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Original article

Canine visceral leishmaniasis caused by *Leishmania infantum* in Senegal: risk of emergence in humans?

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Abstract

In the context of global warming and the risk of spreading arthropod-borne diseases, the emergence and reemergence of leishmaniasis should not be neglected. In Senegal, over the past few years, cases of canine leishmaniasis have been observed. We aim to improve the understanding of the transmission cycle of this zoonosis, to determine the responsible species and to evaluate the risk for human health. An epidemiological and serological study on canine and human populations in the community of Mont Rolland (Thiès area) was conducted. The data showed a high seroprevalence of canine leishmaniasis (>40%) and more than 30% seropositive people. The dogs' seroprevalence was confirmed by PCR data (concordance > 0.85, Kappa > 0.7). The statistical analysis showed strong statistical associations between the health status of dogs and seropositivity, the number of positive PCRs, clinical signs and the number of *Leishmania* isolates. For the first time, the discriminative PCRs performed on canine *Leishmania* strains clearly evidenced that the pathogenic agent is *Leishmania infantum*. The results obtained show that transmission of this species is well established in this area. That the high incidence of seropositivity in humans may be a consequence of infection with this species is discussed.

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Keywords: *Leishmania infantum*; Human; Dog; West Africa; Prevalence

1. Introduction

The threat of infectious diseases is a global concern and not simply a local problem. Indeed, in the context of global warming and the risk of arthropod-borne disease spread,

emergence and reemergence of leishmaniasis should not be neglected. This disease is prevalent in Africa, particularly in its most severe and lethal form, visceral leishmaniasis (VL). On the African continent, this clinical form is classically described in the north and east of the continent. In North Africa, the pathogenic agent is *Leishmania infantum* and the main reservoir is dogs. In East Africa, the main pathogenic agent is *Leishmania donovani* and the disease is considered an anthroponosis, although dogs were described as residual

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zoonotic reservoirs in certain areas [1]. Several human visceral cases were reported in sub-Saharan countries such as Ivory Coast and Gambia, but these reports remain anecdotal with no evidence of an autochthonous transmission cycle [2,3].

In Senegal, several studies have described the circulation of cutaneous leishmaniasis caused by *Leishmania major* [4,5]. The vector and reservoirs are well identified [6,7] and the disease is efficiently treated by public health services. Nevertheless, human visceral leishmaniasis has never been described in this country. The first cases of canine leishmaniasis were described in Senegal by Lafont and Heckenroth in 1915 [8] and further studied, but no human cases were evidenced [9]. More recently, only a few canine strains were identified as *L. donovani* species using the MLEE technique based on five enzymes [10]. Furthermore, entomologic studies showed that the usual vectors of the *L. donovani* complex were absent in these areas [11].

In this epidemiological context, four questions naturally arise: (a) is *L. donovani* definitely the species responsible for canine leishmaniasis in this area? (b) Are humans in contact with parasites and if so, how frequent are these contacts? (c) Are there human clinical cases in this area? (d) Is there a risk for human health? This article describes the first detailed epidemiological survey, with a serological, parasitological and clinical investigation in the community of Mont Rolland on a population of symptomatic and asymptomatic dogs and on human populations in contact with dogs. Finally, we discuss the epidemiological meaning of this unexpected canine leishmaniasis focus and the risk for human health.

2. Materials and methods

2.1. Study area, canine and human populations

From 2005 to 2008, six field studies were conducted in the rural community of Mont Rolland, close to Thiès (70 km from Dakar). This community comprises 18 villages with a total population of 16,000 inhabitants and around 500 dogs.

For this study, 160 dogs from 20 different parts of 17 villages were enrolled for the serological, clinical and parasitological survey. These dogs are domestic and the owners' consent was obtained. Fig. 1 shows the distribution of localities where dogs were sampled. In the course of one field study, in addition to sampling dogs, we enrolled people for a serological and clinical analysis in each compound after obtaining their consent. A total of 133 people with at least one dog in their peridomestic area were included in the study. This study was conducted in agreement with the local public health services of Mont Rolland. This protocol received the administrative authorization of the Ministry of Health of Senegal.

2.2. Dog study

2.2.1. Samples

Each dog was clinically examined by a veterinarian to check the dog health and for the most frequent clinical signs observed in canine leishmaniasis: weight loss, periorbital loss of hair, ulcers, onychogryphosis and exfoliative dermatitis.

