastigotes. *Leishmania*-specific IFAT and ELISA gave positive results and the 14-, 18-, 21-, 23- and 31-kDa bands seen in an *L. infantum*-specific western blot were indicative of patent leishmaniasis (Marty *et al.*, 1995). A sample of serum was, however, PCR-negative for leishmanial DNA (Fissore *et al.*, 2004). The patient was treated with liposomal amphotericin B (3 mg/kg.day on days 1–5, 14 and 21) and responded favourably, with no adverse effects.

DISCUSSION

Rare cases of isolated lymphadenopathy caused by *L. infantum* have been reported before (Peachey *et al.*, 1994; Harms *et al.*, 2001; Aoun *et al.*, 2002). Their physio–pathological significance is open to debate. Aoun *et al.* (2002) raised the possibility that such lymphadenopathy resulted from the aborted visceral dissemination of the parasite or, alternatively, from local extension of cutaneous leishmaniasis. The one French and two Italian cases described above, each of whom had *Leishmania* infection that only seemed to affect one or more superficial lymph nodes, illustrate the narrow border between paucisymptomatic leishmaniasis caused by *L. infantum* and the asymptomatic carriage of the parasite. Apart from their lymphadenopathy, these three cases were asymptomatic or nearly so (Case 2 had asthenia and Case 3 had suffered from low-grade fever). Case 2 was found to have *Leishmania* DNA in his peripheral blood (perhaps indicating aborted visceral dissemination) but his parasitaemia (below one parasite/ml) was as low as seen in many asymptomatic individuals (Le Fichoux *et al.*, 1999; Alborzi *et al.*, 2008; Riera *et al.*, 2008). Although Dereure *et al.* (2003) described two cases of *Leishmania* infection with enlarged superficial lymph nodes — a patient with malignant haemopathy and a pregnant woman — both were immunocompromised. None of the three cases described here displayed overt immunosuppression and their immuno-competence probably allowed containment of the parasite within the lymph nodes. As yet, however, the risk of such paucisymptomatic leishmaniasis evolving towards full-blown VL cannot be predicted. *Leishmania* infection with isolated lymphadenopathy may be mistaken for metastatic adenopathy or malignant haemopathy. In all three cases described here, malignant haemopathy was initially suspected. Histological examination was expected to provide a diagnosis and was conducted as a priority. In *Leishmania*-endemic areas or in travellers visiting such areas, investigation of a lymphadenopathy should include checks for leishmaniasis (Harms *et al.*, 2001; Aoun *et al.*, 2003). Isolated lymphadenopathy caused by *L. infantum* should always be treated because of the risk of visceral extension.

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5 MC is a variant of endocrine carcinoma and should be classified according to endocrine carcinoma of lung or gastrointestinal tract into well-differentiated or poorly differentiated endocrine carcinoma. MC is associated with hormone secretions and is responsible for causing hormonal manifestations. Therefore, in the next WHO classification it should be classified with other endocrine carcinomas for uniformity in diagnosis, prognostic and therapeutic purposes.

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Localized leishmanial lymphadenitis: an unusual manifestation of the disease in an immunocompetent patient

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Sir: Leishmaniasis describes a globally widespread group of parasitic diseases that are transmitted by the bite of infected female phlebotominei sandflies. *Leishmania infantum* is the major causative agent in the Mediterranean area.[1] It may cause a spectrum of clinical diseases, including cutaneous, mucocutaneous and visceral Leishmaniasis with or without lymph node involvement.[2,3] Isolated lymphadenitis is uncommon.[4] Here, we report a case of isolated leishmanial lymphadenitis, an uncommon cause of lymphadenitis that should be considered in the differential diagnosis of unexplained lymphadenopathy in endemic countries.

A 28-year-old White man presented with an indolent left supraclavicular lymph node of 6 months' duration. Physical examination revealed a 20-mm, mobile, firm adenopathy in the left supraclavicular region and another one (10 mm) in the left axillary region. The rest of the examination was unremarkable. Laboratory results were as follows: white blood cell count, 7338 leucocytes/mm^3 (37% neutrophils, 43% lymphocytes). Viral (Epstein–Barr virus, human immunodeficiency virus, hepatitis B virus, hepatitis C virus, cytomegalovirus) and parasitic (toxoplasmosis) serological tests were negative. Serum protein electrophoresis was unremarkable. Computed tomography of the chest, abdomen and pelvis showed some mediastinal lymphadenopathy. The initial clinical impression was lymphoma and the supraclavicular lymph node was excised. Imprints with May–Grünwald–Giemsa stain showed, within the cytoplasm of macrophages, ovoid bodies ranging from 2 to 3 μm with pale cytoplasm and a darkly stained nucleus (Figure 1A). Histological examination showed follicular lymphoid hyperplasia with considerable variation in size and shape (Figure 1B). The follicles were surrounded by an ill-defined mantle. Sinuses were filled with clusters of monocytoid cells. The interfollicular areas were partially replaced by well-demarcated, non-caseating granulomas, composed of epithelioid cells and scattered multinucleate giant cells. Numerous ovoid structures measuring 2–3 μm in diameter were observed in the cytoplasm (Figure 1C) and, interestingly, in the cytoplasm of some mono- or bi-nucleated cells located within the germinal centres (Figure 1D). The absence of Bcl-2 protein immunoreactivity within germinal centres confirmed a reactive follicular hyperplasia. The mon-

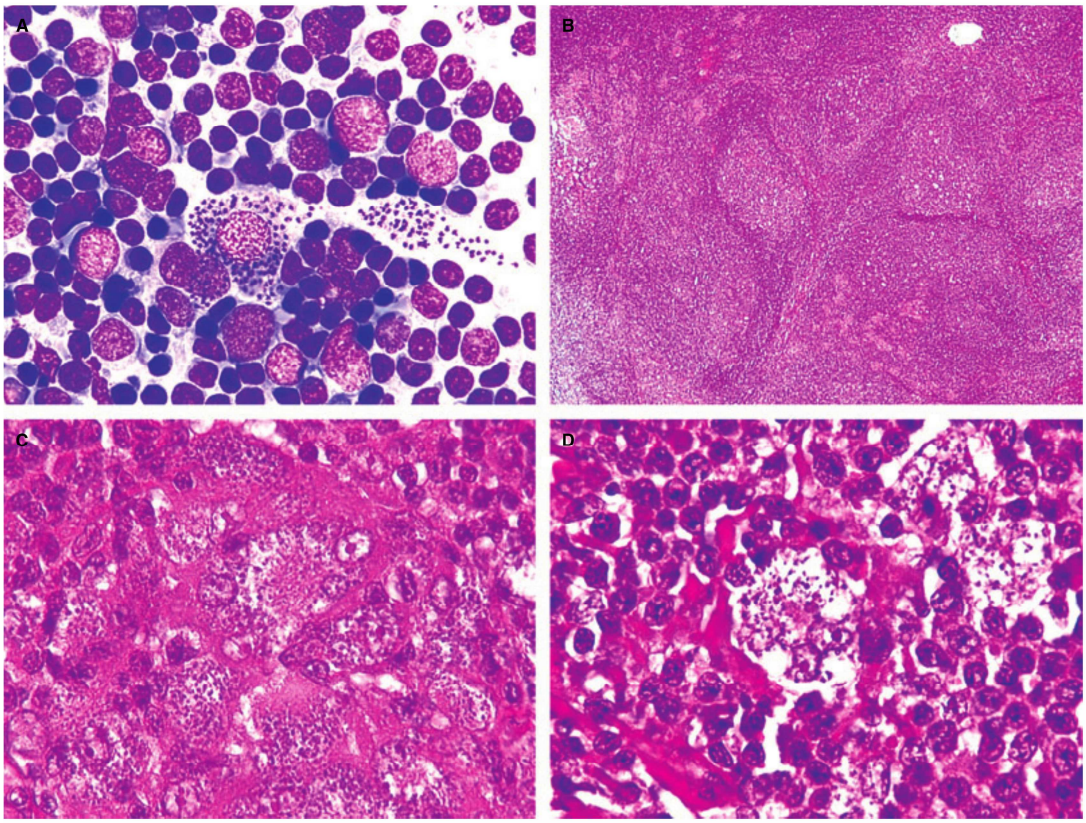

Figure 1. (A) Lymph node imprint: macrophage showing numerous 2–3 μm intracytoplasmic bodies, with dark-stained nuclei, some of them harboring a lateral kinetoplast (MGG x 1000). (B) Lymph node histological section, low magnification: follicular hyperplasia (HES × 40). (C) Interfollicular granuloma composed of parasited epithelioid and giant cells (HES × 1000). (D) Parasited germinal center cell mimicking follicular dendritic cell (HES × 1000).

ocytoid cells expressed B-cell markers (CD20, CD79a) and stains for both κ and λ light chains. The diagnosis of leishmaniasis was again confirmed by parasite DNA research on peripheral blood using real-time polymerase chain reaction (PCR). Antibodies to *L. infantum* (enzyme-linked immunosorbent assay, Western blot) were compatible with leishmaniasis disease. The patient was treated with liposomal amphotericin B (Ambisome®, GILEAD Sciences, Paris, France; 10 mg/kg per day). The PCR controls done on days 14 and 36 were negative.

Isolated leishmanial lymphadenitis may mimic clinically a lymphoma or, because of the location at the junction of the ductus thoracicus, visceral involvement. The clue to histological diagnosis is the recognition of intracytoplasmic Leishman bodies measuring 2–3 μm in diameter. Visualization of the nucleus and kinetoplast is the basis for proper identification of these parasites. Imprints with Giemsa stain are best for morphological identification.[5] This contributes to distinguish leishmanial lymphadenitis from several other infectious diseases, in particular from sporotrichosis and histoplasmosis.[6] Toxoplasma organisms are smaller and not intracellular. Mycobacterium lymphadenitis is similar histologically; however, the organisms are acid-fast bacilli. In our report, the most important as well as the most difficult determination in the case of exaggerated reactive lymphoid hyperplasia, is to distinguish it from follicular B lymphoma. However, distinct germinal centres with tangible-body macrophages, ill-defined mantle zones and negativity of Bcl-2 protein within the follicles contribute to the potential for erroneous

diagnosis. Of particular interest in our case was the presence of parasitized cells, randomly scattered in the centrofollicular areas. The cells were confirmed as macrophages rather than follicular dendritic cells by positivity for CD68 and negativity for CD23 and CD35 on immunohistochemistry. To our knowledge, this surprising finding has not been previously reported.

We report this case to highlight the existence of this unusual clinicopathological presentation of leishmaniasis in an immunocompetent patient. Leishmanial lymphadenitis must be systematically considered when confronted with any adenopathy of unknown origin in endemic areas.

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Histopathogenesis of endometrium with asynchronous glands in dysfunctional uterine bleeding

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Sir: We have recently demonstrated that calretinin reactivity of the endometrial stromal cells (ESC) is strong and diffuse in a zonal pattern in the normal functionalis layer (FL) of the normal cycling endometrium. Reactivity for calretinin of the endometrium was absent or weak in the breakdown phase and increased in the proliferative phase. By the time of ovulation, calretinin immunoreactivity involved most of the thickness of the FL. Calretinin reactivity reached its maximum during the secretory phase, then lost it with shedding.[1–4] CD34 is a marker of endothelial cells and haematopoietic stem cells, and was found to be reactive in the basalis (BL) of the endometrium.[5,6]

In dysfunctional uterine bleeding (DUB), the irregular shedding of endometrium containing both secretory and asynchronous glands of proliferative type is poorly understood. This pattern of endometrial change is thought to be related to the delayed regression of the

Table 1. Summary of 38 cases in the study

		Pattern of ESC Calretinin reactivity in FL			
Type of asynchronous glands	Total *N*	Normal	Diffuse or focal decrease	Absent	CD34 reactivity in ESC, FL
Proliferative with mitosis (Group 1)	4	1	3	0	2
Weakly proliferative (Group 2)	10	1	8	1	7
Weakly secretory or inactive (Group 3)	24	4	17	3	12
Total	38	6	28	4	21

N, number of cases; ESC, endometrial stromal cells; FL, functionalis layer.

FORME CLINIQUE ATYPIQUE : ATTEINTE MUQUEUSE DE LA LEISHMANIOSE A *L. INFANTUM*

A côté des adénopathies isolées, que l'on doit différencier d'un lymphome, d'autres formes atypiques de la leishmaniose à *L. infantum* peuvent s'observer. C'est le cas des formes avec atteinte muqueuse isolée, le diagnostic différentiel étant alors une néoplasie ou une vascularite. Du fait de la chronicité des lésions qu'elle induit, la leishmaniose à *L. infantum* a souvent comme diagnostic différentiel des processus cancéreux. Ces formes muqueuses isolées ne sont pas rares puisque sur les 3 CHU Marseille, Montpellier et Nice, entre 1997 et 2009, 10 cas de leishmaniose muqueuse à *L. infantum* ont été diagnostiqués chez des hommes. Pour 6 de ces cas, les patients étaient immunodéprimés (séropositifs pour le VIH ou traités par immunosuppresseurs), trois des quatre patients restant étaient fumeurs. Il existe une vulnérabilité spécifique du sujet masculin puisqu'il est rapporté dans la littérature sur la leishmaniose muqueuse 35 cas chez le sujet mâle sur les 40 décrits. Les atteintes muqueuses touchent surtout le larynx, mais le pharynx, la bouche, le nez et le cavum sont également atteints. Le typage des souches impliquées ne met pas en évidence de zymodème particulier. Le zymodème MON-1 est prédominant mais aussi MON-24, MON-27, MON-80 ou MON-111 sont à l'origine de cette expression clinique particulière. La sérologie de la leishmaniose semble suffisamment sensible pour confirmer ces cas, contrairement aux données de l'histologie responsable de faux négatifs (4 faux négatifs sur 50 cas). Le polymorphisme des lésions anatomo-pathologiques peut prêter à confusion si l'on ne voit pas les leishmanies. Ainsi, en cas de lésion muqueuse chronique inexpliquée chez un patient vivant en zone d'endémie, une sérologie de la leishmaniose devrait être réalisée ainsi qu'une PCR si l'histologie de ne permet pas d'exclure le diagnostic de lésion muqueuse à *L. infantum*. Le risque de viscéralisation de ce type de lésion n'étant pas négligeable, le traitement entrepris doit prendre en compte la possibilité de lésions viscérales passées inaperçues.

MUCOSAL *LEISHMANIA INFANTUM* LEISHMANIASIS: SPECIFIC PATTERN IN A MULTICENTRE SURVEY AND HISTORICAL CASES

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Mucosal *Leishmania infantum* leishmaniasis: Specific pattern in a multicentre survey and historical cases

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KEYWORDS
Mucosal leishmaniasis;
Leishmania infantum;
Immunodeficiency;
Topical administration;
Chronic rhinitis

Summary *Objective: Leishmania infantum* mucosally restricted leishmaniasis was rarely reported, so that diagnostic and treatment strategies remain debated. A long-term multicentric survey appeared thereby necessary.
Methods: Cases were prospectively collected over 12 years in 3 academic hospitals of Southern France. Predisposing factors, clinical findings, diagnostic procedures, treatment and outcome were compared to medical literature.
Results: Ten new cases and 40 historical reports were collected. Respectively 10/10 and 35/40 patients were adult males. Immunodeficiency was frequent (5/10 and 18/40). No previous cutaneous lesion was reported. Leishmaniasis affected mostly larynx (5/10 and 19/40), but also mouth (2/10 and 19/40) and nose (3/10 and 5/40). Lesions were highly polymorph. *Mucosa* histological examination provided respectively 1/10 and 2/40 false negative results, contrary to serum immunoblotting and PCR on mucosal biopsy. Although local response was always

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satisfactory even using topical treatment, subsequent visceral spreading was observed in 2/10 and 1/40 cases.
Conclusion: L. infantum mucosally restricted leishmaniasis exhibits a specific pattern, marked by tropism for adult males, high clinical and histological polymorphism. Immunoblot screening and PCR confirmation of suspected lesions are necessary because of direct examination occasional false negative results. The risk of visceral spreading sustains systemic therapy.
Summary: Leishmania infantum mucosal leishmaniasis mostly affects adult males, half of them immunodeficient. Clinical and histological polymorphism makes the diagnosis difficult, stressing the need for immunoblot screening and *mucosa* PCR analysis of suspected cases. Possible visceralization sustains systemic therapy.

Introduction

Mucocutaneous leishmaniasis is well-known as *espundia*, caused by New World *Leishmania* species, especially *Leishmania braziliensis*. *Espundia* develops within a few months to several years after a cutaneous leishmaniasis. It provokes severe rhino-facial destructions and may relapse despite parenteral treatment.[1] Around the Mediterranean, mucosally restricted leishmaniasis is known for half a century.[2] The disease is due to *Leishmania infantum*, which most often causes visceral leishmaniasis, and seldom cutaneous lesions.[3] Conversely, other Old World species such as *Leishmania major* cause only mucosal extension of cutaneous lesions.[4] In 2003, a review of the literature gathered 31 cases of *L infantum* mucosally restricted leishmaniasis (LIML).[5] However, since 2003, this condition has been increasingly reported.[6–18] Until now, no study reported more than 3 consecutive cases, so that many points remain controversial: predisposing conditions are badly determined, diagnostic efficiency of pathological examination is uncertain,[5,7,8,17] and local treatments are sometimes used although parenteral treatment remains the reference.[5,14] Overall, LIML seems insufficiently evoked: misdiagnosis as neoplasia or vasculitis sometimes leads to undue therapeutics such as radiotherapy or steroids.[5,8,9,16,17] To raise awareness and clarify remaining issues, we conducted a 12-year-long study among the 3 academic hospitals in French endemic zone and updated the literature review.

Material and methods

Cases were collected in all French academic hospitals bordering the Mediterranean: Marseille, Montpellier (National Reference Center for *Leishmania*), and Nice. A prospectively filled-in registry was used from 1997 to 2009. Criteria for inclusion were primarily the presentation of mucosal lesion due to *Leishmania*, then the absence of initial visceral involvement as demonstrated by a negative direct examination of bone marrow, and finally the identification of *L. infantum* or the absence of travel in areas endemic for other *Leishmania* species. ELISA on serum and PCR on mucosal tissue were performed as previously described.[19,20] Immunoblotting was done using LeishWesternBlotIgG from LDBIO Diagnostics®. Strains were typed using multilocus enzyme electrophoresis (MLEE).[21] After informed consent was obtained, TNF promoter of recent patients from Marseille was sequenced using Applied Biosystems™ 3130 Genetic Analyzer to investigate the presence of the mutation associated with New World mucocutaneous leishmaniasis.

The literature was reviewed using MEDLINE (keywords: "leishmaniasis" and "mucosal", "mucocutaneous", "oral", "nasal", "laryngeal", or "tonsil"). We also checked references in the articles collected to detect additional cases and to avoid duplication of reported cases. Reports without documented bone marrow examination were excluded, so as not to include undiagnosed visceral leishmaniasis. Reports with extra-tegumentary lesions were excluded.

Results

Of the 10 patients, 9 lived in endemic foci of Southern France. One patient took holidays there. All patients were adult males, aged between 21 and 65 years (median: 44). Five patients were immunocompromised: 3 HIV infections (when documented, CD4 counts superior to 200/mm^3), one azathioprine and prednisone treatment for renal transplantation, one chronic lymphoid leukemia. Three patients reported smoking. At time of diagnosis, only single lesions were found. Lesions were ulcerative (4/10), polypoid (2/10), infiltrative (2/10), or nodular (2/10). LIML affected larynx (5/10), including three vocal cord lesions (3/10), nose (3/10, Fig. 1), soft palate (1/10), or cavum (1/10). One lesion was painful. One patient with subsequent visceral spreading then presented with multiple mucosal lesions. For all patients, neither history nor scar of cutaneous leishmaniasis was reported. Delay between first sign and diagnosis ranged from 1 week to 3 years (median: 3 months) (Table 1).

Direct examination of mucosal biopsy showed only nonspecific findings in one patient diagnosed by PCR on mucosal biopsy and serology: even with Giemsa staining, only malpighian metaplasia and polymorph inflammation were seen, without granuloma. Direct examination showed *Leishmania* amastigotes in the nine other cases. *In vitro* culture was positive in 4/8 patients, isolating *L. infantum* zymodeme MON-1. Immunoblot was positive in 8/8 cases, while ELISA was negative in 1/8 cases. In case 1, serology was positive for a serum sampled 10 years before the first manifestation, without clinical manifestation evocative of mucosal or visceral leishmaniasis at that time. PCR on mucosal biopsy was positive in 5/5 cases.