Popliteal node aspirates were sampled when the nodes were enlarged and 5–10 ml of blood was taken from either the saphenous or jugular vein. Vitamins, an antibiotic and ivermectin were administered depending on the dogs' clinical status. After blood centrifugation, plasma and buffy coats were separated. Plasmas were used for the serological diagnostic test (DiaMed-IT LEISH[®], Cressier sur Morat, Switzerland). This test is based on the rK39 antigen and has been previously demonstrated to be mainly suitable for detection of symptomatic human VL [12] and of both clinical and asymptomatic canine VL [13]. We conducted the tests according to the manufacturer's protocol. For the result reading, besides the negative tests coded “–”, we defined three levels of positivity: slight (“+” the band was less pronounced than the internal control), normal (“++” the band was similar to the internal control) and strong (“+++” the band was more pronounced than the internal control). This classification makes it possible to evaluate the test efficacy in detecting leishmaniasis disease in Senegalese dogs as well as asymptomatic or mild cases. Buffy coats were used for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen).

2.2.2. Strain isolation and preservation

Each popliteal lymph node aspirate was sowed on two NNN tubes with 0.75 ml of penicillin diluted in physiological serum (100,000 UI/ml). Cultures were placed at 26 °C. The tubes were observed under the microscope after 3 days and twice a week for 4–6 weeks. Positive tubes were cultured according to standard culture protocol for cryostabilization, DNA extraction and parasite pellet preservation.

2.2.3. PCR

Two different PCR methods were used. The first one, described by Noyes et al. [14], was used to diagnose *Leishmania* DNA in dog buffy coats. This nested PCR is very sensitive and useful for leishmaniasis diagnosis since it targets kDNA minicircles. Furthermore, in the Old World, it can distinguish the *L. donovani* complex from the *L. major* and *Leishmania tropica* species, but cannot differentiate *L. donovani* from *L. infantum*. The second one targets the cysteine protease b (*cpb*) gene and distinguishes *L. infantum* from *L. donovani* and cannot amplify the other *Leishmania* species such as *L. major* or *L. tropica*, as published by Hide and Bañuls [15]. Since the latter is not very sensitive, it was only performed on DNA obtained from the culture of canine parasites. The procedures were performed according to protocols published elsewhere. We used several reference strains in order to identify our samples: *L. major* (MHOM/IL/81/Friedlin), *L. tropica* (MHOM/SU/74/K27), *L. infantum* (MHOM/MA/67/ITMAP263) and *L. donovani* (MHOM/ET/67/HU3).

2.2.4. Statistical analysis

The concordance between the DiaMed-IT LEISH[®] and PCR results was calculated as follows: concordance = results in agreement / (results in agreement + results in disagreement). Kappa statistics were used to compare the two diagnostic tests. Kappa is an index that compares agreement against what can

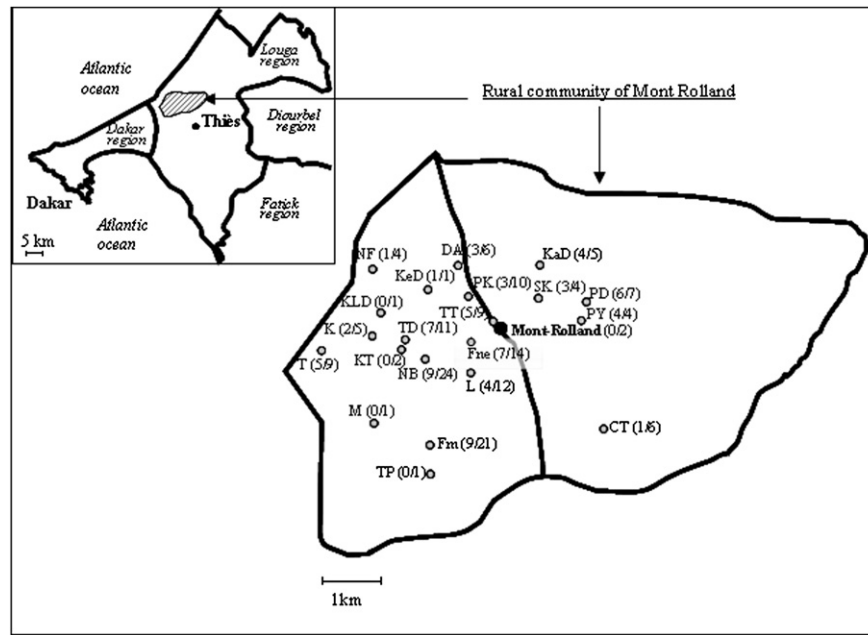


Fig. 1. Maps showing the sampling area in Senegal. This map shows the location of the rural community of Mont Rolland and shows all the districts and villages (only initials) where canine samples were isolated. The numbers in brackets are the number of seropositive dogs out of the total number of dogs by location.