Three patients received liposomal amphotericin B, six received meglumine antimoniate, and one left without treatment. One patient discontinued meglumine antimoniate after 7 days because of pancreatitis, but responded well. No local relapse was described during follow-up (median: 8

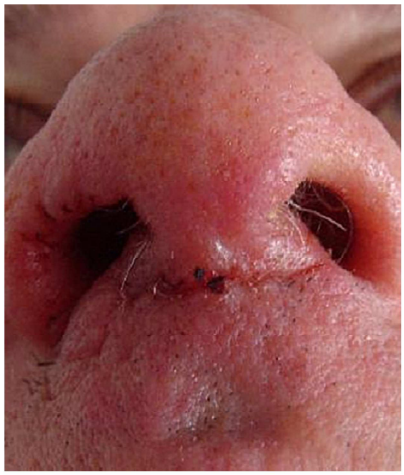

Figure 1 Endonasal mucosal leishmaniasis (Case 5).

months, ranging from 0 to 5 years). One patient developed visceral leishmaniasis after 2 years despite a good initial response to 14 days of meglumine antimoniate. In another patient, PCR in two successive blood samples showed asymptomatic carriage 6 months after treatment with meglumine antimoniate for 28 days. The untreated patient developed visceral leishmaniasis 6 months later.

No topical treatment was used. Case 5 received inhaled steroids (fluticasone) because of misdiagnosis as allergic rhinitis, with consecutive worsening of the disease.

TNF promoter sequencing of case 4 showed NT_007592.14:g.22401358G>C homozygote single nucleotide variation and case 5 showed NT_007592.14:g.22400733C>T heterozygote single nucleotide variation.

Historical cases

A review in 2003 had retrieved 51 cases of allegedly isolated mucosal involvement, but visceral involvement was not excluded in 20 cases.[5] Seventeen additional alleged cases have been reported since.[6–18,22] Overall, bone marrow examination was lacking for 28/68 cases,[5,6,9–12,16,18,22] so that a total of 40 cases were used to elaborate the present report.

Infections occurred in Spain (17/40), Italy (13/40), France (5/40), Malta (1/40), and Morocco (1/40).[5,8,9,13–15,17] The place of infection was unknown for 3 patients who had traveled to North Africa, Italy, and Brazil, to Spain and Israel, and to France, Italy, and Yugoslavia.[5] Strains belonged to zymodemes MON-1, MON-24, MON-27, MON-111, or MON-183.[5,7,14]

Patients were aged between 28 and 71 years (median: 58 years), 35/40 patients were male, and 18/40 patients were immunocompromised: 9 HIV infection (median CD4 count: 235/mm^3; range: 140 to 353/mm^3), 3 organ transplantation, 5 steroid therapy, and one non Hodgkin lymphoma.[5,9,13] Furthermore, 9 patients reported smoking, and 3 received inhaled steroid therapy.[5,8,13,15,23]

Eight patients had multiple lesions.[5] Location of the lesion varied: larynx (19/40), including 9 vocal cord lesions; mouth (17/40), including 3 tongue lesions, 3 tonsil lesions, and one cheek lesion; nose (5/40); and pharynx (2/40).[5,7–9,13–15,17] Aspect of the lesion varied: ulceration (14/40), pseudotumor (10/40), infiltrative swelling (8/40), nodule (5/40), polyp (3/40), and papule (1/40).[5,7–9,13–15,17] Six patients reported pain.[5,9,13,17]

Difficulties in diagnosis were frequently observed: delay between first manifestation and diagnosis was described in 31 cases, ranging from 3 weeks to 4.5 years (mean value: 12 months). Misdiagnosis as neoplasia or autoimmune disease led sometimes to unnecessary radiotherapy or steroid treatment.[5,8,17]

Concerning diagnostic procedures, mucosal sample histologic examination was initially negative in two cases,[8,17] but positive in all other. *In vitro* culture was positive in 14/23 cases. Positive PCR on mucosal biopsy was reported once.[7] Serology was positive in 23/29 cases (using mainly IFAT but also ELISA, agglutination test, counterimmunoelectrophoresis): IFAT was falsely negative in 3 cases and hemagglutination in another (technique unknown for the two others).[5,7,8,13–15,17]

Topical treatments (*in situ* meglumine, *in situ* paromomycin, surgery, or cryotherapy) were successfully applied in 7 cases (follow-up up to 2 years).[5,14] Various systemic treatments were also used: meglumine (21/40), associated with pentamidine for one patient, and with allopurinol for another[5,7–9,13,15]; liposomal amphotericin B (6/40), associated in one case with ketoconazole (1/40)[5,7,15]; antimony tartrate (2/40)[5]; solustibosan (1/40)[5]; itraconazole associated with allopurinol, with terbinafine, and with topical paromomycin (1 case each)[5,17]; aminosidine (1/40).[23] All systemic treatments were initially successful except aminosidine in one case (14 mg/kg for 17 days).[23] Two patients relapsed locally, one after pentavalent antimoniate, and the other after glucantime plus allopurinol. Another patient presented with subsequent visceral leishmaniasis, 9 months after glucantime (850 mg).[5]

Discussion

LIML is not exceptional: it represented 2.3% of autochtonous leishmaniases in France between 1999 and 2007 according to the National Reference Center.[24] Despite more frequent reports, leishmaniasis is rarely suspected in the Old World in case of isolated mucosal lesion.[6–18] This neglect causes diagnostic delays or undue therapeutics.[5,8,9,17,case 5]

Pathophysiology remains unclear. It is unlikely that LIML results from *in situ* inoculation. Besides, unlike *espundia*, there is no initial cutaneous lesion and so LIML might result from hematogenic parasitic spreading after a bite by infected sandfly. Hence, local or general predisposing factors other than mild immunosuppression remain to be determined.

LIML affects mostly inhabitants of endemic zones, but sometimes also vacationers.[5,8,9,16,17,case 10] All cases occurred in the Western Mediterranean basin. This distribution, which roughly corresponds to that of *Phlebotomus ariasi* and *Phlebotomus perniciosus*,[25] remains unexplained. The factors causing the mucosally restricted presentation of *L. infantum* infection are unknown. Adult males represented 10/10 new cases, and 35/40 historical cases. Such male predominance has never been pointed out. Possible prolonged

Table 1 Characteristics of cases of mucosally restricted leishmaniasis due to *L. infantum*.

Patient	1	2	3	4	5
Age	58	35	40	38	54
Sex	M	M	M	M	M
Concomitant illness	Renal transplantation	HIV 203 CD4/mm^3	HIV 299 CD4/mm^3	None	None
Clinical findings (delay to diagnosis)	Pain dysphagy soft palate ulceration (1 month)	Dysphonia ulcerative vocal cord lesion (2 weeks)	Dysphonia polypoid laryngeal lesion (1 week)	Infiltrative rhinitis (1 year)	Nasal obstruction papulo-nodular and infiltrative lesion (3 years)
Mucosa examination:					
DE	Positive	Positive	Positive	Negative	Positive
Culture	Positive	Negative	Not done	Negative	Positive
PCR	Positive	Not done	Not done	Positive	Positive
Serology	Positive: ELISA + WB	Not done	Positive ELISA + WB	Positive ELISA + WB	Positive ELISA + WB
Treatment	IM meglumine antimoniate 850 mg/d for 7 days	IM meglumine antimoniate 850 mg/d	IM meglumine antimoniate 20 mg/kg/d for 14 days	IV liposomal amphotericin B 250 mg/d D1,2,3,4, 5, 19,33,48,63	IM meglumine antimoniate 20 mg/kg/d for 28 days
Outcome (Follow-up)	Cure (5 years)	Cure (none)	Visceral relapse (2 years)	Cure (10 months)	Locally cured, asymptomatic carriage with positive PCR in blood sample after 6 months
Techniques used to dismiss visceral leishmaniasis	DE + culture + PCR	DE + culture + PCR	DE + culture + PCR	DE + culture + PCR	DE + culture + PCR

Patient	6	7	8	9	10
Age	65	49	40	21	58
Sex	M	M	M	M	M
Concomitant illness	HIV (ND)	None	None	None	Chronic lymphoid leukemia
Clinical findings (delay to diagnosis)	Chronic purulent ulcerative rhinitis (4 months)	Vocal cord nodular lesion (3 months)	Dysphonia vocal cord ulcerative lesion (2 months)	Polypoid epiglottic lesion (asymptomatic)	Infiltrative cavum lesion (2 months)
Mucosa examination:					
DE	Positive	Positive	Positive	Positive	Positive
Culture	Contamination	Negative	Not done	Positive	Positive

(*continued on next page*)

Table 1 (*continued*)

PCR	Positive	Not done	Not done	Positive	Not done
Serology	Positive ELISA + WB	Positive WB Negative ELISA	Positive ELISA + WB	Positive ELISA + WB	Positive IFAT
Treatment	IM liposomal amphotericin B 10 mg/kg/d for 2 days	IM meglumine antimoniate 850 mg/d For 21 days	IV liposomal amphotericin B 3 mg/kg/d D1,2,3,4,5,10	None then IM meglumine antimoniate 20 mg/kg/d for 21 days	IM meglumine antimoniate 20 mg/kg/d for 28 days
Outcome (Follow-up)	Cure (none)	Lost to follow-up	Cure (1 month)	Visceral leishmaniasis and multiple mucosal lesions, cured after treatment (8 months)	Cure (1 year)
Techniques used to dismiss visceral leishmaniasis	DE + culture	DE + culture	DE + culture + PCR	DE + culture + PCR	DE + culture + PCR

M: male; HIV: human immunodeficiency virus; PCR: polymerase chain reaction; DE: direct examination; IFAT: indirect fluorescent antibody test; WB: western blot; IV: intra venous; IM: intra muscular.

incubation period or publication bias does not convincingly explain the absence of pediatric cases, and certainly not the overwhelmingly unbalanced sex ratio. Conversely, children are affected by *L. infantum* visceral or cutaneous leishmaniasis, and male/female ratio is 2/1 and 1/1, respectively.[26,27] Further studies should determine endocrinal or immunological predisposing factors in adult males.

LIML is associated in half the cases with mild immunodepression, such as HIV infection with 200—500 CD4/mm^3, and steroid or other immunosuppressive treatments. Conversely, in visceral leishmaniasis, which also affects immunodeficient patients in half of cases, risk rises only when immunodeficiency is more severe, at least in case of HIV-*L. infantum* coinfection: 79—100% of visceral leishmaniasis coinfected patients have less than 200 CD4/μL.[1] Additionally, local factors such as tobacco smoking or inhaled steroid therapy were rarely encountered and do not appear to play a major role in the occurrence of LIML. Besides, the *espundia*-associated TNF promoter — 308G>A nucleotidic substitution[28] was absent in 2/2 cases.

LIML strains do not differ from viscerotropic or cutaneous *L. infantum* strains: in our series, 4/4 strains belonged to zymodeme MON-1, also responsible for 97% of local visceral leishmaniasis.[3] Elsewhere, other viscerotropic or dermotropic zymodemes were involved, such as MON-24, MON-27, MON-80 or MON-111.[5—7,14]

Specific clinical findings are scarce. Incubation period appears long: last exposure dated from 16 years in one case[23] and serology was retrospectively positive for 10 years in case 1. LIML exhibits large polymorphism: lesions are either polypoid, infiltrative, ulcerative, or papulo-nodular.[5—11,14—18] Most frequent location is larynx, but pharynx, mouth, nose, and cavum are also involved. Conversely, *espundia* usually appears as a small-sized hyperemic inflammatory granuloma of the nose, rapidly evolving to an extending ulcer.[1] Chronic rhinitis was observed in three of our cases, but rarely reported before.[17] The actual frequency of leishmaniasis in case of chronic rhinitis is probably underestimated since biopsies are not always taken and histopathology may ignore the diagnosis. LIML should therefore be evoked in endemic zones, especially when response to conventional treatments is unsatisfactory.

Initially, the 10 new patients presented with a single lesion. In one case, however, multiple lesions subsequently appeared, in association with visceral spreading of the parasite. Conversely, 8/40 historical cases presented multiple lesions, without visceralization.[5] Unlike cutaneous disease, lesion can be painful, so that this characteristic must not serve to rule out the diagnosis.[5,9,13,17] History of cutaneous leishmaniasis was absent in 50/50 cases. This feature, opposite to *espundia*,[1] complicates the diagnosis.

Disease severity is variable. Local evolution seems benign, though functional symptoms such as dyspnea may appear: a laryngeal leishmaniasis was not treated for 23 years, without further evolution.[26] On the other hand, visceral spreading is possible: three patients developed visceral leishmaniasis and one developed asymptomatic carriage. Even though comparative strain typing could not be performed, the possibility of early reinfection seems very unlikely in a region where visceral leishmaniasis incidence is very low.[26] Besides, as *Leishmania* PCR was initially negative on a bone marrow sample for these 3 patients, an

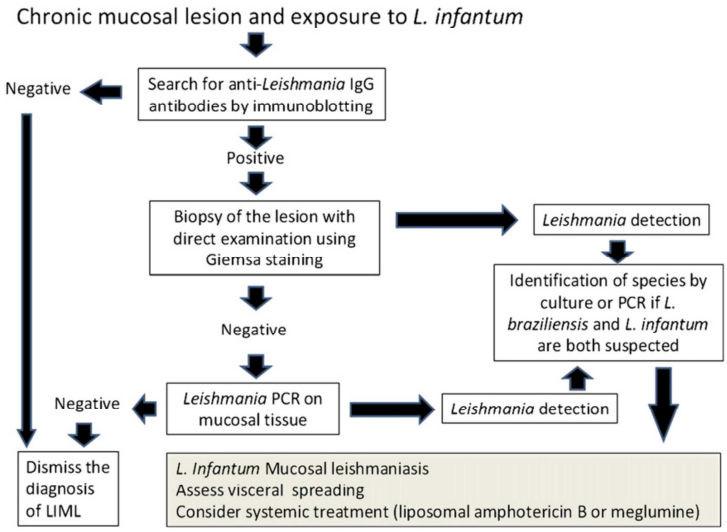

Figure 2 Proposed algorithm for the diagnosis of mucosally restricted leishmaniasis due to *Leishmania infantum*.

undiagnosed initial visceral spreading is very unlikely. Overall, a continuum may exist between LIML and visceral *L. infantum* infection as mucosal lesion can also be concomitant[5] or subsequent[29] to visceral disease, conversely to South American species.[1] In case of lymphoid involvement such as tonsillitis, one should fear even more visceral involvement, as a patient with normal bone marrow examination exhibited tonsillitis and hepatic involvement.[30]

Serology appears sensitive: in the current series, immunoblot and ELISA were positive in 8/8 and 7/8 cases, respectively. Previous reports showed an 80% sensitivity using mainly IFAT, and no false negativity of immunoblot.[5,8,9,15,17] Hence, immunoblot should be consistently proposed to screen chronic mucosal lesion patients in endemic zone. Negativity might reliably invalidate leishmaniasis, whereas positivity should be interpreted cautiously as it concerns up to 16% of healthy subjects in Southern France because of asymptomatic carriage or immunologic scars of past infection.[20]

Conversely to New World mucocutaneous leishmaniasis,[1] earlier reports claimed a 100% sensitivity of mucosal biopsy direct examination.[5] Actually, false negativity occurred in case 4 and in three historical cases.[5,8,17] Furthermore, a large histological polymorphism makes the diagnosis difficult when parasites are undetected.[31] Inversely, PCR false negativity was never reported. One of the new LIML cases was diagnosed thanks to seric immunoblotting and PCR on mucosal tissue despite a normal direct examination of a mucosal biopsy. This case highlights the risk of underdiagnosis associated with the conventional diagnosis strategy, based only on direct examination of mucosal tissue. So, in case of chronic mucosal lesion in *L. infantum* endemic zone, search of anti-*leishmania* IgG antibodies by immunoblotting should be consistently performed, and PCR assays should be done when LIML is suspected but not confirmed by the direct examination of mucosal tissue. Besides, in South America, in those geographical areas where *Leishmania braziliensis* and *L. infantum* occur sympatrically,[32] identification of the species by PCR or culture should be done to determine evolutive risks, and thereby appropriate treatment and follow-up. To summarize this new strategy aiming at avoiding diagnostic delays and misdiagnosis, a new algorithm is proposed here (Fig. 2), taking into account the reliability of serologic and molecular tools.

LIML responds initially well to treatments and scars are rare,[5] so that the issue is the prevention of local relapse and visceral spreading, which occur despite prolonged parenteral treatments.[5,11,18] Harmless lesions should hence also be treated to prevent visceral spreading.

Topical treatments were sometimes used.[5,14] However, the risk of visceral spreading supports consistent administration of parenteral treatment such as liposomal amphotericin B as in visceral leishmaniasis. Topical treatment should probably be restricted to contraindication or intolerance to parenteral treatment. Various protocols of parenteral treatments were used, so that it is not possible to define precisely how it should be administered. Miltefosine, the only oral medication for leishmaniasis, was not evaluated in LIML and cannot be recommended yet. Cautious prolonged follow-up is essential, since local or visceral relapse can occur, even after parenteral treatments.

As a conclusion, the current long-standing multicentre prospective series establishes that *L. infantum* mucosally restricted leishmaniasis exhibits a specific pattern characterized by slow and mild local evolution, clinical and histological polymorphism, and good response to treatment. Additionally, this study highlights the risk of visceral spreading. New concepts are thereby emerging: first, serology and PCR proved useful, given that histological examination may not reveal the disease; second, the possibility of visceralization raises concerns about topical treatments.

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Conflict of interest

None.

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ETUDE PAR LES MICROSATELLITES DE SOUCHES DE *L. INFANTUM* ISSUES DE PORTEURS ASYMPTOMATIQUES

Les porteurs asymptomatiques constituent une part très importante des sujets infectés par *L. infantum*. Malgré cela à ce jour, très peu de souches ont pu être isolées de porteurs asymptomatiques. En effet, du fait de la très faible parasitémie, l'isolement de souches nécessite de cultiver de grande quantité de sang en culture afin de réussir à isoler des souches. Grace à une enquête réalisée en 1996-1997 chez les donneurs de sang de Monaco, de grandes quantités de cellules mononucléées périphériques issues de porteurs asymptomatiques ont été mises en culture. Neuf souches de *L. infantum* issues de neuf sujets différents ont ainsi été isolées. La comparaison de ces souches issues de porteurs asymptomatiques avec d'autres souches fait l'objet d'un article qui est en re-soumission à *International Journal of Parasitology*. Afin de comparer ces souches avec d'autres souches de diverses origines (humaines, canines et de phlébotomes), un génotypage par les microsatellites a été réalisé en utilisant 33 marqueurs microsatellites différents. La comparaison porte sur 36 souches dont les 9 issues de porteurs asymptomatiques. Ces souches proviennent de la zone d'endémie allant de la région Languedoc Roussillon, la région Provence Alpes Côtes d'Azur et la Corse. Il s'agit là de la première étude de comparaison génétique des souches issues de porteurs asymptomatiques avec d'autres souches permettant d'appréhender les facteurs liés aux souches dans la virulence de *L. infantum*. Par cette étude, les microsatellites mettent en évidence des sous populations à l'intérieur des populations de souches MON-1 confirmant la diversité génétique intra MON-1. Parmi les 9 souches issues de porteurs asymptomatiques, 7 ont la même structure génétique et les 2 autres, identiques entre elles, sont très proches des précédentes. De plus, il semble y avoir une différence entre les souches issues de porteurs asymptomatiques et les souches issues de patients symptomatiques, et plus particulièrement

les sujets séropositifs pour le VIH. Ce résultat suggère que la structure génétique de la souche aurait une influence sur l'expression de la pathologie.

ARTICLE RE – SOUMIS

PARASITIC GENOTYPES DIFFER IN LEISHMANIASIS PATIENTS COMPARED TO ASYMPTOMATIC RELATED CARRIERS

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Parasitic genotypes differ in leishmaniasis patients compared to asymptomatic related carriers.

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Abstract.

For numerous infectious diseases affecting humans, clinical manifestations range from asymptomatic forms to severe pathologies. The originality of this study was to focus on asymptomatic carriers (AC) of *Leishmania infantum* in Southern France. The fundamental interest of these AC is that they can be a reservoir of potentially pathogenic microorganisms. It remains to be established whether the parasitic genomes from AC differ from those of patients. Multilocus microsatellite typing (MLMT) was used to investigate the genetic variation among 36 French strains of *L. infantum*. Nine parasitic strains of AC isolated from blood donors were compared to 27 strains of *L. infantum* belonging to zymodemes, MON 1, 33 and 183. These strains were isolated from humans HIV positive or not with visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), from canine leishmaniasis (CanL) or from phlebotomine sandflies. MLMT data generated using 33 locus were analyzed by a Bayesian model-based clustering algorithm and constructing phylogenetic tree based on genetic distances. Both analyses structured the MON-1 sample into two main clusters. Furthermore, genetic analysis demonstrated that these nine AC strains are divided into 2 clusters grouped with the MON-1 strains. One cluster with 7 strains is related but different to human symptomatic strains from Alpes-Maritimes region whereas the other cluster has the two remaining strains together with CanL strains as well as one strain from VL patient. Genetic diversity among AC was very weak since the 9 *Leishmania* strains belong to only 2 genotypes. Genetic differentiations were evidenced between AC strains and non AC strains and especially between AC and HIV+ populations. Our data explore for the first time the genetic diversity among *Leishmania infantum* from asymptomatic human carriers and reveal a weak polymorphism compared to *Leishmania* parasites isolated from human patients.

Index Keywords: Asymptomatic carriers, cryptic infection, reservoir, *Leishmania infantum*, molecular epidemiology, multilocus microsatellite typing (MLMT), Southern France, phylogenetic analysis

Introduction

Leishmaniases, caused by the *Leishmania* parasite, are a worldwide endemic disease, with an estimated disease burden of 2,357,000 disability-adjusted life years and 59,000 deaths per year (WHO, 2002). These diseases range in severity from a healing skin ulcer to an overwhelming visceral leishmaniasis. Zoonotic visceral leishmaniasis, caused by the protozoan parasite *Leishmania infantum*, is a sandfly-borne disease found in the Mediterranean area, Asia, and Latin America. This species is associated with benign cutaneous leishmaniasis (CL) as well as severe visceral leishmaniasis (VL), fatal without treatment. In the Mediterranean Basin, dogs are the principle peridomestic reservoir of human VL. Because control programs based on the elimination of infected dogs have failed to halt or prevent epidemics of urban visceral leishmaniases due to *L. infantum* in Brazil (Madeira Mde et al., 2004), other secondary reservoirs such as humans may be important in propagating the infection. Parasite circulation in peripheral blood has been reported in asymptomatic leishmaniasis (persons or animals who are infected with infectious microorganisms but display no symptoms) detected either by PCR with presence of DNA and/or by culture (Guevara et al., 1993; Le Fichoux et al., 1999; Garcia-Garcia et al., 2006; Fakhar et al., 2008; Riera et al., 2008; Scarlata et al., 2008; Michel et al., 2011). Most scientific studies of infectious diseases have focused on diseased individuals, from an epidemiological or clinical point of view. However, it has been demonstrated that asymptomatic carriers (AC) represent a large part of the human population with infectious diseases in endemic areas (Costa et al., 2002; Riera et al., 2004). This must be considered to understand the dissemination pathways of *L. infantum* parasites and their capacity for surviving in hosts (Bañuls et al., 2011). A previous study on blood donors isolated nine *L. infantum* strains from AC living in Southern France (Le Fichoux et al., 1999). Besides these rare strains isolated from AC, most *L. infantum* parasites are isolated from VL patients. In Southern Europe, *Leishmania*/HIV co-infections have become increasingly frequent, with up to 9% of AIDS cases developing VL, particularly in France (Desjeux and Alvar, 2003). In France the incidence of VL in HIV+ patients dropped from 12 in every 10,000 people per year to 7 per 10,000 per year after 1996, the year in which Highly Active Anti-Retroviral therapy was introduced in this country (del Giudice et al., 2002). One of the most powerful and discriminative DNA-based methods for strain differentiation and population genetics is the analysis of highly variable, co-dominant microsatellite markers. Recently, multilocus microsatellite typing (MLMT) has been used successfully to differentiate *L. infantum* populations in the Mediterranean region of Europe.

This method enabled differentiation even at the intra-zymodeme level, as shown for the predominant MON-1 zymodeme of *L. infantum* (Ochsenreither et al., 2006; Montoya et al., 2007; Kuhls et al., 2008). Here, a panel of 33 microsatellite markers was used to investigate genetic structure and gene diversity among strains of *L. infantum* isolated from healthy blood donors and to compare them with other *L. infantum* strains (isolated from sandflies, dogs, or human patients) from Southern France. To our knowledge this is the first genetic study on "asymptomatic" *Leishmania* strains. Our results will be discussed regarding the potential contribution of AC in leishmaniasis transmission. The possibility of leishmaniasis transmission from an asymptomatic blood donor to a patient will also be discussed regarding the public health risk.

2. Materials and methods

2.1. Leishmania *strains*

Table 1 lists the W.H.O code, geographical origin, clinical manifestation, and Multi-Locus Enzyme Electrophoresis (MLEE) identification for the 36 local strains of *L. infantum*. All the strains were isolated in Southern France and provided by the French National Reference Center of *Leishmania* (Figure 1). Thirty three belong to zymodeme MON-1, two to MON-33, and one to MON-183 (used as outgroup). Parasites were isolated either from VL and CL HIV patients (8 strains), VL (9 strains), CL (5 strains), canine leishmaniasis (3 strains), *Phlebotomus perniciosus* (1 strain), *Phlebotomus ariasi* (1 strain), or healthy human blood donors (9 MON-1 strains described in (Le Fichoux et al., 1999). Promastigotes were maintained at 26°C in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal calf serum, 2mM glutamine, 100U/ml penicillin, and 100µg/mL streptomycin in disposable flasks. Parasites were harvested in logarithmic phase by centrifugation (5,000g for 30 min at 4°C) and washed twice in PBS (Phosphate-Buffered Saline). Parasite pellets were kept at -80°C until DNA extraction. DNA was extracted by phenol/chloroform (Sambrook, 1989), and DNA concentration was estimated by spectrophotometry at 260 nm.

2.2. Microsatellite Genotyping

Microsatellite analysis was done using 33 microsatellite markers (Table 2). Twenty-eight microsatellite loci are already published (Rossi et al., 1994; Russell et al., 1999; el Tai et al., 2000; Jamjoom et al., 2002; Ochsenreither et al., 2006; Kuhls et al., 2007); five were developed by the authors. The 36 strains under study were amplified according to the following conditions. Every 30µL of reaction mix was composed of 10pmol of each primer,

the forward being labeled, 100ng template DNA, 1nmol of each dNTP, 3µL buffer 10X, and 1.5 unit of Taq Polymerase (Taq Polymerase, 5U/µl, Roche Diagnostics, France). Amplifications were carried out in a thermal cycler using the following conditions: 35 cycles of 94°C for 30 s, annealing temperature of each locus (see Table 2) for 1 min, 72°C for 1 min, and a final extension step of 72°C for 30 min. Size analysis of the fluorescence-labeled PCR-products was carried out on an ABI Prism 3130XL genetic analyzer (Applied Biosystems, France) and data were stored and analyzed with GeneMapper analysis software (version 4.0, Applied Biosystems, France). Genescan 500 LIZ (Applied Biosystems, France) was used as internal size standard. All 36 *Leishmania* strains were genotyped at all 33 loci.

2.3. Data analysis

2.3.1. Phylogenetic and population STRUCTURE analysis

Data were analyzed with Genetix version 4.05.2 (2004) and FSTat Version 2.9.3.2 softwares (Goudet et al., 2002), which compute estimates and test the significance of various parameters of population genetics. Genetic polymorphism was measured by the number of alleles per locus (*N*a) and by Nei's unbiased genetic diversity within subsamples H_s (Nei and Chesser, 1983). F_{ST} measures the relative inbreeding in subpopulations that is attributable to the subdivision of the total population into subpopulations of limited size. F_{ST} thus also measures genetic differentiation between subpopulations. Data were heterogeneous regarding year of sampling, clinical manifestations, geographical origin, host species, and patient's sex and age. To assess the possible contribution of these factors to genetic differentiation, we compared F_{ST} obtained with different sampling strategies. We constructed a neighbor-joining tree to examine the relationships between the AC population and the others by using the PHYLIP software (version 3.5c; J. Felsenstein, Department of Genetics, University of Washington, Seattle, 1993). The neighbor-joining tree (Saitou and Nei, 1987) was constructed through calculations of Cavalli-Sforza genetic distance from allelic frequencies, and the robustness of tree topology was obtained by bootstrap resampling of loci. The tree was edited using TreeDyn software (Chevenet et al., 2006).

2.3.2 Clustering analysis

MLMT data were analyzed by a Bayesian statistics-based method implemented in STRUCTURE v.2.3.3 (2010, January) (Pritchard et al., 2000) to explore the structure of AC population versus non-AC populations. STRUCTURE uses Bayesian Monte-Carlo Markov Chain (MCMC) sampling to identify the optimal number of clusters *K* for a given multi-locus

dataset without needing to identify population subunits *a priori*. The parameters used were the admixture model with length of burn-in period of 200,000 iterations, followed by 200,000 of MCMC repeats after burn-in. Based on multilocus genotype data, the individuals were divided into *K* subpopulations with *K* ranging from 1 to 11 and ten independent runs were performed for each value of *K*. The methods of Evanno et al. (2005) and Garnier et al. (2004) were employed to assess the optimal value of *K* (i.e. the optimal number of clusters in the dataset) corresponding to the peak in the DeltaK graph (Garnier et al., 2004; Evanno et al., 2005).

3. Results

3.1. Global genetic diversity and allelic polymorphism

We obtained clear electrophoregrams for all genotypes at all 33 loci investigated, with only one or two alleles per strain at each locus. A total of 128 alleles based on 31 polymorphic microsatellite markers were identified for 36 strains of *L. infantum* (MON-1, MON-33 and MON-183) from Southern France since two loci were monomorphic (LIST7023 and LIST7030). The number of alleles per locus (*N*a) ranged from 1 to 8, the most polymorphic being LibTG (Table 2), that is, a mean value of 4.13 alleles per locus. Within MON-1, the 33 strains encompassed 50 different alleles using 18 polymorphic microsatellite markers. The number N_a ranged from 2 to 6, the most polymorphic being LibTG, LibTA, and Lm4TA, that is, a mean value of 3.18 alleles per locus. The observed heterozygosity (H_o) is quite weak; it ranged from 0-0.181 (overall 0.042) for the whole sample and varied between 0-0.104 (overall 0.018) for the MON-1 population. The mean genetic diversity, H_s, was 0.345 (0.177-0.684) for the whole sample and 0.367 (0.043-0.619) within the MON-1 population.

3.2. Phylogenetic reconstruction and clustering analysis

The neighbor-joining tree (Figure 2) designed from MLMT genetic distances underlined three main clusters. MON-183 genotype was used as outgroup considering its allelic profiles. The first cluster was composed of the non-MON-1 strains (2 MON-33) and was separated from the MON-1 strains with a strong bootstrap value (100%). Thus, the MON-1 sample appeared monophyletic. The second cluster (C2) was composed of 16 MON-1 human strains isolated in the Alpes-Maritimes (AM), Var, and Corsica regions and sustained by a bootstrap of 58%. This group contained two subclusters: (i) two VL strains (bootstrap value: 60%) and (ii) fourteen strains from human (7 human patients and 7 asymptomatic carriers) (bootstrap value: 91%). Seven of the 9 strains isolated from asymptomatic carriers (AC) were in the latter cluster and constituted a single genotype. The third cluster (C3) sustained by a bootstrap of

59% contained three subclusters: (i) the 6 MON-1 strains isolated from Hérault region (bootstrap 69%), (ii) 5 MON-1 human strains from AM and Corsica regions (bootstrap 53%), and (iii) the 3 canine strains with the 2 remaining AC strains and a VL strain. The latter group constituted a single genotype. Interestingly, the nine AC strains were poorly polymorphic since they revealed only 2 genotypes, one was AC specific (7 strains) whereas the second one (2 strains) was identical to the 3 canine strains of our sample and a VL case. In addition to the genetic distance-based approach, the model-based algorithm was used to infer the population structure of our French *L. infantum* sample. MLMT data were analyzed with the STRUCTURE software to infer the population structure of (i) the whole sample (36 strains) and (ii) MON-1 population (33 strains). According to Evanno et al. (2005) and Garnier et al. (2004), within the whole sample, the most probable *K* numbers are *K*=3 and *K*=5. In the first case, population 1 was composed of the 3 non MON-1 strains (2 MON-33 + 1 MON-183), population 2 of the MON-1 cluster C2 (14 strains), and population 3 of the MON-1 cluster C3 (17 strains) (Figures 2 and 3). The STRUCTURE software did not allow classifying the strains LEM3492 (CL) and LEM5458 (VL) which were a mixed genotype of the populations 2 and 3. For *K*=5, population 1 (non MON-1) was subdivided into two subpopulations (MON-183 in one part (population 1b) and MON-33 in the other part (population 1a)) and population 3 was subdivided into two subpopulations since the group (population 3b) of the 3 canine strains with the 2 remaining AC strains and a VL strain was separated from the other strains (population 3a). Within MON-1, the most probable K number is *K*=3 with populations 2, 3a, and 3b (data not shown) (Figure 3). Results obtained by genetic distance and clustering analysis were congruent and both dispatched the nine AC strains into two separated populations. Genetic differentiation among various populations was tested using FSTat Version 2.9.3.2 software (Goudet et al., 2002) (Table 3). Concerning geographical differentiation, strains from Hérault were isolated from strains from the Alpes-Maritimes, Var, and Corsica (data not shown) as well as from the AM considered alone (Table 3) and were characterized by a specific homozygous allele (98bp) for locus Li22-35. Year of isolation, sex, and age of the patient were tested but no significant results were obtained (data not shown). These parameters did not appear as significant subdividing factors. F_{ST} was significant between the human and canine populations from AM (F_{ST}: 0.3462, *P*-value: 0.05) but we studied only three canine strains and they showed the same genotype. Thus, no conclusion could be drawn from this result, which has to be confirmed with a larger canine sample.

3.3. Comparison AC versus non AC strains

The nine AC strains were grouped with the MON-1 strains and were divided into two populations. For the 33 locus, the AC population revealed 42 alleles whereas the other MON-1 strains (24 strains) revealed 67 alleles. The degree of polymorphism in the AC population was much lower than among *L. infantum* MON-1 of Southern France but also considering only the samples from the Nice region. This remains true if we consider only strains from 1997 or 2007 (the most abundant in our sample) which have a higher degree of polymorphism that AC population. Seven strains presented the same genotype and belonged to population 2 defined by STRUCTURE within cluster C2 (14 human strains). There were no specific alleles able to discriminate them among the other populations. However, three alleles were predominantly found in these seven AC strains (and were homozygous for these three loci) compared to the other MON-1 strains (26 strains), for which these loci were heterozygous except for LIST7026 (LIST7026 205bp: 1 and 0.192, Lm4TA 75bp: 1 and 0.077, LibTA 236bp: 1 and 0.077 (allele frequencies respectively for the 7 AC and other MON-1 strains). The two remaining AC (LEM3280 and LEM3273) strains from population 3b defined by STRUCTURE (K=5) were grouped with the three canine strains and a VL case within cluster C3. Four homozygous loci provided specific alleles for population 3b: Li45-24 100bp, Lm4TA 73bp, LibTA 234bp, and LIST7038 122bp. There were no specific alleles able to discriminate the nine AC strains among the other populations. However, three homozygous alleles were predominantly found in the AC population compared to the other 24 MON-1 strains: Lm4TA 75bp: 0.778 and 0.083, LibTA 236bp: 0.778 and 0.083, and LIST7026 205bp: 0.778 and 0.208 (allele frequencies respectively for AC and other MON-1 strains). LIST7026 was homozygous for the whole sample. Population genetics analyses using FSTat Version 2.9.3.2 software (Goudet et al., 2002) were performed considering the whole AC sample (9 strains) as a population (table 3). Differentiations were evidenced between the AC population and the non-AC population (24 strains) (F_{ST}: 0.2211, P-value: 0.050). Because there was a genetic differentiation between the Hérault and Alpes-Maritimes samples, F_{ST} were recalculated considering only the Alpes-Maritimes sample. The AC population was differentiated from the non-AC population (F_{ST}: 0.1659, P-value: 0.050) and this was mostly due to the differentiation between the AC population and the HIV+ population (F_{ST}: 0.3340, P-value: 0.025). Note that we obtained a marginally significant differentiation between the AC population and the CL population (2 strains) (F_{ST}: 0.3303, P-value: 0.083) but this result must be confirmed on a larger sample.

4. Discussion

Genetic structure

The genetic structure and diversity of strains of *L. infantum* from asymptomatic carriers (AC) and symptomatic hosts (dogs and humans) from the South of France were for the first time investigated and compared. The MLMT approach employing 33 microsatellite markers was previously shown to be highly discriminatory for *Leishmania* typing and even for strains of *L. infantum* belonging to the zymodeme MON-1, which predominates in the Mediterranean basin (Ochsenreither et al., 2006; Kuhls et al., 2008). The mean number of alleles per locus (N_a) was 4.13 alleles per locus. This agrees with a previous study for which the mean was 5.6 alleles per locus within a European sample of *L. infantum* and 4.6 within their European MON-1 subsample (Kuhls et al., 2008). In our sample, the level of heterozygosity was low as demonstrated by the H_o and the H_e. This agrees with all the previous studies, which demonstrated an overall heterozygote deficiency in all the studied *Leishmania* species (Kuhls et al., 2007; Amro et al., 2009; Rougeron et al., 2009; Rougeron et al., 2011). A previous population genetics analysis showed that this deficiency could be explained by the frequent occurrence of mating between individuals with related strains (i.e. endogamy, see (Rougeron et al., 2010). This suggests that each *L. infantum* genotype can propagate for long time if conditions are favorable and if they are not eliminated (by the immune system of hosts for example) during the cycle. Furthermore, our analysis revealed a geographical structuring within our sample, Hérault versus Alpes-Maritimes. This kind of structuring has already been observed when considering the European countries instead of regions (Kuhls et al., 2008) as well as for another *Leishmania* species in Ethiopia (*L. aethiopica*) (Schonian et al., 2000). Hérault and Alpes-Maritimes are only 200 miles apart but are separated by the Camargue (Rhone delta). This area could act as a barrier to gene flow by isolating Phlebotomine populations.

Comparison between AC and symptomatic Leishmania *strains*

It is important to study *Leishmania* in healthy subjects to improve our knowledge of clinical manifestation, transmission of leishmaniasis, the pathways of parasite dissemination, and its capacity for surviving in human hosts. By using the Bayesian statistics-based clustering method STRUCTURE and by constructing a neighbor-joining tree based on microsatellite genetic distances, strains isolated from AC are grouped with the zymodeme MON-1 and reveal a weak genetic polymorphism (2 genotypes) compared to those isolated from symptomatic humans. Several hypotheses could explain this lack of polymorphism relative compared to

"symptomatic" *Leishmania*. For example, (i) AC genotypes could result from a recent clonal propagation and have not yet diverged or (ii) the selective pressure to maintain this avirulent form in its human host is very high. Despite the low diversity of the AC population, we found indications for population substructures. This study included all the *Leishmania infantum* from AC available worldwide but an effort must be made in the future to confirm these data on a bigger sample. In addition, AC strains were assigned to two subpopulations. One AC population included seven AC strains and pertained to population 2 and the second one, population 3b, included two AC strains, the three canine strains, and one strain from a human case of VL. There was no link between these two AC strains and the VL strain since they were cultured and studied in two different universities. This separation into two clusters cannot be explained regarding epidemiological data since there was no correlation with isolation date, donor's sex, and donor's age. The 9 AC strains were isolated from blood donors between April and November 1996 in the same area (Alpes-Maritimes). Recent studies have previously used strains isolated from blood and revealed genetic diversity (personal communication). Thus, there is no culture bias due to the blood which could explain the lack of diversity observed among AC parasites. Interestingly, the three canine strains of our sample belonged to population 3b. To validate this cluster, further investigation on a bigger canine sample is needed, but it suggests that the "asymptomatic" human genotypes are able to propagate and to infect dogs on top of humans. It is known that there is no structuring between CL, VL, and HIV+ populations and our data confirmed this result (Kuhls et al., 2008; Kuhls et al., 2011). However, an interesting correlation was observed between *Leishmania* genotypes and clinical manifestation since genotypes from AC are differentiated from those of symptomatic patients. This genetic differentiation is mainly due to the HIV+ subpopulation and, but much less so to the CL subpopulation. Our hypothesis is either HIV patients were infected by *Leishmania* parasites before HIV infection or AIDS stage or some genotypes could be unable to cause disease even in immunocompromised patients. Western blotting has shown that in zones of endemic leishmaniasis, such as the south of France, about 10% of HIV+ individuals showed the presence of antileishmanial antibodies (Kubar et al., 1998). The authors suggested that these individuals were AC. Furthermore, it has been proposed that AC result from both parasite inoculation by vector and control of the parasite spreading by the host immune system; only in a very few cases, parasite growth is not controlled by the immune system, resulting in clinical VL (Bogdan et al., 1993). In this case, we can wonder if these AC strains have pathogenic properties able to produce clinical manifestation in humans. To explore the last hypothesis, pathogenicity of AC and nonAC strains must be compared in vivo in immunocompromised

mice. It is important to note that the 9 donors have a medical follow in the hospital of Nice from their blood donation in 1996 and any of them have develop leishmaniasis. Considering *Plasmodium falciparum*, responsible for malaria, some authors showed that, even in HIV+ patients, the percentage of clinical malaria (4%) was still lower than the percentage of malaria parasitemia (12%) (Whitworth et al., 2000). In this case, 8% of HIV+ patients remained asymptomatic for malaria infection. In any case, these AC strains must be considered as a source of material to identify parasite factors involved in the observed clinical polymorphisms.