be expected by chance. Fisher exact tests were used to assess the association between clinical groups and seropositivity, PCR positivity, strain isolation, or leishmaniasis clinical signs.

2.3. Human study

2.3.1. Human blood samples

One hundred and thirty-three persons (1–82 years of age; mean, 40.5 years) were enrolled in this study. The inclusion criteria were the presence of at least one dog in the peri-domestic area and informal consent. Parental consent was obtained for children. We sampled a drop of blood on filter paper that was then dried and stored at room temperature until Western blot analysis (from 1 to several months later). The filter paper was cut to take only the drop. This blood confetti was plunged in 400 μ l of PBS 1 \times corresponding to around 20 μ l equivalent serum.

2.3.2. Western blot analysis

The 133 samples were treated using the Western blot technique developed by Marty et al. [16]. This method can detect specific antibodies of *L. infantum* antigens in sera. Western blot analysis with acute clinical leishmaniasis attributable to *L. infantum* should present the simultaneous presence of antibodies against five antigens with molecular masses of 14, 18, 21, 23 and 31 kDa [16,17]. In asymptomatic immunocompetent individuals, only the 14- and/or 18-kDa bands were detected by Western blotting [18]. All seropositive individuals (with a minimum of one band, 14 or 18 kDa) were called to the Mont Rolland health centre for a detailed clinical examination by a medical doctor and for a rapid diagnosis of disease using a DiaMed-IT LEISH[®] test.

3. Results

3.1. Dog study

3.1.1. Clinical status and serology

We classified the dogs according to their overall health after a detailed clinical examination regardless of serological and molecular results. The canine population was thus subdivided into three groups: group 1 corresponding to dogs with no clinical signs ($n = 68$), group 2 corresponding to dogs with mild clinical signs (benign cutaneous lesions, enlargement of lymph nodes, onychogryphosis, slight weight loss or slight depilation with somewhat compromised general health; $n = 55$) and group 3 with dogs in very poor health (several severe clinical signs together, such as serious cutaneous lesions and enlargement of lymph nodes and onychogryphosis, weight loss and substantial depilation with poor general health; $n = 37$) (see Table 1). In groups 2 and 3, 58 dogs

Table 1

Three groups of dogs were created according to their health status (group 1 corresponding to healthy dogs, group 2 corresponding to dogs with few clinical signs and group 3 to dogs in poor health). For each group, we determined the number of seropositive dogs, the number of dogs with potential leishmaniasis signs, the number of dogs with positive PCR, the number of *Leishmania* isolates. All isolated strains were identified as *Leishmania infantum*.

	Number of dogs	Number of seropositive dogs	Number of dogs with potential signs	Number of positive PCRs	Number of <i>L. infantum</i> isolates
Group 1	68 (42.5%)	15 (22%)	0	10 (14.7%)	3 (4.4%)
Group 2	55 (34.4%)	30 (54%)	23 (37.8%)	20 (36.4%)	10 (18.2%)
Group 3	37 (23.1%)	29 (78.3%)	35 (94.3)	27 (73%)	20 (54.1%)
Total	160	74 (46.3%)	58 (36.3%)	57 (35.6)	33 (20.6%)

presented a minimum of three clinical signs mainly observed in canine leishmaniasis such as skin lesions, cachexia, onychogryphosis, alopecia, periorbital rings of alopecia and lymph node enlargement.

In the entire canine population, 74 dogs were seropositive with DiaMed-IT LEISH[®], distributed into the three clinical groups (see Table 1). Forty-four dogs revealed a strong signal (+++, more pronounced than the internal control), 14 dogs a normal signal (++, similar to the internal control), 16 dogs a mild signal (+ less pronounced than the control) and 86 dogs no signal (–) (see Table 2). Eight dogs belonging to group 3 revealed negative serological tests. This result is not surprising since other diseases can have clinical signs similar to leishmaniasis such as demodicosis, manges and ehrlichiosis. These clinical confusions could explain the contradictory results obtained in our study.

3.1.2. Polymerase chain reaction results, parasite isolation and identification

The nested-PCR of Noyes et al. [14] performed on the DNA extracted from the canine buffy coats showed 57 positive samples out of 160 (35.6%) (see Table 1). Fifty-five of these nested PCR-positive samples were also seropositive dogs (see Table 2). Only two samples were seronegative and nested PCR-positive. The nested PCR profile comparison with the *Leishmania* DNA references suggested that the parasites belong to the *L. donovani* complex (Fig. 2A) and not to the *L. major* or *L. tropica* species.

The cultures performed from the lymph node biopsies of dogs resulted in 33 isolates. The 33 dogs belonged to the three clinical groups with a majority of the strains from group 3 corresponding to dogs in poor health (see Table 1). All these isolates were cryopreserved. Since nested PCR cannot differentiate *L. infantum* from *L. donovani*, we used the PCR based on the *cpb* gene developed by Hide and Bañuls [15] on the cultured strains. The electrophoresis profiles clearly demonstrated that they all belonged to the *L. infantum* species (see example in Fig. 2B).

3.1.3. Statistical analysis

On the basis of the data presented in Table 2, we found good agreement between the PCR results and serology with a concordance of 0.87 and a Kappa of 0.73. Fisher exact tests showed that the number of seropositive samples (A), *Leishmania* clinical signs (B), number of positive PCR samples (C) and number of isolated strains (D) significantly increased from group 1 to group 3 (Fig. 3). We also tested the association between the signal intensity of DiaMed-IT LEISH[®] tests and

Table 2
Comparison of diagnostic PCR and DiaMed-IT LEISH results. These values were used for concordance calculation. Concordance was equal to 86.9%. Q1

PCR results	DiaMed-IT LEISH test		
	Positive	Negative	Total
Positive	55	2	57
Negative	19	84	103
Total	84	86	160