Role of AC in transmission dynamics of leishmaniasis

The role of asymptomatic infection in the transmission dynamics of infectious diseases has been studied for various pathogens such as influenza or *Shigella* (Nelson et al., 1968; Hsu and Hsieh, 2008; Patrozou and Mermel, 2009). These asymptomatic infections seem involved in transmission dynamics. Other authors suggested that for a given pathogen (not especially *Leishmania*), avirulent strains could have evolved to infect safe hosts whereas virulent strains would be adapted to infect hosts already infected by avirulent strains (Alizon and van Baalen, 2008). Using PCR, we found a very high AC rate in both human and canine populations, with respectively up to 70% and 40% in Southern France (Hide et al., unpublished data). We do not know the target organs of human AC strains or if their amastigotes are intracellular or free, or their transmission capacity. However, we know that asymptomatic infection can continue at least 6 months (Le Fichoux et al., 1999). There are only a few studies concerning the capacity of human AC to infect sand flies. For example, Costa *et al*. showed that no sand flies acquired infection from 27 asymptomatic persons (with positive leishmanin skin) (Costa et al., 2000). In a study from Brazil involving dogs, 28% of seropositive symptomatic dogs were infectious to *Lutzomyia longipalpis* sand flies, whereas only 5.4% of the asymptomatic dogs were infectious (Michalsky et al., 2007). Recently, the same results were found by Soares et al (Soares et al., 2011). On the contrary, some authors showed that infectivity to *Phlebotomus perniciosus* sandflies was independent of the extent of symptoms in infected dogs (asymptomatics versus polysymptomatic dogs) (Molina et al., 1994). In conclusion, the high AC rate and the genetic structure of *Leishmania* from AC must be taken into account to estimate the role of human AC populations as a potential reservoir. As we have the 9 parasites from AC, one way to answer this question would be explored their infectivity in *Phlebotomus* sandfly.

Risk of transfusion-transmitted leishmaniasis

From the standpoint of public health, there is no *Leishmania* screening before blood transfusion, yet leishmaniases are endemic in the South of France. Transmission can occur during organ transplant (Basset et al., 2005) and a case of transfusion-transmitted VL has recently been revealed due to *L. mexicana* in Colombia (Mestra et al., 2011). Other transfusion-transmitted leishmaniases have been reported and reviewed in Dey and Singh, 2006. In addition, an anthroponotic cycle has emerged among intravenous drug users, in which syringes replace the sand fly vector (Cruz et al., 2002). However, concerning blood donors, French blood products have been deleukocyted since August 1998; in 2007, the French Institute for Public Health Surveillance (InVS) considered that deleukocytation has vastly lowered the risk of blood donation infection by *Leishmania* (French Institute for Public Health Surveillance (InVS) - Brouard, 2007). Another study showed that leucodepletion (less efficient than deleukocytation) effectively reduces parasitemia, thus minimizing the potential risk of *Leishmania* transmission through blood transfusions in endemic areas (Riera et al., 2008).

Conclusions

This is the first genetic study on *Leishmania* parasites isolated from asymptomatic carriers (blood donors). Our data show for the first time that *Leishmania* parasites isolated from asymptomatic human carriers differs from parasites isolated from human patients, even in a species that has very little genetic variation such as *Leishmania infantum*. The AC genotypes revealed a weak polymorphism and pertained to zymodeme MON-1. The strongest genetic differentiation was observed between AC population and HIV population. This opens new research orientations since these strains (especially the genotype shared by seven AC strains) can bring precious information for understanding the outcome of leishmaniases in humans.

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Figure legends

Figure 1: Geographic origin of the 36 *L. infantum* strains. 1A : Southern France; 1B : Nice region.

Figure 2: Left, neighbor-joining tree inferred from the Cavalli-Sforza genetic distance for the data of 33 microsatellite markers and 36 *L. infantum* strains. The values above the branches indicate the percentage with which a given branch is supported in 100 bootstrap replications. *Leishmania* strains isolated from AC are underlined; right, population structure inferred by Bayesian clustering for *K*=3 and *K*=5.

Figure 3: Estimated population structure and substructure of *L. infantum* as inferred by the STRUCTURE program**.** Results are based on MLMT of 33 microsatellite markers obtained for the 36 *L. infantum* strains studied. A) In the bar plots, each strain is represented by a single vertical line divided into *K* colors, where *K* is the number of populations assumed. Each color represents one population, and the length of the color segment shows the strain's estimated proportion of membership in that population. Isolates are organized by membership coefficients. According to *deltaK,* the most probable numbers of populations in the data set are three and five. B) Derived graph for *deltaK* shows a peak at *K* = 3 and *K* = 5, indicating the existence of three and five populations in the investigated

Table 1: *Leishmania infantum* strains used in this study.

Strain	W.H.O code	Town(department)[a]	Clinic[b]	HIV status	MON zymodeme[c]	STRUCTURE Population[d]
LEM2355	MHOM/FR/91/LEM2355	Toulouse (31)	VL	+	183	1b
LEM356	MHOM/FR/82/LEM356	Amélie les Bains (66)	CL	-	33	1a
LEM3495	MHOM/FR/97/LPN160	Southern France	CL	-	33	1a
LEM576	IPER/FR/1984/LEM576	St Clément la Rivière (34)	N/A	N/A	1	3a
LEM595	IARI/FR/1984/LEM595	St Clément la Rivière (34)	N/A	N/A	1	3a
LEM3114	MCAN/FR/95/LPN122	Falicon (06)	CanL	N/A	1	3b
LEM3115	MCAN/FR/95/LPN123	St Pancrace (06)	CanL	N/A	1	3b
LEM3116	MCAN/FR/95/LPN124	Aspremont (06)	CanL	N/A	1	3b
LEM3280	MHOM/FR/96/LPN131	Monaco (98)	AC	-	1	3b
LEM3270	MHOM/FR/96/LPN134	Monaco (98)	AC	-	1	2
LEM3273	MHOM/FR/96/LPN136	Monaco (98)	AC	-	1	3b
LEM3282	MHOM/FR/96/LPN137	Monaco (98)	AC	-	1	2
LEM3283	MHOM/FR/96/LPN138	Monaco (98)	AC	-	1	2
LEM3296	MHOM/FR/96/LPN142	La Turbie (06)	AC	-	1	2
LEM3295	MHOM/FR/96/LPN143	Menton (06)	AC	-	1	2
LEM3294	MHOM/FR/96/LPN144	La Turbie (06)	AC	-	1	2
LEM3313	MHOM/IT/96/LPN145	Ventimiglia (Italy)	AC	-	1	2
LEM1098	MHOM/FR/87/LEM1098	Nebian (34)	CL	-	1	3a
LEM3496	MHOM/FR/97/LPN161	Gattieres (06)	CL	-	1	3a
LEM5459	MHOM/FR/07/LPN313	Luceram (06)	CL	-	1	2
LPN83	MHOM/FR/92/LPN83	Lavasina (2B)	CL	+	1	3a
LEM663	MHOM/FR/85/LEM663	Clermont l'Herault (34)	VL	-	1	3a
LEM716	MHOM/FR/85/LEM716	Nebian (34)	VL	-	1	3a
LEM75	MHOM/FR/78/LEM75	Béziers (34)	VL	-	1	3a
LPN105	MHOM/FR/94/LPN105	Bastia (2B)	VL	+	1	2
LPN119	MHOM/FR/95/LPN119	Cannes (06)	VL	+	1	2
LPN129	MHOM/FR/96/LPN129	Vallauris (06)	VL	+	1	3a
LPN132	MHOM/FR/96/LPN132	Mouans Sartoux (06)	VL	-	1	3b
LEM3484	MHOM/FR/97/LPN155	Nice (06)	VL	-	1	2
LEM3491	MHOM/FR/97/LPN158	Colomars (06)	VL	-	1	2
LEM3492	MHOM/FR/97/LPN159	Fayence (83)	VL	-	1	2/3a
LEM5458	MHOM/FR/07/LPN312	Grasse (06)	VL	-	1	2/3a
LEM5460	MHOM/FR/07/LPN314	Peymeinade (06)	VL	+	1	3a
LEM5535	MHOM/FR/07/LPN316	Nice (06)	VL	-	1	2
LPN92	MHOM/FR/93/LPN92	Nice (06)	VL	+	1	3a
LPN94	MHOM/FR/93/LPN94	Nice (06)	VL	+	1	2

Table 1: *Leishmania infantum* strains used in this study.

N/A not applicable. MHOM humans; MCAN *canis familiaris*, IPER *Phlebotomus perniciosus*, IARI *Phlebotomus ariasi*. [a] French departments are noted: Hérault (34), Alpes-Maritimes (06), Haute-Corse (2B), Haute-Garonne (31), and Var (83).

[b] Clinical forms are noted: visceral leishmaniasis (VL), cutaneous leishmanisis (CL), canine visceral leishmanisis (CanL), and asymptomatic carrier (AC).

[c] Zymodemes were determined with the MLEE method performed by the W.H.O reference center of Montpellier (MON) (Rioux et al., 1990). [d] Populations according to STRUCTURE analysis (*K*=5).

Table 2: Characteristics of the 33 microsatellite loci used in this study for *Leishmania infantum* genotyping:

Microsatellite marker	Dye label	GenBank Access no.	Size range (bp)	Ta (°C)	Na	References
LiBTG*	6-FAM	nd	219-257	58	8	Fisa, unpublished data
LiBTA*	VIC	nd	226-246	58	7	Fisa, unpublished data
Lm4TA*	NED	nd	65-85	58	7	Ochsenreither 2006
LIST7026*	NED	AF427874	201-231	56	6	Jamjoom 2002
Li22_35*	VIC	AM050045	90-106	58	6	Ochsenreither 2006
Li45_24*	NED	AM050048	88-108	58	6	Ochsenreither 2006
TubCA*	6-FAM	nd	74-84	58	6	Ochsenreither 2006
LIST7039*	PET	AF427887	199-215	58	5	Jamjoom 2002
Li71-33	6-FAM	AM050053	104-136	57	5	Ochsenreither 2006
Rossi2*	VIC	X76393	140-160	57	5	Rossi 1994
LIST7021*	6-FAM	AF427869	228-246	54	4	Jamjoom 2002
LIST7029	6-FAM	AF427877	172-182	56	4	Jamjoom 2002
LIST7033*	6-FAM	AF427881	196-226	58	4	Jamjoom 2002
LIST7035*	PET	AF427883	188-202	56	4	Jamjoom 2002
LIST7037*	6-FAM	AF427885	178-194	58	4	Jamjoom 2002
Li71-7	6-FAM	AM050051	90-98	58	4	Ochsenreither 2006
Li72-20*	VIC	AM050057	87-95	50	4	Ochsenreither 2006
DPB1	NED	AF182167	141-147	59	3	Hide, PhD
DPB2	PET	AF182167	231-235	59	3	Hide, PhD
HG	6-FAM	AF170105	191-199	55	3	Hide, PhD
ITS1	6-FAM	AJ000288	288-314	55	3	el Tai, 2000
LIST7024	VIC	AF427872	198-224	59	3	Jamjoom 2002
LIST7025*	6-FAM	AF427873	171-179	56	3	Jamjoom 2002
LIST7027	PET	AF427875	177-185	59	3	Jamjoom 2002
LIST7031*	PET	AF427879	166-174	54	3	Jamjoom 2002
LIST7034	NED	AF427882	139-197	54	3	Jamjoom 2002
LIST7038*	NED	AF427886	122-130	56	3	Jamjoom 2002
Li71-5/2*	VIC	AM050050	104-108	54	3	Ochsenreither 2006
CS20	NED	nd	86-96	58	2	Khuls, 2007
LIST7028	VIC	AF427876	140-152	58	2	Jamjoom 2002
Rossi1	6-FAM	X76394	104-110	59	2	Rossi 1994
LIST7023	PET	AF427871	153	55	1	Jamjoom 2002
LIST7030	NED	AF427878	178	59	1	Jamjoom 2002

Table 2: Characteristics of the 33 microsatellite loci used in this study for *Leishmania infantum* genotyping: locus, *: polymorphic loci within zymodeme MON-1, dye label, GeneBank

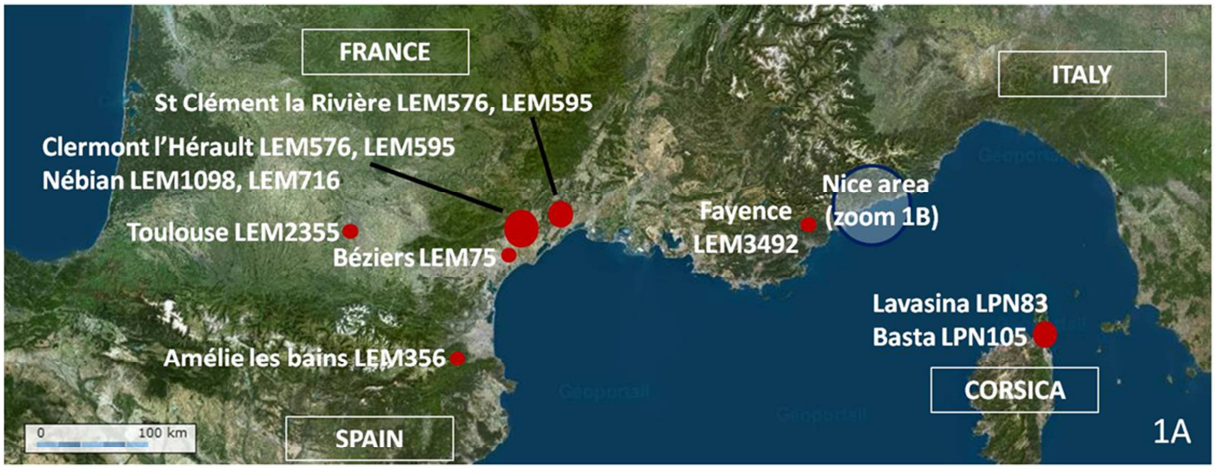
accession number, allele size (bp), thermocycling conditions (annealing temperature, T_a), genetic variation (allele number within the whole sample), N_a; References.

Table 3: Differentiation measures (F_{ST}) and testing (*P*-value)

Comparison	Subsamples	F_{ST}	*P*-value
Clinic	**AC vs Symptomatic (All)**	**0.2211**	**0.050**
	AC vs Symptomatic (humans)	0.2135	0.050
	AC vs Symptomatic (humans from AM)	**0.1659**	**0.050**
	AC vs VL	0.2097	0.030
	AC vs CL	0.3808	0.016
	AC vs HIV+	0.2876	0.016
	HIV+ vs LC/LV	-0.0070	0.400
	AC vs VL (from AM)	*0.0523*	*0.308*
	AC vs CL (from AM)	0.3303	0.083
	AC vs HIV+ (from AM)	**0.3340**	**0.025**
Geography	**Hérault vs AM**	**0.3643**	**0.016**
	Hérault vs AM (humans)	0.3843	0.050
Host	**Humans vs dogs (from AM)**	**0.3462**	**0.050**

Table 3: Differentiation measures (F_{ST}) and testing (*P*-value) between different *Leishmania infantum* MON-1 strains according to the clinical manifestations, geographical origins, and host species and controlling for the other factors (possible only on some occasions). F_{ST} considering time, sex, and age of the patient are not significant (data not shown). asymptomatic carriers (AC); Alpes-maritimes (AM).

Figure 1


FRANCE
ITALY
St Clément la Rivière LEM576, LEM595
Clermont l'Hérault LEM576, LEM595
Nébian LEM1098, LEM716
Toulouse LEM2355
Béziers LEM75
Fayence
LEM3492
Nice area
(zoom 1B)
Lavasina LPN83
Basta LPN105
Amélie les bains LEM356
CORSICA
SPAIN
0 100 km
1A

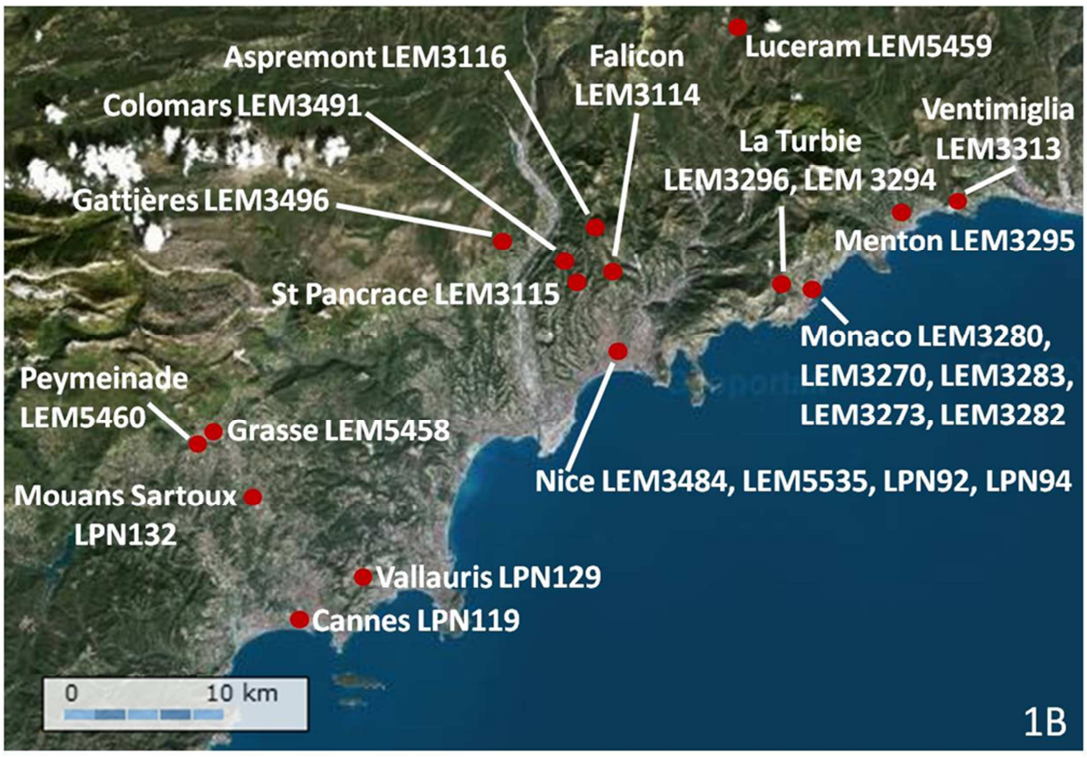

Aspremont LEM3116
Falicon
LEM3114
Luceram LEM5459
Colomars LEM3491
Ventimiglia
LEM3313
La Turbie
LEM3296, LEM 3294
Gattières LEM3496
Menton LEM3295
St Pancrace LEM3115
Monaco LEM3280,
LEM3270, LEM3283,
LEM3273, LEM3282
Peymeinade
LEM5460
Grasse LEM5458
Nice LEM3484, LEM5535, LPN92, LPN94
Mouans Sartoux
LPN132
Vallauris LPN129
Cannes LPN119
0 10 km
1B

Figure 2

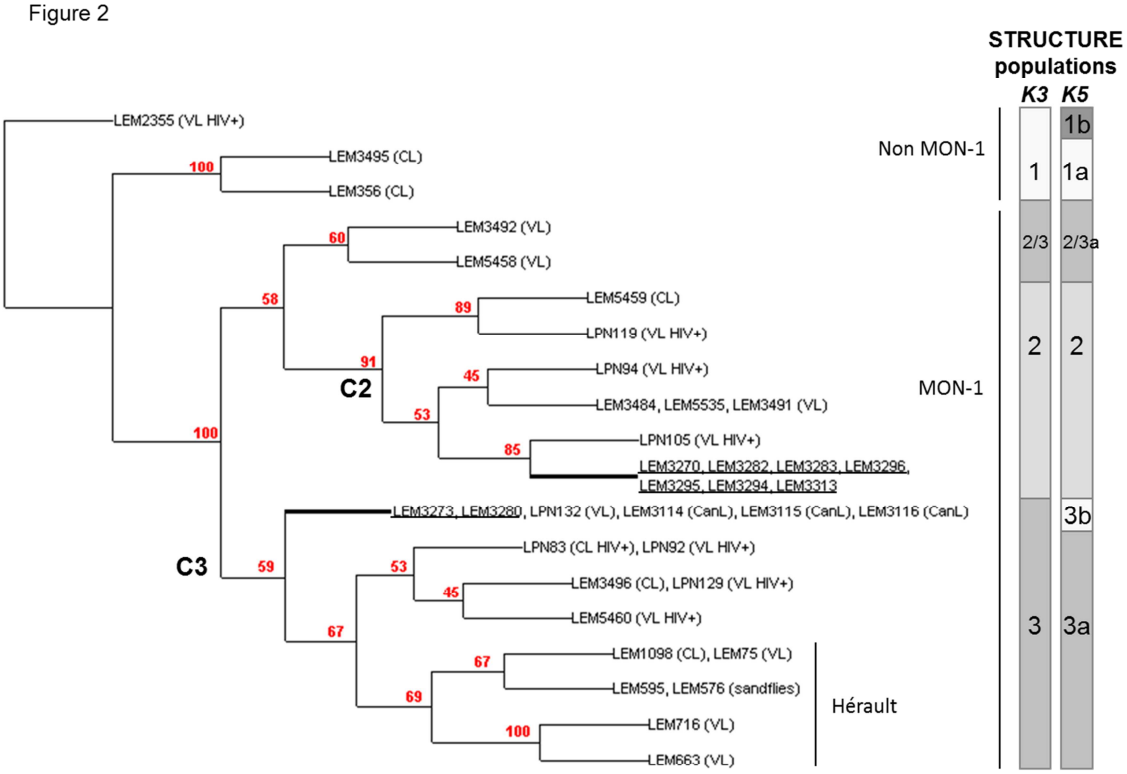
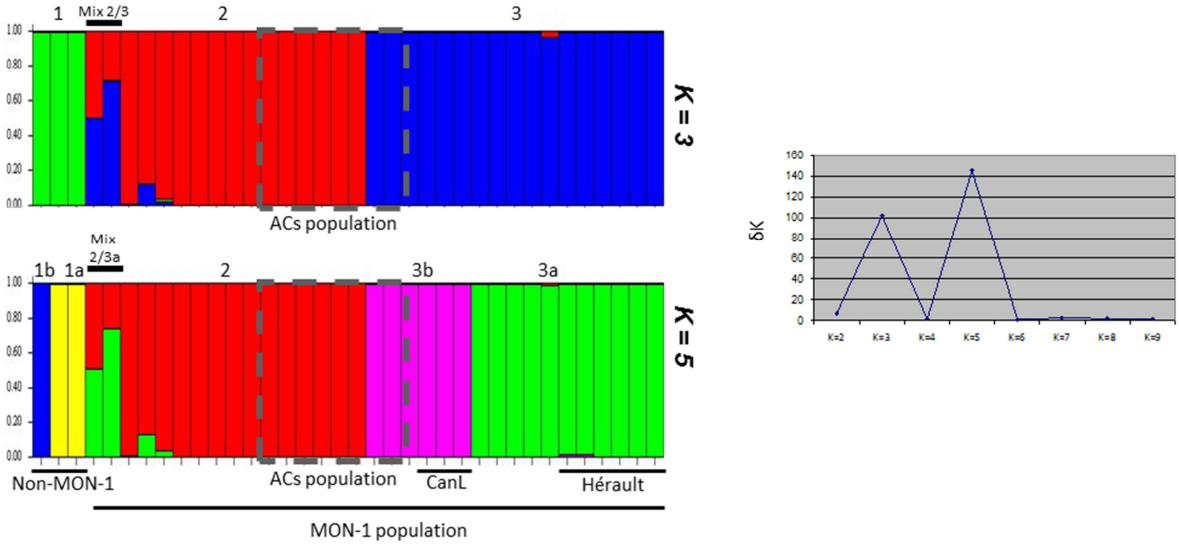


Figure 3



TRANSMISSION DE LA LEISMANIOSE A *LEISHMANIA INFANTUM* ET HETEROGENICITE DES ENVIRONNEMENTS DANS LE SUD-EST DE LA FRANCE

L'étude de l'environnement associé à la transmission de la leishmaniose viscérale est un concept récent qui trouve son origine dans le fait qu'à côté des environnements ruraux classiquement connus pour être un milieu favorable au phlébotome (forêt de chênes et de châtaigniers), des cas de leishmaniose sont endémiques dans des environnements urbains. Une étude de l'environnement associé à 328 cas de leishmaniose viscérale entre 1993 à 2009 a été réalisée sur la région Provence Alpes Côte d'Azur (PACA). Plusieurs environnements ont été définis ainsi que des zones à risque de transmission. Dans la région PACA, les cas de leishmaniose sont surtout présents dans les zones rurales et péri-urbaines représentées par collines autour de Nice et dans un environnement urbain continu pour le foyer marseillais. Les zones situées entre et autour de ces deux foyers présentent un risque plus faible de développer une leishmaniose viscérale. Ainsi, pour une même pathologie, transmise par un même vecteur (*Phlebotomus perniciosus*) impliquant un même parasite (*L. infantum* zymodème MON-1), il est retrouvé des environnements de transmission très différents selon que l'on habite à Marseille ou autour de Nice. Il est important de tenir compte de ces données dans une perspective de contrôle de la transmission des leishmanies ainsi que pour identifier les futures zones de transmission suite au réchauffement climatique. Il sera intéressant d'étudier si l'étude du polymorphisme des souches par une méthode plus discriminante que l'analyse isoenzymatique met en exergue une différence entre les parasites issus des deux foyers. A cette fin, une étude génétique par les microsatellites des souches niçoises et marseillaises est en cours de réalisation. Elle permettra de plus de rechercher d'éventuelles différences sur la structure génétique des populations de leishmanies propres à chaque foyer.

HETEROGENEITY OF ENVIRONMENTS ASSOCIATED WITH TRANSMISSION OF VISCERAL LEISHMANIASIS IN SOUTH-EASTERN FRANCE AND IMPLICATION FOR CONTROL STRATEGIES

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PLOS NEGLECTED TROPICAL DISEASES

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PLoS NEGLECTED TROPICAL DISEASES

Heterogeneity of Environments Associated with Transmission of Visceral Leishmaniasis in South-Eastern France and Implication for Control Strategies

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Abstract

Background: Visceral leishmaniasis due to Leishmania infantum is currently spreading into new foci across Europe. Leishmania infantum transmission in the Old World was reported to be strongly associated with a few specific environments. Environmental changes due to global warming or human activity were therefore incriminated in the spread of the disease. However, comprehensive studies were lacking to reliably identify all the environments at risk and thereby optimize monitoring and control strategy.

Methodology/Findings: We exhaustively collected 328 cases of autochthonous visceral leishmaniasis from 1993 to 2009 in South-Eastern France. Leishmaniasis incidence decreased from 31 yearly cases between 1993 and 1997 to 12 yearly cases between 2005 and 2009 mostly because Leishmania/HIV coinfection were less frequent. No spread of human visceral leishmaniasis was observed in the studied region. Two major foci were identified, associated with opposite environments: whereas one involved semi-rural hillside environments partly made of mixed forests, the other involved urban and peri-urban areas in and around the region main town, Marseille. The two neighboring foci were related to differing environments despite similar vectors (P. perniciosus), canine reservoir, parasite (L. infantum zymodeme MON-1), and human host.

Conclusions/Significance: This unprecedented collection of cases highlighted the occurrence of protracted urban transmission of L. infantum in France, a worrisome finding as the disease is currently spreading in other areas around the Mediterranean. These results complete previous studies about more widespread canine leishmaniasis or human asymptomatic carriage. This first application of systematic geostatistical methods to European human visceral leishmaniasis demonstrated an unsuspected heterogeneity of environments associated with the transmission of the disease. These findings modify the current view of leishmaniasis epidemiology. They notably stress the need for locally defined control strategies and extensive monitoring including in urban environments.

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Introduction

Visceral leishmaniasis (VL) due to Leishmania infantum remains a public health problem in the Mediterranean basin: despite underreporting, European reference centres record more than 400 cases each year [1]. Less frequently, cutaneous and mucosal manifestations may occur [2]. While overall VL incidence strikingly decreased since highly active antiretroviral therapy have been used to treat HIV infection [3], VL is currently emerging in several new foci, notably in Northern Italy [4–7]. Autochthonous animal infection was even reported in South Germany [5].

VL transmission requires that the parasite (Leishmania infantum), the sandfly vector (Phlebotomus perniciosus or Phlebotomus ariasi in France), the canine reservoir, and the human host meet [8]. In Mediterranean countries, such occurrence was reported to be strongly associated with specific rural environments [7,9]: in the French rural focus of the Cevennes Mountains, Leishmania transmission by P. ariasi was showed 40 years ago to be associated with one ecological niche made of oak forest and chestnuts groves on the hillsides [10]. These findings were confirmed in other countries such as Morocco [11].

In South America, L. infantum VL epidemics were also reported in urban environments associated with building sites, garbage dumps, residual vegetation cover, and presence of various domestic animals such as rabbits, pigs and chicken [12–15]. In Europe, where sandfly species differ, urban transmission was reported notably in Athens, Lisbon, and Madrid [16–19].

The recent spread of L. infantum around the Mediterranean Sea was attributed to vegetation changes and movements of vectors or reservoir hosts due to global warming or to human activities

Author Summary

As Leishmania infantum was reported to be spreading in Europe, we conducted an exhaustive collection of visceral leishmaniasis cases in Provence-Alpes-Côte d'Azur, the most active focus in France, from 1993 to 2009. The analysis of the 328 cases showed no spread inside the study area and a three-fold decrease of yearly incidence notably because cases associated with AIDS became less frequent. Distribution of the disease showed two distinct foci strongly associated with specific environments. One focus, close to the border with Italy, was associated with areas characterized by scattered habitation and mixed forest in the foothills as previously acknowledged. Oppositely, the other focus was centered in urban areas of Marseille. These results modify our view on the epidemiology of visceral leishmaniasis in Europe by highlighting the ability of the parasite to spread into urban environments. These findings stress the need for continuation of monitoring and prevention efforts and demonstrate that control strategy should be locally defined.

[5,6,20,21] whereas host factors such as the diffusion of new immunosuppressive treatments appeared marginal [22]. However, comprehensive studies about this suspected relation between environment and VL spread remain scarce despite calls for integrated monitoring [23,24].

Provence-Alpes-Cotes d'Azur (PACA) is a region covering 31,400 km2 in South-Eastern France inhabited by 4.500.000 people (figure 1). Leishmania transmission has been reported in PACA for 100 years [9]. Nowadays, PACA is the most active French VL focus: from 1999 to 2009, 132 of the 195 VL cases reported in mainland France occurred in PACA while the highest incidence numbers in France (6.6 VL cases per 1.000.000 inhabitants per year) were observed in the Nice Department (Figure 1) [25]. Besides, canine leishmaniasis has been spreading in PACA for the last ten years [26]. Only limited descriptions of the environments associated with VL transmission in PACA have been provided yet [9]. Specifically, none addressed urban transmission although VL was reported in the city of Marseille in the 1970s [27]. As PACA exhibits a wide range of Mediterranean natural environments including foothills as in the emerging VL focus in neighbouring Italy [7], it appeared to be a relevant area to study ongoing epidemiological trends. To allow optimization VL control strategies, we conducted this retrospective study over 17 years.

Materials and Methods

Objectives

The present study aimed to exhaustively collect cases of visceral leishmaniasis in PACA and test the hypothesis that the distribution of the disease was related to specific environments.

Collection of cases

VL cases in PACA were exhaustively collected from 1993 to 2009. First, specific registries from the parasitological Departments of the two PACA academic hospitals (Marseille and Nice) were consulted. It is noteworthy that only these two laboratories perform Leishmania PCR and serology in PACA. Then, all departments of infectious diseases, general medicine, internal medicine, and pediatrics from the 81 PACA hospitals were contacted by phone to identify additional cases. After that, the microbiological laboratories of PACA hospitals were contacted by phone to look for missing cases. Finally, data obtained from Medical Information Departments of PACA hospitals enabled to confirm the consistency of the database. Cutaneous leishmaniasis, relapses and imported diseases were excluded. Age, gender, immunological status, time of diagnosis and place of residence were anonymously collected. Because our work did not imply any intervention (either diagnostic or therapeutic) but only relied on a retrospective collection of anonymous cases, we did not submit our research protocol to an ethical committee, in accordance with French laws.

Geographical and environmental data

Geographical and environmental data included town boundaries and population, dog density, digital terrain model, wind resource potential, minimal temperatures, and land cover (using PACA CORINE land cover data obtained by comparing of remote sensing data [Landsat┐ images] and aerial pictures from 1999 and 2006 [www.eea.europa.eu/ publications/ COR0-landcover]). Land cover data was analysed using a 200 m wide buffer around places of residence. Land cover description was simplified to include the following 15 categories: 1) continuous urban area (i.e. buildings, roads and artificially surfaced area cover more than 80% of the ground, non-linear areas of vegetation and bare soil are exceptionally observed) 2) discontinuous urban area (i.e. buildings, roads and artificially surfaced area cover 50% to 80% of the ground, presence of non-linear areas of vegetation and bare soil); 3) scattered habitation; 4) industrial, commercial, and transport units; 5) mine, dump and construction sites; 6) green urban areas; 7) agricultural areas; 8) broad-leaved forest; 9) coniferous forest; 10) mixed forest; 11) transitional woodland/ shrub; 12) moors and heathland; 13) open spaces without vegetation; 14) other natural spaces; 15) water bodies.

Statistics

Spatial distribution of VL was first investigated using the Kulldorff's spatial scan statistic [28]. The SaTScan software (Kulldorf, Cambridge, UK, www.satscan.org) systematically moves a circular scanning window of increasing diameter over the studied region and compares observed case numbers inside the window to the numbers that would be expected under the null hypothesis (random distribution of cases). The maximum allowed cluster size corresponded to 50% of the population. The statistical significance for each spatial cluster was obtained through Monte Carlo hypothesis testing, i.e., results of the likelihood ratio were compared with 999 random replications of the dataset generated under the null hypothesis as recommended [29]. To avoid any misinterpretation due to methodological biases (mainly border effect and cluster shape effect), spatial clustering was also explored using SpODT (Spatial Oblique Decision Tree) [30]. This method, adapted from CART (classification and regression tree), builds oblique partitions of the study region providing spatial classes of homogeneous risk. Statistical significance was calculated using Monte Carlo inference as recommended.

Second, we investigated environmental characteristics underlying this spatial distribution. Univariate analysis was performed on environmental characteristics, using Fisher exact test. Because of the strong colinearity between these variables (prohibiting classical regression methods), the environmental characteristics were gathered in order to define environmental classes associated with VL. For that purpose, Multiple Correspondence Analysis (MCA) was carried out to generate an integrative description of the environments by defining a limited number of environmental classes. Hierarchical Ascendant Classification (HAC) was then performed to obtain the most homogeneous and the most distinctive classes (groups) according to similarity. The effect of

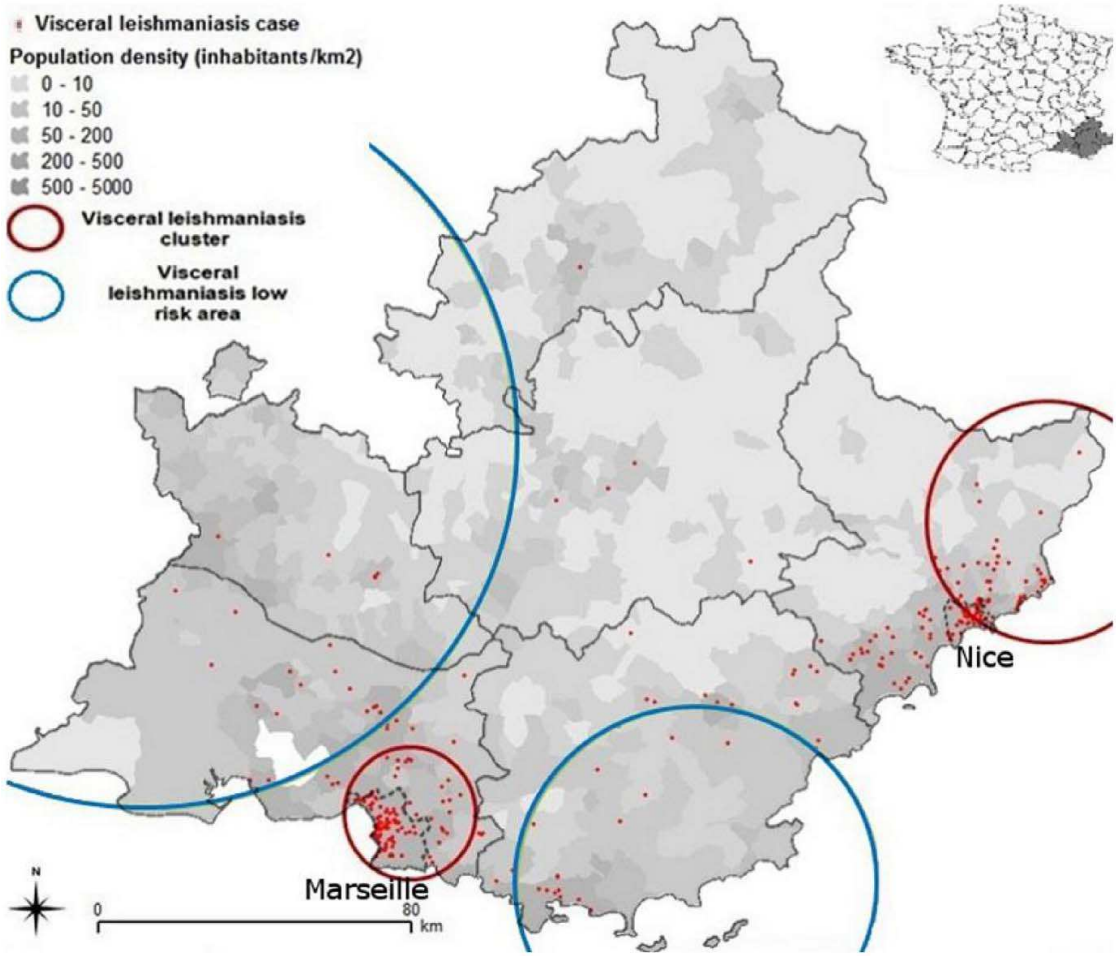

Figure 1. Visceral leishmaniasis clusters and low risk areas in Provence-Alpes-Cote d'Azur using SatScan.
doi:10.1371/journal.pntd.0001765.g001

the obtained classification on VL was tested using a logistic regression model. The absence of residual spatial autocorrelation of this final model was assessed by the Moran coefficient [29]. The analyses were all performed using R 2.11.1¬ (The R Foundation for Statistical Computing, 2009).

Study design

The study was first conducted over the whole PACA region. Cases were linked to a georeferenced digitized map according to their home address using Quantum Gis 1.6.0H. The spatial distribution of VL was analysed by SatScan and SpODT using communal population numbers, i.e. all PACA inhabitants without reported VL were taken as controls. As environment study needed to be performed at an individual level, controls were then randomly selected from the 2008 telephone book: 1 control was selected per 10.000 inhabitants in each of the six departments of PACA without matching criterion. Environment around the places of residence of cases and controls was analysed as previously described using a 200 m wide buffer for land cover data extraction.

A specific study was then conducted focusing on the two regional main towns: Marseille (852,395 inhabitants) and Nice (347,060 habitants). To increase statistical power, additional controls were selected to obtain a ratio of two controls per one case. Spatial clustering and environmental risk factors were analysed as previously described.