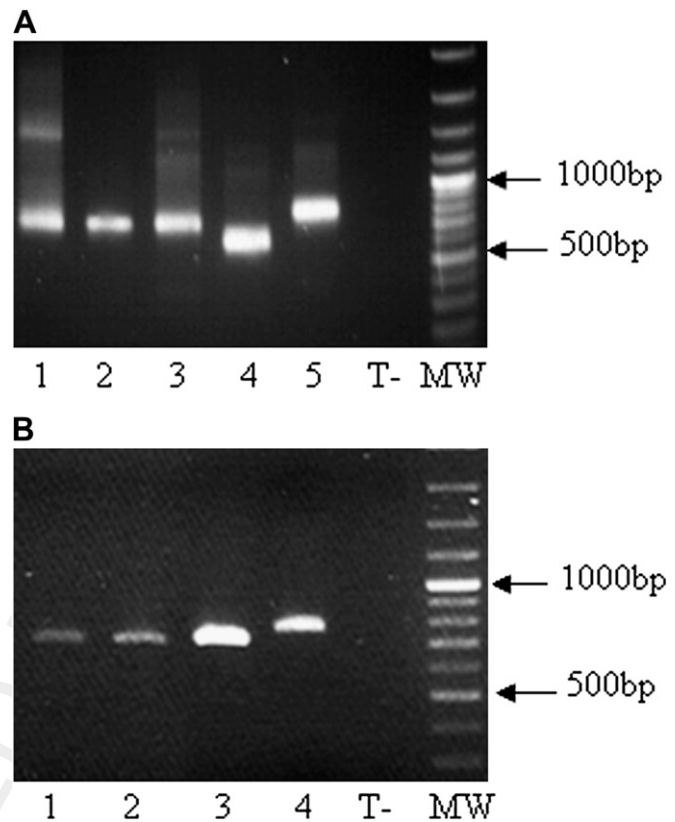


Fig. 2. A. The profiles obtained with nested PCR designed by Noyes et al. [14], which detects the positive dogs and differentiates *Leishmania donovani* complex from *L. tropica* and *L. major* species. T, negative control; MW, molecular weight; Line 1, Senegalese dog; line 2, DNA from a child's cutaneous lesion; line 3, *L. infantum* reference strain (MHOM/MA/67/ITMAP263); line 4, *L. major* reference strain (MHOM/IL/81/Friedlin); line 5, *L. tropica* reference strain (MHOM/SU/74/K27); T-, negative control; MW, molecular weight (100 bp). B. The profiles obtained with the PCR designed by Hide and Bañuls [15], which differentiates *L. donovani* (741 bp) from *L. infantum* (702 bp). Lines 1 and 2, two Senegalese canine strains; line 3, *L. infantum* reference strain (MHOM/MA/67/ITMAP263); line 4, *L. donovani* reference strain (MHOM/ET/67/HU3); T-, negative control; MW, molecular weight (100 bp).

the dog clinical groups by odds ratio estimation with 95% confidence intervals (see Fig. 4). The dogs in group 1 presented a significantly higher probability of having no signal (–) than the other dogs and a significantly lower probability of having strong positivity (+++) than the other dogs. An opposite trend was observed for the dogs in group 3, which displayed a significantly lower probability of having no signal (–) than the other dogs and a significantly higher probability of having strong positivity (+++) than the other dogs. It is worth noting that the other signal intensities (+ and ++) can be observed in the three clinical groups, but the ++ intensity is more present in group 3 and the + intensity in group 2.

3.2. Human study

All the people enrolled were clinically examined and we detected no visceral leishmaniasis symptoms. One 10-year-old child presented multiple cutaneous lesions. Since we were

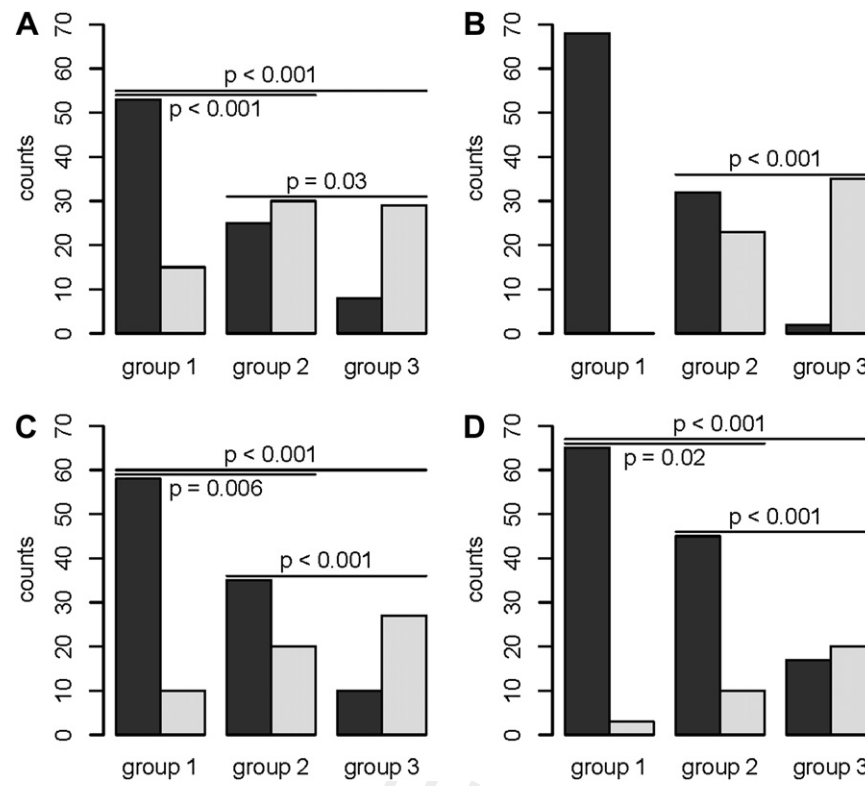


Fig. 3. Results of Fisher exact tests. The four sections present the differences between dog clinical groups (group 1, healthy dogs; group 2, dogs with few clinical signs; group 3, dogs in poor health) and the number of seropositive samples (A), *Leishmania* clinical signs (B), the number of positive PCR samples (C) and the number of *Leishmania* isolates (D). The black bars represent the number of negative samples and the grey bars the number of positive samples in each group, the horizontal lines indicate the significant differences and their probability between groups. All comparisons show significant differences between the three groups except for the *Leishmania* clinical signs between groups 1 and 2.