Results

Demographic features

328 VL cases were collected (figure 2). Overall number of incident cases was 19.3 cases/ year, decreasing from 31.2 cases/ year between 1993 and 1997 to 11.4 cases/ year between 2005 and 2009. Male were more often affected than female (220 cases, 67%), especially in case of HIV coinfection: 81% of HIV-infected patients were male. Median age was 36 years (0.4–90), with 87 patients (26.5%) under the age of 15 years including 73 (22.2%) under the age of five. One-hundred-sixty-two patients (49.4%) were immunodeficient, mostly because of HIV coinfection (133

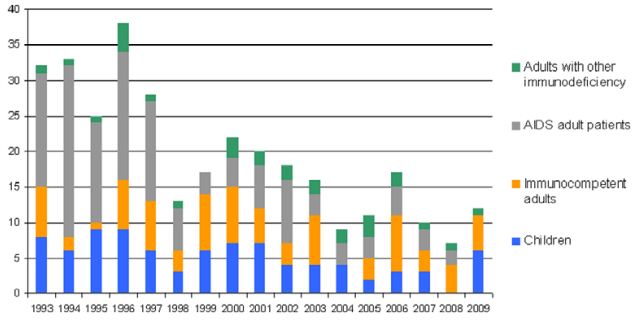

Figure 2. Visceral leishmaniasis cases diagnosed each year in Provence-Alpes- Cote d'Azur.
doi:10.1371/journal.pntd.0001765.g002

cases, 40.5%). Immunodeficiency mostly affected adult VL patients (66%) but was rarely found in children (2%).

VL mean yearly incidence varied between the departments from 6/ 1,000,000 inhabitants in the Nice department neighbouring Italy to 0.4/ 1,000,000 in the mountainous northern department (Figure 1). The exact home address was obtained for 306 of the 328 collected cases (93.3%). In most cases, absence of address was due to a homeless status (14 cases) or because patients were leading a nomadic existence (2 cases).

VL spatial distribution

SatScan results similarly showed a heterogeneous repartition (figure 1): two spatial clusters were identified accounting for 60.4% of cases. The most affected spatial cluster was located in a rural hillside area near Nice, the second regional main town. In this cluster, the main cities were affected only in the discontinuous urban areas or scattered habitations surrounding them. This cluster did not include the cities located closed to the seashore. These densely populated areas were indeed associated with significantly lower incidence numbers. This spatial cluster accounted for 64 cases (OR: 2.44, p, 10^{29}). The second spatial cluster included as well continuous as discontinuous urban areas in and around Marseille. This spatial cluster included 116 cases (OR: 1.88, p, 10^{25}). Between these two spatial clusters, a hilly area of intermediate risk included 78 cases. The rest of PACA was at low risk for leishmaniasis: Rhône River (valley and delta), coastal plains, and Alps Mountains (OR: 0.09, p, 0.05). SpODT confirmed these results (p, 10^{25}). Distribution did not differ between patients with and without HIV coinfection.

Environmental characteristics analysis

Odds-ratios associated with specific environmental characteristics showed a contrast between the two spatial clusters according to univariate analysis (table 1): in the Nice spatial cluster, VL was significantly associated with scattered habitation and mixed forest; in the Marseille spatial cluster, VL was associated with the absence of agricultural areas.

Classification method (MCA) allowed identifying four environmental classes (Figure 3). The characteristics associated with each pattern are presented in Table 2. Numbers of cases and Odd-ratios associated with the various environment classes are presented in Table 3. Overall, the highest risk was associated with environmental class 3 associating scattered habitation, mixed forest, intermediate slope (15–30%), and intermediate monthly mean minimum temperature (0–3uC). An additional association was found in the Marseille focus between VL risk and environmental class 1 associating continuous urban area, absence of agricultural areas, low altitude (, 50 m) and higher monthly mean minimum temperature (. 3uC). Environmental class 1 was the most frequently found in VL cases in the focus in and around Marseille. Environmental classes explained VL distribution: when they were taken into account, no spatial autocorrelation was found anymore (Moran coefficient = 0.0039, p = 0.14).

Urban analysis

Distribution analysis using SatScan (Figure 4) and SpODT showed that, in Nice, VL cases were clustered in the foothills areas where there are no continuous urban areas (OR: 3.47, p, 10^{22}) while they were significantly less frequently found downtown (OR: 0.27, p = 0.02). In Marseille, VL homogeneously involved most of the continuous urban areas of the city centre and surrounding discontinuous urban areas. Spatial distribution did not differ between patients with and without HIV coinfection.

Environment analysis similarly showed that VL risk was higher in Nice if scattered habitation (OR: 5.7 [1.4–27.8], p = 0.01) or mixed forest (OR: 15.5 [3.0–154.5], p, 10^{23}) were observed near the place of residence. In Marseille, these associations were not observed.

Discussion

This study benefits from several strengths. A large number of cases could be collected thanks to an excellent regional collaboration between 81 health facilities. Compared to the results of spontaneous reporting to the national reference centre [25], 27 additional cases could be identified between 1999 and 2009 (159 vs 132), illustrating the underreporting bias associated with passive monitoring methods. Because VL is a disease that always needs hospital settings to be diagnosed and treated, it can be assumed that the collection of cases was exhaustive or almost exhaustive. This enabled to rule out possible selection biases associated with passive collection of cases or thorough investigation focusing on limited territories. Additionally, the multiple geographical analyses enabled to assess for the first time the statistical significance of the observed clusters while ruling out a possible bias due to method specifications. Finally, the study design focusing on human diseases brought us to identify areas where the intensity of transmission led to a significantly higher incidence of human cases. The possible cases of infection far from the place of residence might have resulted in a loss of statistical power but they did not impact our study enough to prevent us from identifying significant clusters of cases. Though essential to define public health policies, such information could not be obtained from studies about canine

Table 1. Significant association between risk of visceral leishmaniasis and environmental characteristics according to univariate analysis.

		Marseille focus		Nice focus	
Environmental characteristic	Category	OR (CI)	p	OR (CI)	p
Land cover: mixed forest	Presence	NS	NS	4.9 (2.2–11.8)	, 10^{25}
Land cover: scattered habitation	Presence	NS	NS	2.8 (1.6–5.0)	, 10^{23}
Land cover agricultural areas	Presence	0.5 (0.3–0.9)	0.02	NS	NS
Altitude			, 0.01		, 10^{25}
	, 50 m[a]	1		1	
	50–300 m	2.2 (1.4–3.6)		3.7 (1.9–7.1)	
	300–1000	NS		3.3 (1.5–7.6)	
Slope			0.04		, 10^{26}
	, 15%[a]	1		1	
	15%–30%	2.7 (1.1–7.5)		3.6 (1.9–7.2)	
	. 30%	NS		7.0 (2.8–19.3)	
Monthly minimum temperature			NS		, 10^{23}
	. 3uC[a]	NS		1	
	0–3uC	NS		3.1 (1.7–5.6)	
	, 0uC	NS		NS	
Average wind velocity	High: 3.1–5 m/s	0.6 (0.3–0.9)	0.01	NS	NS

[a]taken as reference class for Odd-Ratio calculation.
NS: No significant difference, OR: Odd-Ratio, CI: 95% Confidence Interval.
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leishmaniasis or asymptomatic carriage. Most human infections by L. infantum are indeed not associated with visceral leishmaniasis [8,31,32]. Our results are therefore complementary to those previously published.

The demographic features of present VL patients corresponded to previous descriptions [3,25]. Specifically, pre-school children accounted for a minority of cases as usually in Europe, contrary to North Africa where VL mostly affects children under the age of three years [8]. Besides, almost half of the patients were immunodeficient, mostly because of HIV infection. Contrary to some regions such as northern Italy [4], VL incidence decreased in PACA since the 1990s. This overall decrease of VL incidence was largely related to a decrease of HIV/ VL coinfections due to the availability of highly active antiretroviral treatments. Such evolution was observed in most European countries [3].

Our first finding of interest was that two limited foci of VL accounted for 2/ 3 of VL cases in PACA. These results modify our view of VL epidemiology in France, which is one of the main VL foci in Southern Europe [1]. Human VL foci appeared more limited in the current study than in previous reports based on a passive collection of human [33] or canine [26] leishmaniases. Contrary to what was observed in Italy [5,7], no significant spread of human VL was found in PACA. Yet, a recent spread of canine leishmaniasis was reported in France [26]. This discrepancy suggests that human VL incidence was low in areas with recent introduction of L. infantum, highlighting the need for protracted monitoring. The monitoring system should therefore probably be based on mandatory rather than on spontaneous notification of human cases to increase its sensitivity, as differences in accuracy of passive and active monitoring were demonstrated by the 17% more cases identified with our active collection of cases compared to the spontaneous reporting to French National Reference Centre. However, the apparent spread of canine leishmaniasis might also be related to an improvement in the recognition and notification of canine cases as previous studies were based on unexhaustive collection of cases [26]. Overall, our findings did not confirm that human VL is currently spreading in PACA as it was observed in other European areas, notably in Italy.

Our results also revealed that VL transmission occurred in different environments in two foci though located 150 km apart despite identical parasite (L. infantum zymodeme MON-1), predominant vector (P. perniciosus), reservoir (dog), and human host [26,33]. The focus north of Nice was associated with scattered habitation and mixed forest in the foothills as previously described [9]. Oppositely, the focus in and around Marseille was mostly associated with urban environment including continuous urban areas. The biology of P. perniciosus remains partly unknown [8,34], but it was showed that P. perniciosus breeding sites can be found in heterogeneous biotopes from gaps among rocks to rubbish, basement and animal shelters which can explain the heterogeneous environments associated with VL transmission [34,35]. The environmental differences between the two VL foci in PACA could be related to specific parasitic or vector subspecies. Because molecular studies proved able to distinguish sandflies on an infra-species scale [36], further entomologic studies might be of interest to investigate the vectors populations in these two foci. Previous publications did not report that such differing environments were associated with L. infantum transmission by P. perniciosus in France [9,10,11,27]. A recent environmental risk mapping showed that VL transmission could occur in distinct environments in France, but it related each of them to a specific vector (i.e., P. perniciosus or P. ariasi) and failed to identify urban transmission [26]. Besides, sandflies were also found in northern territories where they sometimes caused canine leishmaniasis outbreaks [26]. This heterogeneity of involved environments is of major importance as current risk mapping strategies often rely on limited entomologic studies [24]. Results from retrospective studies about canine leishmaniasis in Europe confirmed that environment

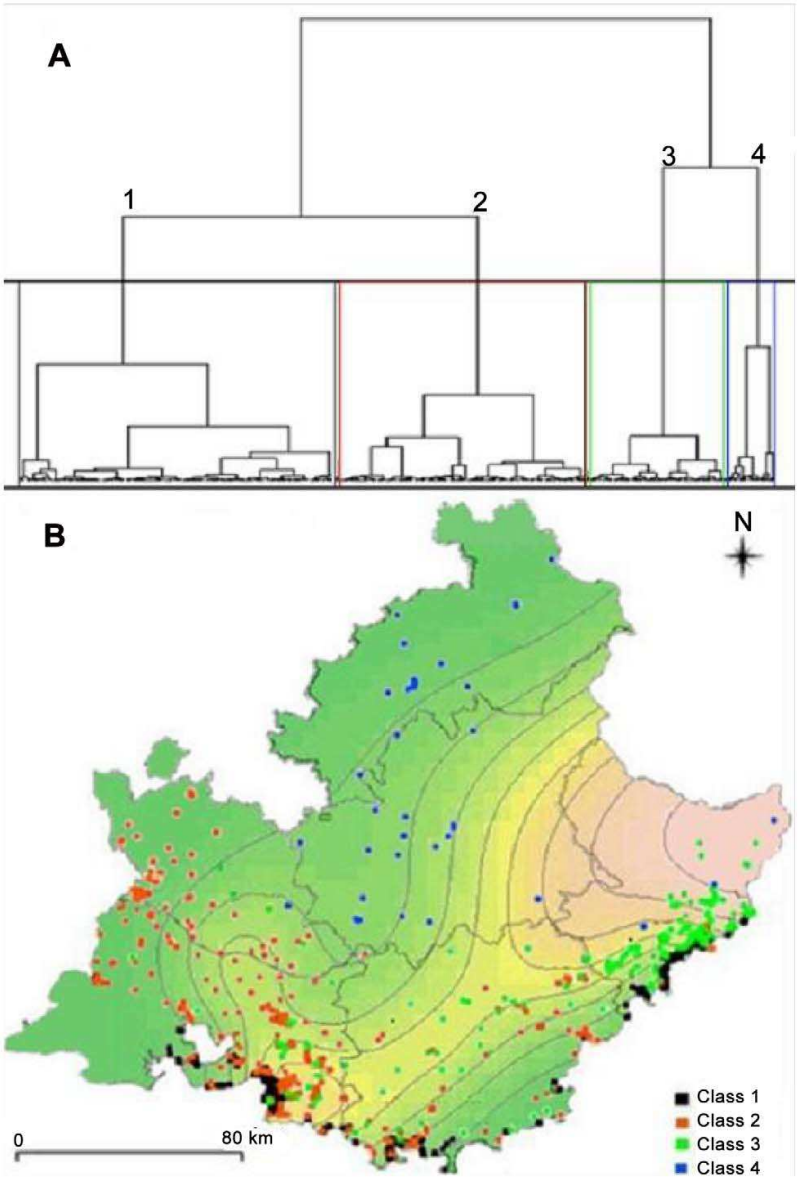

Figure 3. Environmental classes determined by multiple correspondence analysis. Hierarchical ascendant classification determined 4 environmental classes presented on a dendrogram (A) and on a map (B) of controls and visceral leishmaniasis cases produced using interpolation method based on spline functions [42].
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largely determined the distribution of canine leishmaniasis including in emerging foci [37]. These studies supported that heterogeneous environments were involved by showing that models based on overall data were less accurate than those based on local data.

Our results support the former hypothesis [10] that VL foci are distributed following the presence of vectors and not the density of the canine reservoir. Such result is worrisome as sandflies appeared to be spreading and might spread further North in France and in Central Europe. In particular, climatic conditions might become increasingly suitable because of global warming [21,26]. However, this situation could change because of current campaigns advocating the use of deltamethrin-impregnated dog collars [38] and dog immunization [39]. In the future, VL distribution could depend on the frequency of their use as well as on the vector distribution.

Table 2. Main characteristics associated with the environmental classes determined by the hierarchical ascendant classification.

Environmental class	Main characteristics
Class 1	Continuous urban area
	Absence of agricultural areas
	Low altitude (, 50 m)
	Higher monthly mean minimum temperature (. 3uC)
Class 2	Intermediate monthly mean minimum temperature (0–3uC)
	High mean velocity of wind (3.1–5 m/s)
	Low slope (, 15%)
	Presence of agricultural areas
Class 3	Scattered habitation
	Mixed forest
	Intermediate slope (15–30%)
	Intermediate monthly mean minimum temperature (0–3uC)
Class 4	Low monthly mean minimum temperature (, 0uC)
	High (. 300 m) and very high altitude (. 1000 m)
	Scattered habitation

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The continuous urban transmission of VL in Marseille is a striking result in the current context of reported Leishmania spread [1,5,6], especially as it did not appear to be limited to areas with individual houses and important residual vegetal cover as reported in the 1970s [27]. A recent seroepidemiological study also described a homogeneous risk of Leishmania infection over the whole city of Marseille without predominance in discontinuous urban areas [40]. This result was also corroborated by the high rate of asymptomatic carriage found among Marseille healthy inhabitants [31]. This urban transmission was not observed in a recent study based on a passive collection of canine leishmaniasis cases in France [26] because Marseille veterinarians do not notify leishmaniasis cases to the national reference centre. Therefore, to allow setting up optimal monitoring and control strategies, awareness should be raised over the ability of L. infantum to fulfil its cycle in continuous urban areas.

Table 3. Association between risk of visceral leishmaniasis and class of environment observed around the place of residence.

Environmental class	Whole region			
	Cases	Controls	OR (CI)	p
Class 1	113	185	1.9 (1.3–2.7)	, $10^{-2.3}$
Class 2[a]	71	217	1	-
Class 3	116	54	6.6 (4.3–10.1)	, $10^{-2.15}$
Class 4	7	31	NS	NS
	Focus north of Nice			
	Cases	Controls	OR (CI)	P
Class 1	37	69	NS	NS
Class 2[a]	1	7	1	-
Class 3	79	31	17.8 (3.0–341)	, $10^{-2.2}$
Class 4	2	1	NS	NS
	Focus in and around Marseille			
	Cases	Controls	OR (CI)	P
Class 1	71	80	1.7 (1.1–2.6)	0.02
Class 2[a]	59	111	1	-
Class 3	16	5	6.0 (2.2–19.0)	, $10^{-2.3}$
Class 4	0	0	-	-

[a]Class 2 was taken as reference class for Odd-Ration calculation.
NS: No significant difference, OR: Odd-Ratio, CI: 95% Confidence Interval.
doi:10.1371/journal.pntd.0001765.t003

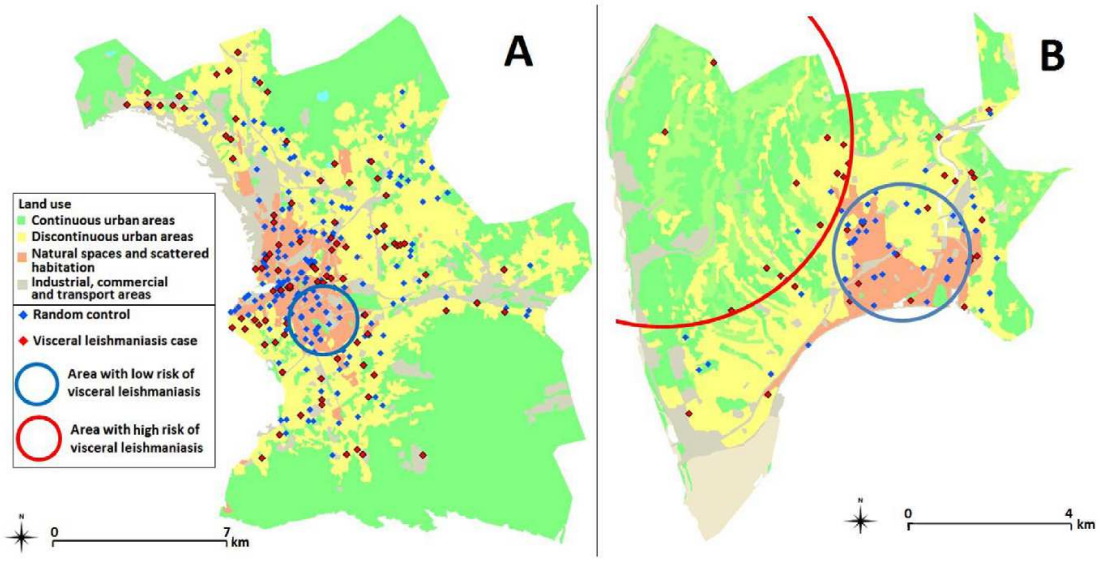

Figure 4. Visceral leishmaniasis high risk and low risk areas in Marseille (A) and Nice (B) using SatScan.
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Urban transmission was already incriminated in Athens, Greece [16], where it seemed to involve peri-urban environments made of discontinuous urban areas among quarries. This urban transmission in downtown Athens appeared of lower intensity than that observed in Athens suburbs according to a study of canine seroprevalence [17] contrary to our findings in Marseille. Urban transmission was also observed in Madrid, Spain, where canine seroprevalence was as high (around 5%) in peri-urban than in rural areas [18]. In Lisbon, Portugal, presence of infected vectors was demonstrated inside the city and canine seroprevalence appeared to increase from 5.5% in 1980 to 19.2% in the early 2000s [19], raising concerns about a progressive increase of VL transmission in the city. In Italy, P. perniciosus was observed in new residential urban districts [41]. All these studies did not allow for tracing of transmission to downtown rather than peri-urban environments, and mostly focused on canine leishmaniasis which is much more widely distributed than human VL.

The specific environments associated with a higher risk of VL transmission in the Marseille urban focus need to be further investigated. The negative correlation with higher wind velocity was unsurprising because sandflies do not easily fly in case of wind [8,36]. Similarly, the apparent lower VL risk associated with agricultural areas around Marseille could be related to mechanical or chemical destruction of sandflies' breeding sites [8]. However, these associations were not confirmed by multivariate analyses and should therefore not be overinterpreted. Besides, these associations were not observed in the Nice focus. Interestingly, most affected areas in Marseille were located inside the perimeter of a major city renovation project. P. perniciosus breeding sites were previously found in abandoned buildings and in animal shelters such as those of watch dogs [34] and the numerous rats observed in these areas were sometimes suggested to be a possible reservoir [35]. Besides a higher risk of VL associated with construction and waste sites was described in South America [12,13,14] but such result cannot be extrapolated to Europe because vectors differ. Identifying the environments associated with this urban transmission is all the more important as response strategy based on environmental vector controls proved effective elsewhere [13].