unable to grow the parasites from the sample taken on the unhealed lesions, we tested it for *Leishmania* using nested PCR after DNA extraction. The pattern obtained showed an infection by a parasite from the *L. donovani* complex excluding *L. major* and *L. tropica* species. The DiaMed-IT LEISH[®] result was negative. From the 133 sera tested using the Western blot technique, no typical patterns of five bands were demonstrated [16,17]. Thirty-three percent (44/133) of the sera samples revealed either the 14-kDa band and/or the 18-kDa band, described as a typical profile of either previous contact or asymptomatic carriage. To confirm the absence of leishmaniasis symptoms, the 44 seropositive persons were called to the health centre for a detailed clinical check and a DiaMed-IT LEISH[®] test. None of these individuals had any specific symptoms of visceral leishmaniasis and all the DiaMed-IT LEISH[®] tests performed were negative.

4. Discussion

This study ascertains for the first time that the pathogenic agent of canine leishmaniasis in Senegal is *L. infantum*. Several aspects make the Senegalese leishmaniasis focus atypical and worrisome: (i) an unusual localization of *L. infantum* in sub-Saharan Africa; (ii) a hyperendemicity of canine leishmaniasis and (iii) a potential risk for human health.

The previous MLEE characterization based on five isoenzymatic loci of Senegalese canine isolates proposed *L. donovani* species *sensu lato* [19]. In our study, the nested PCR [14] confirmed that the 33 isolates from dog buffy coats belong to the *L. donovani* complex and the specific PCR published by Hide and Bañuls [15] clearly identifies the *L. infantum* species for the 33 isolates.

Until now, the human cases of cutaneous leishmaniasis in Senegal were attributed to *L. major*, whose biological cycle is well known [4,5,7]. Nevertheless, this species had never been detected in Mont Rolland and primary PCR results in approximately 1000 sandflies captured in this area did not show any evidence of the presence of *L. major* (Senghor et al., unpublished data). On the other hand, *L. infantum* transmission was only suspected for canine leishmaniasis since no human case has been described. The results obtained in this study, with the molecular confirmation of *L. infantum* species, high seropositivity in dogs (46.3%) and humans (33.3%) strongly suggest that the *L. infantum* life cycle is well established in this area. Nevertheless, a previous entomologic study did not evidence any known vectors for *L. infantum* [7]. This strongly suggests that *L. infantum* may be transmitted in this area by an unusual species of sandfly. Besides the absence of classical vectors of *L. infantum* in this focus, localization of this species in sub-Saharan West Africa is unusual. Indeed, canine visceral leishmaniasis caused by *L. infantum* has mainly been described

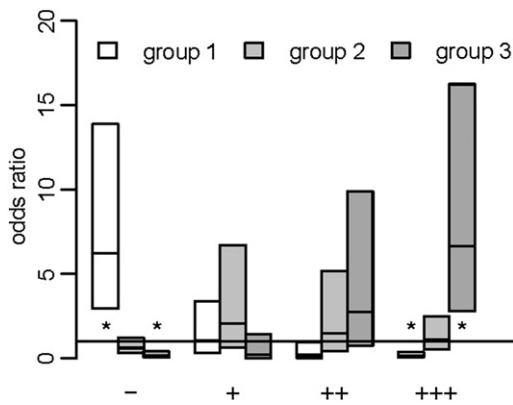


Fig. 4. Odds ratio estimation with 95% confidence intervals of having an association between signal intensity of the DiaMed-IT LEISH[®] test and the dog clinical groups (group 1, healthy dogs; group 2, dogs with few clinical signs; group 3, dogs in poor health). The signal intensities in the DiaMed-IT LEISH[®] were categorized as follows: “-”, no signal; “+”, slight signal (the band was less pronounced than the internal control); “++”, normal signal (the band was similar to the internal control); and “+++”, strong signal (the band was more pronounced than the internal control). The mean odds ratio values are indicated by a bar inside each box. Only the significant probabilities were noted. The results show that a negative signal was statistically observed in group 1 and absent in group 3 and that the strong signal was statistically observed in group 3 and absent in group 1.

in the north of the African continent, i.e. in the Mediterranean basin. This focus appears as an island when regarding the distribution of this species in Africa, suggesting disease establishment by the introduction of an infected host (e.g. dogs or humans) followed by parasite transmission by an autochthonous sandfly species. Another possible hypothesis is that this disease is endemic in West Africa but still not detected since only a few cases of visceral leishmaniasis have been documented. Detailed genetic and phylogenetic analysis of the strains and a comparison with European and Northern African strains could provide a better understanding of the establishment of this parasite in this area.