As a conclusion, the use of new geographical and statistical tools allowed revisiting the close relation between parasite transmission and environment and thereby improving our understanding of VL epidemiology. While the strong link between VL risk and the previously incriminated environment was confirmed, it was found that VL could indeed involve other environments including continuous urban areas. These results raise concern about a possible underestimation of the current and future spread of L. infantum around the Mediterranean Sea. By suggesting the risk of a higher future burden than previously expected, our findings plead for the continuation of current strategies for control as those taking place in the current European program EDENext (www.edenext.eu). Our results specifically underline the need for local definition of control strategies and for extensive monitoring including in urban environments.

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Author Contributions

Conceived and designed the experiments: BF JG RP. Performed the experiments: BF JG. Analyzed the data: BF JG FF CP CM PM RP. Contributed reagents/materials/analysis tools: BF FF CP CM CP RP. Wrote the paper: BF JG FF PM RP.

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DISCUSSION

Au cours de cette thèse, il a surtout été abordé la diversité et la complexité des leishmanioses à *L. infantum*. Cependant, plusieurs autres espèces sont présentes à travers le monde avec des expressions cliniques préférentielles. Il est décrit des espèces viscérotropes qui vont entraîner une leishmaniose viscérale, des espèces dermotropes entrainant des lésions cutanées et des espèces ayant un tropisme muqueux. Certaines espèces comme *L. infantum* et *L. braziliensis* sont connues pour avoir un double tropisme : un tropisme viscéral préférentiel et un tropisme cutané plus rare pour *L. infantum*, et un tropisme cutané et muqueux pour *L. braziliensis*. Cependant, dans certaines conditions, des formes muqueuses sont décrites pour *L. infantum* et des formes viscérales sont exprimées chez des patients avec des espèces dermotropes comme *L. major*. Il existe donc, comme classiquement décrit, une grande diversité d'expressions cliniques au sein des espèces de *Leishmania*, mais aussi une diversité d'expression clinique intra-espèce. Nous nous sommes intéressés plus précisément à la leishmaniose à *L. infantum*. Plusieurs entités cliniques sont présentes et se côtoient sans que l'on sache précisément qu'elle est le rôle de l'hôte ou du parasite, voire du vecteur dans la survenue d'une leishmaniose-maladie. Les études de prévalence ont montré que le portage asymptomatique est la forme clinique de leishmaniose la plus fréquemment retrouvée que ce soit dans l'Ancien ou le Nouveau Monde. Selon les techniques de dépistages utilisées, la prévalence peut aller de 0,6% à 71,3%. Malgré cette forte prévalence très peu de souches issues des porteurs asymptomatiques ont pu être isolées en culture. Actuellement, seulement 9 souches, isolées par notre équipe, sont disponibles au niveau mondial. Elles ont pu être obtenues grâce à un travail avec la banque de sang de Monaco (Kubar et al., 1998). Ces 9 souches proviennent de la mise en culture d'une grande quantité de cellules mononucléées périphériques provenant de sujets ayant été suspectés comme porteurs asymptomatiques par la présence en Western blot

des bandes 14 et/ou 18 kDa (Kubar et al., 1998). L'étude de ces souches par les microsatellites, démontre qu'elles sont très peu polymorphes et qu'il n'existe que deux génotypes différents. Sept souches ont un génotype unique et différent de toutes les autres souches testées, et les deux autres ont un génotype différent des sept premières, mais commun avec des souches issues de leishmanioses canines et d'une leishmaniose viscérale chez un sujet séronégatif pour le VIH. Les neuf souches issues des porteurs asymptomatiques ont été isolées dans la région de Monaco, dans une bande étroite située entre Vintimille (Italie) et La Turbie (France). Cinq souches proviennent de patients vivant à Monaco, deux souches de La Turbie, une souche de Menton et une de Vintimille (Italie). De plus cet isolement a été réalisé au cours de la seule année 1996. Il y a de ce fait un biais d'échantillonnage géographique et un biais lié à l'année d'isolement des souches. Cependant, il est retrouvé des différences génétiques entre les sept souches issues des porteurs asymptomatiques et identiques entre elles et d'autres souches comprises dans un rayon de 20 kilomètres autour de Monaco (Falicon, Nice, Ventimille). De plus, les deux autres souches issues de porteurs asymptomatiques sont génétiquement identiques avec des souches isolées d'un humain et de trois chiens malades. Ces dernières souches (humain et chien) ayant été isolées avant 1996 à l'intérieur ou à l'extérieur de ce rayon de 20 kilomètres centré par Monaco. Ainsi, même s'il y a des biais géographiques et d'année d'isolement des souches, ceux-ci ne semble pas être à l'origine des résultats. Les souches issues de porteurs asymptomatiques diffèreraient des autres souches indépendamment de l'année et du lieu d'isolement. Jusqu'à présent l'étude des souches par les microsatellites n'a pas mis en évidence de relation entre l'expression clinique et un génotype particulier (Botilde et al., 2006; Kuhls et al., 2011). Le concept de souches induisant plutôt un portage asymptomatique est donc très novateur et mérite d'être confirmé par l'étude d'un plus grand échantillon. Afin de minimiser les biais précédemment cités, les souches issues de porteurs asymptomatiques seront comparées à toutes les souches isolées au

CHU de Nice de 1978 à nos jours. S'il semble exister des facteurs de « virulence » ou de « non virulence » liés à la génétique des souches, ceux-ci pourront être explorés par un séquençage complet du génome des souches issues de porteurs asymptomatiques.

A côté du risque d'une expression clinique de l'infection en fonction des souches il existe un risque de transmission et donc un sur-risque de leishmaniose viscérale en fonction du lieu d'habitat. En effet, l'étude de l'environnement associé au risque de leishmaniose viscérale dans la région Provence Alpes Côte d'Azur montre clairement deux foyers distincts : le foyer marseillais avec une transmission urbaine et le foyer niçois avec une transmission rurale dans les communes se trouvant sur les collines adjacentes à l'agglomération. Pour le foyer marseillais, habiter en zone urbaine constitue un risque de leishmaniose viscérale alors que pour le foyer niçois, habiter en zone urbaine constitue un facteur de protection. La comparaison de ces deux foyers sera très utile pour comprendre les facteurs d'endémisation de la leishamniose viscérale méditerranéenne. Depuis le début de l'identification de *L. infantum* par les isoenzymes en 1981 jusqu'à nos jours, les zymodèmes constituent la seule façon standardisée de différencier les souches de *L. infantum* entre elles (Lanotte et al., 1981; Pratlong et al., 2004). Ainsi, une étude portant sur 712 souches isolées dans les 5 foyers classiquement décrits dans le Sud de la France : Pyrénées-Orientales, Cévennes (Ardèche, Aveyron, Gard, Hérault et Lozère), Provence (Bouche du Rhône et Vaucluse), Côte d'Azur (Alpes-Maritimes et Var) et Corse, a permis de mettre en évidence 7 zymodèmes différents (Pratlong et al., 2004). Cependant, le zymodème MON-1 représentait à lui seul 88,5% des cas. La prédominance du zymodème MON-1 se retrouve aussi en Provence. Le zymodème MON-108, peut aussi y être identifié mais plus rarement. Dans le foyer Côte d'Azur, le zymodème MON-1 est toujours majoritaire et le zymodème MON-24 y est parfois retrouvé. Du fait de la prédominance du zymodème MON-1, l'analyse des izoenzymes dans les deux foyers Provence et Côte d'Azur, ne met pas en évidence de polymorphisme important des souches.

Le foyer des Pyrénées-Orientales est plus varié avec 5 zymodèmes différents (MON-1 majoritairement mais aussi MON-11, MON-29, MON-33 et MON-34) (Pratlong et al., 2004). Certains zymodèmes semblent essentiellement dermotropes (MON-11, MON-29 et MON-33) alors que d'autres sont moins clairement associés à une expression clinique propre. Ainsi, MON-24 qui était considéré comme dermotrope a maintenant été retrouvé dans des cas de leishmanioses vicérales. Cette spécificité relative des zymodèmes est moins marquée chez les sujets séropositifs pour le VIH puisque que l'on retrouve chez ces sujets des cas de leishmanioses viscérales dus à des variants dermotropes (MON-29, MON-33 et MON-24). Les zymodèmes permettent ainsi une première approche épidémiologique dans la caractérisation des souches en fonction de l'expression clinique, mais aussi en fonction de l'origine géographique. En effet, si l'on regarde plus précisément la zone allant de l'Italie à l'Espagne, il semble y a voir une gradation dans la diversité izoenzymatique des souches. Le polymorphisme apparaît peu marqué en Italie (sauf en Sicile) et augmente au fur à mesure pour atteindre jusqu'à 10 zymodèmes différents en Catalogne espagnole (Pratlong et al., 2004). Cependant, s'il existe des différences enzymatiques entre les souches, elle est peu marquée pour nos foyers d'intérêt : Marseille et Nice. Pourtant, ces deux foyers sont très différents d'un point de vue géographique. Dès lors nous nous sommes demandés si cela pouvait être expliqué par des différences au niveau du vecteur ou du parasite, non détectées par les techniques mises en œuvre jusqu'à présent. Alors que des études sur le vecteur sont en cours de réalisation, nous nous sommes intéressés à la génétique des populations de souches circulant dans ces deux foyers en réalisant une étude du polymorphisme des microsatellites. Ce travail est présenté dans le chapitre perspectives

PERSPECTIVES

La comparaison des souches marseillaises avec les souches niçoises est en cours de réalisation. Le génotypage des souches niçoises par des marqueurs microsatellites est réalisé et le génotypage des souches marseillaises est en cours de réalisation.

Le travail préliminaire sur les souches niçoises est présenté ci-dessous.

Etude préliminaire dans le foyer des Alpes-Maritimes :

Nous avons recensé tous les cas de leishmaniose à *L. infantum* pris en charge au Laboratoire de Parasitologie-Mycologie du CHU de Nice. L'étude a porté sur 122 souches isolées à Nice entre 1978 et 2011 et cryoconservées au Centre National de Référence des Leishmanioses (CNRL) à Montpellier. Elles correspondent à 35 femmes, 67 hommes (Pour 2 patients il a été isolé respectivement 4 et 2 souches lors de rechutes), 15 chiens et 1 chat ayant développé une leishmaniose viscérale ou cutanée. Parmi la population humaine, les sujets étaient âgés de 4 mois à 93 ans, la moyenne d'âge étant de 33 ans. Ces cas correspondaient à 27 leishmanioses viscérales de l'enfant (≤ 15 ans), 34 leishmanioses viscérales chez le sujet *a priori* non immunodéprimé, 40 leishmanioses viscérales chez des sujets séropositifs pour le VIH, 2 leishmanioses chez des sujets immunodéprimés autres que VIH (Lymphome et greffe rénale) et 3 leishmanioses cutanées. Les souches étaient issues pour 104 cas des Alpes-Maritimes, 7 du département du Var, 2 de Corse, 2 de l'Hérault, 2 de Monaco, 1 de la Drôme et 4 souches avaient une origine incertaine ou étrangère (Amérique du Sud, Espagne, Portugal, Ardèche). L'analyse des isoenzymes de ces souches révèle que 119 appartiennent au zymodèmes MON-1, une souche au zymodème MON-80 chez un sujet séropositif pour le VIH, et 2 souches au zymodème MON-24 chez un sujet séropositif pour le VIH et chez un sujet présentant une

leishmaniose cutanée. Sur l'ensemble des 122 souches, il a été testé 12 locus microsatellites (Tableau 1).

Le matériel et méthodes correspondant à cette étude est présenté en annexe 1.

Tableau 1: Caractéristiques des 12 locus microsatellites utilisés dans l'étude pour génotyper *L. infantum*.

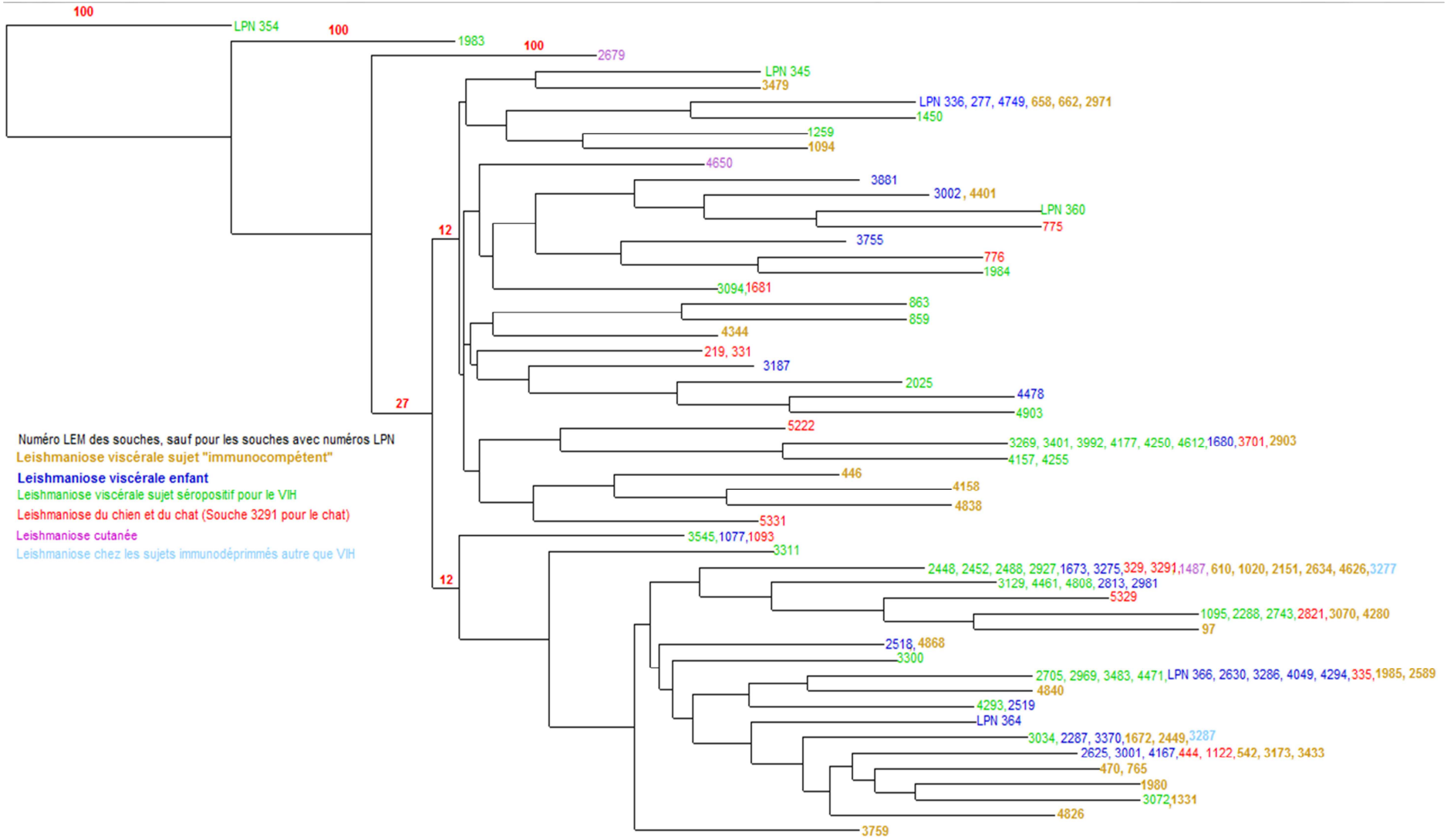
Marqueur microsatellite	Marqueur Fluorochrome	Numéro d'accès GenBank	Fourchette de taille (pb)	Th (°C)	Na	Références
LIST7021*	6-FAM	AF427869	228-246	54	8	(Jamjoom et al., 2002)
LIST7026*	NED	AF427874	201-231	56	6	(Jamjoom et al., 2002)
LIST7031*	PET	AF427879	166-174	54	3	(Jamjoom et al., 2002)
LIST7033*	6-FAM	AF427881	196-226	58	6	(Jamjoom et al., 2002)
Li22_35*	VIC	AM050045	90-106	58	8	(Ochsenreither et al., 2006)
Li45_24*	NED	AM050048	88-108	58	5	(Ochsenreither et al., 2006)
Li71-5/2*	VIC	AM050050	104-108	54	2	(Ochsenreither et al., 2006)
LiBTA*	VIC	nd	226-246	58	7	Fisa, unpublished data
LiBTG*	6-FAM	nd	219-257	58	7	Fisa, unpublished data
Rossi2*	VIC	X76393	140-160	57	5	(Rossi et al., 1994)
TubCA*	6-FAM	nd	74-84	58	4	(Ochsenreither et al., 2006)
LIST7025*	6-FAM	AF427873	171-179	56	3	(Jamjoom et al., 2002)

Caractéristique des 12 locus microsatellites, * polymorphisme intra-MON-1, Ta: température d'hybridation, Na: nombre d'allèle pour ce locus pour l'ensemble des souches.

Grace à ces 12 locus microsatellites testés sur les 122 souches, on a pu mettre en évidence 53 génotypes différents pour les souches MON-1 ou non MON-1. Ce résultat indique qu'il existe, avec les 12 locus utilisés, une grande variation intra MON-1 des génotypes. Ce résultat a déjà été démontré par d'autres équipes avec d'autres marqueurs microsatellites (Ochsenreither et al., 2006; Kuhls et al., 2008). En effet, pour les 119 souches MON-1 on retrouve 50 génotypes différents. Cette diversité est représentée sur un arbre phylogénétique (Arbre 1). On retrouve les trois premières souches : LPN354, LEM1983, et LEM 2679 qui

appartiennent aux zymodèmes MON-80 et MON-24, et qui ont été isolées respectivement de deux sujets séropositifs pour le VIH et d'une leishmaniose cutanée. Les souches suivantes dans l'arbre correspondent au zymodème MON-1.

Arbre 1 : Représentation phylogénétique de la distribution des différents génotypes des 122 souches. Les chiffres en rouge représentent les Bootstrap



Ainsi, les microsatellites discriminent les souches appartenant au zymodème MON-1 des autres zymodèmes. La distribution phylogénique des souches du foyer niçois sera comparée a celle des souches « marseillaises » qui vont être génotypées de la même manière. En effet, l'environnement associé aux deux foyers étant différent, on peut supposer l'existence de différences génotypiques entre les souches, mais surtout on étudiera les déséquilibres dans la distribution spatiale des génotypes entre les deux foyers. En effet, des études montrent que l'on peut mettre en évidence des sous-populations à l'intérieur des souches MON-1 et que ces sous-populations correspondent à des zones géographiques (Kuhls et al., 2011). Deux études comparant par les microsatellites des foyers distincts, ont pu mettre en évidence de nettes différences entre ceux-ci (Bulle et al., 2002; Kuhls et al., 2008). La première étude portait sur 20 souches de Catalogne espagnole, 6 souches madrilènes, 14 souches marseillaises et 10 d'Israël et montrait que les souches provenant de Catalogne espagnole ont un grand polymorphisme génétique tandis que les souches d'Israël sont moins polymorphes (Bulle et al., 2002). La seconde étude correspondait à l'analyse de 66 souches espagnoles, 44 souches portugaises, 16 souches grecques, 7 souches françaises, 2 souches italiennes une souche de Malte, 2 souches turques, 2 souches d'Israël, et une souche de Tunisie. Cette étude a permis de mettre en évidence 3 groupes de souches distincts génétiquement : un premier groupe composé de la Grèce, la Turquie, Israël et la Tunisie ; un deuxième groupe constitué par les Iles Baléares et un troisième groupe incluant l'Espagne et le Portugal (Kuhls et al., 2008). Plus récemment, il a été démontré grâce aux microsatellites que les souches de *L. infantum* du Nouveau Monde ont été importées récemment du Sud de l'Europe (Kuhls et al., 2011). Cependant, ces études de comparaison de foyers très éloignés, utilisent un nombre de restreint de souches ne reflétant pas fidèlement la diversité génétique de chaque foyer. Il sera intéressant de voir si dans les deux foyers marseillais et niçois éloignés de 200 km et séparés entre eux par une zone de faible risque de transmission (le département du Var), il est retrouvé

des différences génétiques entre les souches. Ce travail est en cours de réalisation sur un nombre de souches représentatif de la réalité des deux foyers. Il pourra s'étendre à l'étude des souches du foyer Languedoc- Roussillon. Selon les résultats il sera peut-être pertinent de proposer une caractérisation systématique de toutes les souches prises en charge par le CNRL par les microsatellites en plus du zymodème.