This study shows that canine leishmaniasis occurs at a high frequency in this area with more than 45% seropositive dogs (DiaMed-IT LEISH[®]), among which 29 were severely affected. It is worth noting that our study was not conducted on the whole canine population in the community (but rather on approximately 30% of the dog population). Nevertheless, previous epidemiologic studies in *L. infantum* foci have never shown such high rates of seropositive dogs. For example, recent studies showed seroprevalence of 20.1% in Southeastern Spain and of 14% in Southeastern France [20,21]. In North Africa, the seroprevalence is also lower than in Senegal, with 12% in Tunisia [22] and around 20% in certain regions of Morocco [23]. To date, the highest seroprevalence has been reported in South America, with 36% in Jacobina, Brazil [24]. However, it was assumed that the seroprevalence evaluated using the ELISA and IFAT techniques underestimated the rate of infected dogs in endemic areas [25]. This hypothesis has been further confirmed by PCR-based screening, which showed more than 65% positivity in different areas in France [21] and 67% in Majorca, Spain [26]. Interestingly, in our study, the 0.87 concordance and the 0.73 Kappa between the PCR results

and DiaMed-IT LEISH[®] tests strongly suggest that the estimated prevalence in Senegalese dogs may not be underestimated. The DiaMed-it LEISH[®] tests seem to give a good estimation of seroprevalence in dogs since it detects both symptomatic and asymptomatic dogs. Nevertheless, although the techniques of serology are different in the published studies, the comparison suggests that *L. infantum* circulation in Senegal is very high and strongly suggests a hyperendemic situation of canine leishmaniasis in this area. Different hypotheses could explain this high level of transmission in dogs: (i) dogs are more susceptible in Senegal due to either genetic canine factors (high susceptibility) or the recent emergence of the disease in this area; (ii) the vector specifically feeds on dogs and this could consequently potentiate transmission. Vector identification and the analysis of sandfly blood meals could validate or invalidate this last hypothesis.

In this epidemiological context, one must raise the fundamental question of the risk for human health. Zoonoses are of considerable concern as a source of recurring human infection as well as future epidemics [27]. In leishmaniasis, domestic dogs are the main reservoir of *L. infantum* and play a key role in the transmission to humans. It is noteworthy that in industrialized as well as developing countries where *L. infantum* is transmitted, cases of human visceral leishmaniasis can be observed. Although the incidences of canine and human disease have not been directly correlated in the Mediterranean basin, the presence of infected dogs plays an important role in the long-term maintenance of human VL in specific regions [28]. Nevertheless, different surveys have demonstrated that appreciable increases in canine as well as human cases of VL have been reported in Southern France and Sicily [29,30]. In our study, the 33% human seroprevalence clearly suggests a frequent contact between humans and parasites.

Nevertheless, visceral leishmaniasis has never been detected in the Mont Rolland and Thiès area in the present study and in the Thiès Regional Hospital, or throughout Senegal (Faye B, personal communication).

Discussion with the public health services brought out that although the cutaneous leishmaniasis produced by *L. major* is known in the main medical centres (e.g. Le Dantec Hospital, Dakar), visceral leishmaniasis is completely unknown. Since the symptomatology is not specific in the visceral disease, it is difficult to know whether some cases have remained undetected. Furthermore, the HIV prevalence among adults aged 15 years or more was 835 per 100,000 inhabitants in Senegal in 2005. Consequently, the risk of coinfection is far from insignificant. To date, even if we are not able to say whether these parasites can produce the visceral form of the disease, the case of cutaneous leishmaniasis in a 10-year-old child detected in this study shows that these parasites can be pathogenic for humans.

Finally, many unknowns remain such as the vectors, the localization of transmission (intradomiciliary, peridomestic, or outside of the inhabited zones), the distribution of *L. infantum* in Senegal and the real impact on human health. This study thus demonstrates the need to develop further entomological and ecological investigations to advance the knowledge of the

L. infantum cycle in Senegal and to establish a system of surveillance in order to control the risk of emergence of visceral leishmaniasis cases. Medical doctors and health care personnel in regional hospitals but also in local health centres should be informed.

Acknowledgments

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