CONCLUSION

Ce travail est centré sur les formes cliniques atypiques de la leishmaniose à *L. infantum*. Si la leishmaniose viscérale classique est toujours présente à l'état endémique dans le Sud de la France, les formes asymptomatiques sont les plus nombreuses. En marge de ces deux entités, on retrouve des formes beaucoup moins classiques et dont le nombre est peut être sous évaluées comme la leishmaniose ganglionnaire isolée et la leishmaniose muqueuse. Ces formes inhabituelles doivent être différenciées cliniquement de maladies graves comme les lymphomes ou les processus néoplasiques muqueux. L'influence respective des facteurs liés aux souches et à l'hôte dans le développement de la maladie a aussi été abordée au cours de ce travail. L'étude des microsatellites sur les souches issues de porteurs asymptomatiques met en évidence des génotypes spécifiques absents chez les souches issues de leishmanioses viscérales. L'expression clinique de la maladie pourrait donc être en partie liée au génotype des souches. Par ailleurs, l'étude de l'environnement immédiat des domiciles occupés par des patients ayant présenté une leishmaniose viscérale, a mis en évidence deux foyers de transmission très différents du point de vue géographique. Le foyer marseillais est de nature urbaine tandis que le foyer situé sur les collines autour de Nice est en partie rural et en partie périurbain. Pour aller plus loin dans la compréhension des déterminants à l'origine de ces différences, une étude par les microsatellites des souches de ces deux foyers est en cours de réalisation. Nos premiers résultats confirment le plus grand pouvoir discriminant des microsatellites lorsqu'ils sont comparés aux zymodèmes. Ainsi, bien que la grande majorité des souches de la région PACA-Est appartiennent au zymodème MON-1, nous avons retrouvé, parmi les 122 souches typées, 50 génotypes différents. La réalisation d'une étude identique sur les souches marseillaises permettra de comparer les deux populations de souches.

Le développement d'une leishmaniose à *L. infantum* est plurifactoriel. Au cours de cette thèse, nous avons donc tenté d'élucider la part liée au parasite par l'étude des microsatellites des souches issues de porteurs asymptomatiques et de malades et la part liée à l'environnement via le risque encouru de leishmaniose viscérale en fonction du lieu de vie.

ANNEXES

Annexe 1 : Matériel et Méthodes pour l'analyse des 122 souches par les microsatellites

Les souches en cryotubes provenant du CNRL ont été remise en culture dans du milieu de Schneider et cultivé à 26°C. Lorsque les parasites en culture atteignent leur phase de croissance exponentielle, la culture est centrifugée et le culot est récupéré. Sur ce culot, une extraction d'ADN est réalisée grâce au kit QIAamp DNA Mini (QIAamp DNA Mini, Qiagen, France) selon les recommandations du fabricant.

Les 12 locus testés correspondent à ceux inscrits dans le tableau 1. Chaque 30 µl de mélange réactionnel PCR est composé de : 10 pmol de chaque amorce dont l'amorce sens est marquée, 50ng d'ADN, 1 nmol de chaque dNTP, 3 µl de tampon 10X et 1,5 unités de Taq Polymerase (Taq Polymérase, 5U/µl, Roche Diagnostics, France). Les amplifications sont réalisées sur un automate de PCR avec les conditions suivantes : 35 cycles à 94°C pour 30 secondes, la température d'hybridation de chaque marqueur pour 1 minute (cf tableau 1), 72°C pour 1 minute et une extension finale à 72°C pour 30 min. L'analyse de taille des produits PCR marqués est réalisée sur un automate ABI Prism 3130XL (Applied Biosystems, France) et les données ont été analysées grâce au logiciel GeneMapper (Version 4.0, Applied Biosystems, France). Le Genescan 500 LIZ (Applied Biosystems, France) a été utilisé comme marqueur interne de taille. L'ensemble des 122 souches ont été génotypées avec les 12 locus microsatellites.

La construction de l'arbre phylogénique a été réalisée en utilisant le logiciel PHYLIP (version 3.5c ; J. Felsenstein, Département de Génétique, Université de Washington, Seattle, 1993). L'arbre a été édité en utilisant le logiciel TreeDyn (Chevenet et al., 2006).

Annexe 2 : Détails des 33 microsatellites utilisés dans l'article : « Parasitic genotypes differ in leishmaniasis patients compared to asymptomatic related carriers »

*** Polymorphisme intra-MON-1**

Marqueur microsatellite	GenBank Access no.	Chromosome	Amorce sens (3'→5')	Amorce anti sens (5'→3')	Amorces et séquences amplifies contenant les motifs répétés	References
LiBTG*	nd	1	CTGTTGTACGCCACATCGAC	GCCACACGTGCCTACAAGT	CTGTTGTACGCCACATCGACGTGAAGGGCAACACGAAAAAAGCGAGGAATGAAAGAAGTAGATGAGCACACGCGCGCGCACACACAGGTGTGCTCTGCATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGGGCGTGTGGAAGGTTGTAGAGGGGAGGGAGTGGGAGGGCGATGCGGCAGGCTGCTCTGCTGCATGCAATCCACTGCGTCTGTGTACACTTGTAGGCACGTGTGGC	Fisa, unpublished data
LiBTA*	nd	1	CCTCGTTCATGGCAGAT	TCATAACCGAATTCGTACAC	CCTCGTTCATGGCAGATAGCTCCCTATATTTCACGTCCTCGGAAGTGTATCTCACTTCCCATTTTCTCCACATGATCTCTCTTCCCTCACGCTCTCTCATTACACGAATGTGTCATTTTTGAATTGGGAACCTTTTTTTTTTGCCACACACATACACTTAGTAAAGGTGTATATATATATATATATATATATATATATTTCGGTGCTCGCGATGTCGTCTACAGTGTACGAATTCGGTTATGA	Fisa, unpublished data
Lm4TA*	nd	1	TTTGCCACACACATACACTTAG	GTAGACGACATCGCGAGCAC	GTAGACGACATCGCGAGCACCGAAATATATATATATATATATATATATATACACCTTTACTAAGTGTATGTGTGTGGCAAA	Ochsenreither 2006
LIST7026*	AF427874	13	TGGAGCGAGAAAGGACAAGT	ACACGCAGAGAACTCGGTTT	TGGAGCGAGAAAGGACAAGTAATGGTGCTGAGGAAGGGAGTCGAGGTTGGTGGGGAACCACAACTAAGACGCGAGTGAGGGGTGAGGCACTGATGGCAATTAGGATGGCTACTCAAGCCGGTTCGCGCGTGGGGCGCACACGGATGAGCAGATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTAAACCGAGTTCTCTGCGTGT	Jamjoom 2002
Li22_35*	AM050045	1	CTTGATGTTCGGGTTAGCAAGT	ATGCACACCAAAAATCATGTG	ATGCACACCAAAAATCATGTGGATACGTGTACGTCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCGTGCGGTTGTACTTGCTAAGCCGAACATCAAG	Ochsenreither 2006
Li45_24*	AM050048	17	GCGCCTACAGGCATAAAGGA	CTGGCGCATCAACGGTGT	CTGGCGCATCAACGGTGTTGCGTGTGTATTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGGGTGTGTGGGTGTGGGTGCGGGTGTTCCTTTATGCCTGTAGGCGC	Ochsenreither 2006

TubCA*	nd	34	GGCGTGGTTGCTAAACTGAT	GCCTGCGCACACAGAGAC	GGCGTGGTTGCTAAACTGATACAGGAAAACATACGCACACACACACACACACAAAGAAAAGCGTCTCTGTGTGCGCAGGC	Ochsenreither 2006
LIST7039*	AF427887	30	CTCGCACTCTTTCGCTCTTT	GAGACGAGAGGAACGGAAAA	CTCGCACTCTTTCACTCTTTGCTGCCCTCGCACACCATCTGTGCCTCCTCACATCCCCCAACCTCGCACATCGACACACACACACACACACACACACACACACGTGACATTGTCGATATTGTGGAGCCATCGTCGCTTAGCATGCGTAAGAGCATCTCCTATCACCGCCACCCGCATCTCTTCTTATTTTCCGTTCCTCTCGTCTC	Jamjoom 2002
Li71-33	AM050053	31	CTCCTTTCACACCGCCTCT	GAGAGAAGACGAGACGAAGT	GAGAGAAGACGAGACGAAGTCGCCCACGCCCGTTACAAATACAGAAAAGGCACACACACACACACACACACACATGCCGATAACAAAAAGAGGCGGTGTGAAAGGAG	Ochsenreither 2006
Rossi2*	X76393	14	GAGATACCGAAGCCATCAGC	GCACCCACCTTCGAAGTCTA	GCACCCACCTTCGAAGTCTATTGTACGTGCGTGTGCGCCCACTTCACACACACACACACACACACACACACACACACACACACGTAAAGGCGCAGAGAGAGAGAGAGCGAGAAGTGGACTGGAAGCACACATGACTGCGCTGATGGCTTCGGTATCTC	Rossi 1994
LIST7021*	AF427869	36	CCGAATACACAAGCCTCCTC	TCAGGCTTCGTCGTTTCTTT	TCAGGCTTCGTCGTTTCTTTTTGTTTTCCGCTCCCCTCCCCCTCACATGAACATCGTTTTAGATCGCTCTCTCACGCGACGTCCTTTTCTTCCTTCCTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGATTTCACTGTTTTGCGTCTTTTTTATTGTTTCTTGTGCGTTTATGTGTGCCTGGGGAGAGGAAAAGGGAGGGGAGAGGAGGCTTGTGTATTCGG	Jamjoom 2002
LIST7029	AF427877	30	GCAGAGCTTCTGCTTGGATT	GCATTGCTGTTCTCATCCAC	GCAGAGCTTCTGCTTGGATTTTCTCCCCCTCCCCCACAACACACACACACACACACACACATCCACACACACTGTTGCTAACGGATCGTGCTTATTTCCAATGTCTGCGGTGAGCGCCCGTCCGAGCCCATACCGGGATTGTCTTGGCAGTGTTGTCGTGGATGAGAACAGCAATGC	Jamjoom 2002
LIST7033*	AF427881	25	CATTGCTGAGTGCTGCTAGTG	ATGAGCGTACTGGGCACAC	CATTGCTGAGTGCTGCTAGTGTACACGCACACGGTGACTGAACGACTAGAAAAATGCACGGCGAGACAGCCGATCGGAGGTGACAGCGTGCGCCGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTAAGTGGGGAGTGCGCACTGGCAGTTAAAGGCGTCGCATTGGGTATCGGTGTGCCCAGTACGCTCAT	Jamjoom 2002
LIST7035*	AF427883	23	AAAGGTATGATACGCCTGTGG	ACCGCAAAGAACGGACAT	AAAGGTATGATACGCCTGTGGCGGATCGCACATTGACAAACGTGTGTGTGTGTGTGTGTGTGTGTGGTGGTCCGTGGAGGTGCGAGTCACCTCTTCACGCTCCTACTGTGGGTCTTCACAAACTGTGTTTTTTTTCTTCTTTCCTTTTCTGTCGGCGCATGTGCGGATGTCCGTTCTTTGCGGT	Jamjoom 2002
LIST7037*	AF427885	21	ATGCTGAGCCCATCAAGACT	GATGTCCCCGTTTACTCCAA	GATGTCCCCGTTTACTCCAAACAACACACACACACACACACACACAAAACTATGTCCAAAAATGAGAAAAACACAATAGGTGTAGAAGGTAAACAAAGACAGCAGGAGAAAACATACGTCGCCCCGCTTTTTCGGTTCATTAGAAAACAAGGATCCTTAGTCTTGATGGGCTCAGC??	Jamjoom 2002

Li71-7	AM050051	30	GCTGCAGCAGATGAGAAGG	GTGAGAAGGCAGGGATTCAA	GTGAGAAGGCAGGGATTCAAGCAGGCAGCGCCCCTATCCGCCGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTCTGTGTGCGTTTTCCTTCTCATCTGCTGCAGC	Ochsenreither 2006
Li72-20*	AM050057	31	GATCCCTTCGGATTACTGC	CTGCTAGCGAGGGGATAGG	CTGCTAGCGAGGGGATAGGCTGAAGTACACGCAACCGCTGCGCATAAGAGTGGTGTGTGTGTGTGTGTGTGTGTGTGTGCAGTAATCCGAAGGGATC	Ochsenreither 2006
DPB1	AF182167	8	CATCTCCTAGGTTAAGCGCATC	CTGTTCAGGGAGG CTTGTTC	CTGTTCAGGGAGGCTTGTTCCTTCTCTATGAGGGCTGATATACACACGTATATATATATATATGTATATACCTACCGGTATGGGTATGCGAGAGTCAGGGGGAGGGGGGCGTACGCAGGCGGATGATGCGCTTAACCTAGGAGATG	Hide, PhD
DPB2	AF182167	8	CACATGCACATACGCGTTC	TTGCAAAGGAGGAACCTGAG	????AAGGAGGAACCTGAGGACGAAGGGAGGCAAGCAAACACGCACTCGCACAGCCGTGACGAGGCGCGTCCGATCCGTTATGAAGAGCAAGAGCCCGTATCACCGAGGGCCGTGTATGTCCACGGGCGCGTATGGGTGCGTGTGCGTGCCTCTCTCTCTCTCTCTATATATATATATGTATTCGTACGTGTGTCGGTATCTAGAGCTCGATGGGAACGCGTATGTGCATGTG	Hide, PhD
HG	AF170105	21	CACTGCCTCTTGTCCCTTG	AACATCTCGAGCAGGAAGGA	CACTGCCTCTTGTCCCTTGCAAGTCCCTGCCGGTGTATTGCCCTCCCCCTCCCTCCTCTGTTCTCGCAATACTCGATCCCACGTTATCGATATATATATACCTATATATATATTATATATATATATAGGTATATAACGCCCTCCACTCCCCATCCTGCCCACCGCTCCACCGGCGCCTCTCCTTCCTGCTCGAGATGTT	Hide, PhD
ITS1	AJ000288	?	CTGGATCATTTTCCGATG	TGATACCACTTATCGCACTT	TGATACCACTTATCGCACTTTACTGCGTTCTTCAACGAAATAGGAAGCCAAGTCATCCATCGCGACACGTTATGTGAGCCGTTATCCACACACGCACCCACCCCGCCAAAAAACCGAAACGCCGTATATTTTTTGTATAAACGGACATTTTTGCTTTTGTATAGGCGGTGCGTTATAACGTCGATCGGCCTTTTTTTTACTGCAAATTTTGAGTACAAAACTTTGCTGTGTATGTGGGAAAGGCCTACATATATATACATAGGTCTCCCCGAGTTGTATATGTTTTTTGGGTGTAATCATCGGAAAATGATCCAG	el Tai, 2000
LIST7024	AF427872	30	TAAACTGCATGGTCCCCTCT	ACAAGCACCATCAT CCACAT	ACAAGCACCATCATCCACATAATCGCGCGGAGACGGAGACCGAGACCGCCAACACACACACACACACACACACACGCACAGCAGCGTGGGCGTGTAGTGCCTACTTATCTTGCTCGCTCGCTCTCTTTTTAAGCAGCCAACATACCGCGCAAATGGAGAGCGGCAGCGATTCCTCCGAAGAGGGGACCATGCAGTTTA	Jamjoom 2002
LIST7025*	AF427873	10	GGAGTCGTCTCTCTGTTACGC	ATCGCGTGCATGGGTATT	GGAGTCGTCTCTCTGTTACGCACACACACACACACACACACACGAAACAAGGACGTGTCTCTGCGTCCTTGCGTACTGCACGTTCGCTCTGATGCTGCTCGTTTTAGTTCTCCTCTGTTTTGCGTTTCTCTGTCAAGGACGTCGCAAAACGTCGGCGCAAATACCCATGCACGCGAT	Jamjoom 2002
LIST7027	AF427875	26	CTCTCTCGTCACCACAGCAC	AGGGGACAAGACACAGATGG	CTCTCTCGTCACCACAGCACAGCAGTTTATCTGGCTCTGAATCACACACACACACACACACTTCACCCCACCAGTGTTGCCGCCCTTCCCCCCTCCTCCTCCTTCTTGTCCTGTCATACGTTCGACTTCAGCATATTAGTCGCCTTGTAGGTGTGTGCCATCTGTGTCTTGTCC???	Jamjoom 2002

LIST7031*	AF427879	10	CCACTGG TGGAAAT AGAAAG ACT	GGAGAA CTAAAAC GAGCAGC A	CCACTGGTGGAAATAGAAAGACTATAGATATGTATGAAGCAGCGGAGCGCGGC GGGCGGGAGTCGTCTCTCTGTTACGCACACACACACACACACACACGAAAC AAGGACGTGTCTCTGCGTCCTTGCGTACTGCACGTTCGCTCTGATGCTGCTCGTT TTAGTTCTCC	Jamjoom 2002
LIST7034	AF427882	12	TGCATGC ACGTGGT CTCT	GTTCACC GCCACCA TAATAAA	GTTCACCGGCACCATAATAAATAGCAACGTCATGCAAAGAGCGCGCTTGAGGG AGGGAGGAAGACAGTGACGTGTCCGTGTGTGTGTGTGTGTGTGTGTCCGTGT GTGTGTGTGTGGCAGCAGAGACCACGTGCATGCA	Jamjoom 2002
LIST7038*	AF427886	26	GCGCTTT TCTTTCC CTTCTT	ATGGCGA AGTTGTT TGTGC	GCGCTTTTCCTTCCCTTCTTTGGTGCTCTCGCACTCTCCCTCGTTTAGCAGTTCA GAAAAGAGGTATGTGGAGAACGGGCTCAGGGAGACGAAACACACACACACAC ACACAAACAACGTCGCCAT	Jamjoom 2002
Li71-5/2*	AM050050	35	GCACGGT CGGCATT TGTA	GATAAAC GAGATGG CCGC	GCACGGTCGGCATTTGTACCGCACACACACACACACACAAAGCACGAGAGGAC CGACGGTGTCGAGCGCGCCGTTCGTTTTCTTTTTTTTTCGCGGCCATCTCGTTTA TC	Ochsenreither 2006
CS20	nd	19	CGTTGGC TGTTGAT TGTGTA	GCGTGGC AATCTCC TCATT	CGTTGGCTGTTGATTGTGTATGTGTGTGTGTGTGTGCGTGTGTGTGTGTGTGTGT GTGTGCCGGCGAATGAGGTGATTGCCACGC	Khuls, 2007
LIST7028	AF427876	36	CACTCCA CTGCGTT GGATA	CTTTGAC CGCCGTT CTTT	CACTCCACTGCGTTGGATATCCAACACACCTCTTACATGATAGTGAGAAGTATC GCGCAGAGCGCCCACTTCGCGCCTGGCCCATCATTCTTGAGACGTTTAGTGTGT GTGTGTGTGTGTGTGTGCGTGTGCGCGCAAAGAACGGCGGTCAAAG	Jamjoom 2002
Rossi1	X76394	4	CCCTCCA CCTAGTC ACCTCA	GCCTATG ACAGGG AGCAAGT	GCCTATGACAGGGAGCAAGTAAGGAGGCCATTACACACACACACACACACATA CACACAGAGGGCGGGGGTCGGGGGCGTTATCGTGGCAGTGAGGTGACTAGGTG GAGGG	Rossi 1994
LIST7023	AF427871	33	CTTTGCG TTGCGCA CTAA	GCTTGTG TTCCGTG TGTGTT	GCTTGTGTTCAGTGTGTGTTCAGCGTGTGTGTGTGTACGCACCGTCGACTCTGA TCTTTGTCCTCCCTGCCCCCCTCCCCCGTCCCCTTTGGGCGTAGCTGAGTTTCGA AGGTCGCGGAATCGTACATTGCAGCTTAGTGCGCAACGCAAA	Jamjoom 2002
LIST7030	AF427878	17	TCTCTGC ACGTCTG TGTGTG	TCTTCCT GAAGGG CGATG	TTCTTCCTGAAGGGCGATGGGTGCCACACACACACACACACACACCCGTCCCA GCACTCCCTGCTGAACGCACGGGCGGCGCTGCGGAGCACCACCACTCCCACTT GAGGGCCTCACCAGATCATCCTCCTCAGCCTTTCCTCCAACACGCACGCAACCA CACACAGACGTGCAGAGA	Jamjoom 2002

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