Research Article

Epidemiological Investigation of Canine Leishmaniasis in Southern Morocco

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Dogs are the major reservoir of *Leishmania infantum*, the causative agent of human and canine visceral leishmaniasis in the Mediterranean basin. In Morocco, canine leishmaniasis (CanL) is usually believed to be widespread mainly, if not only, in the northern regions and few data are available about the situation in southern parts of the country. Here, we report the results of a preliminary, clinical, and serological study carried out in 2004–2007, in four provinces of southern Morocco. Serological analyses were processed using two different Elisa techniques, a homemade Elisa test and IDVET commercial kit, and confirmed by two different western blot (WB) tests, homemade and LDBIO commercial kits. We highlighted the presence of CanL infection in southern regions, known until then as free of the disease: 19.8% (48/243) of examined dogs displayed clinical signs compatible with CanL and the seroprevalence was particularly high, respectively, 81.8% and 87.8% by Elisa and western blot tests. Our current developed and validated homemade (Elisa and WB) tools will be cost-effective and useful for next large-scale epidemiological studies on Moroccan leishmaniasis animal reservoir.

1. Introduction

Canine leishmaniasis (CanL) is a zoonotic disease caused by *Leishmania infantum*, a trypanosomatid protozoan, transmitted through infected sand fly (Diptera: Psychodidae) bites. CanL is endemic in the Mediterranean basin, where seroprevalence ranges between ten and 37% [1, 2] and symptoms in dogs occur in various combinations [3]. Both symptomatic and asymptomatic dogs are sources of the parasite and phlebotomine sand flies play an active role in the transmission of *Leishmania* to humans [4].

In northern Morocco, natural CanL was first reported in the region of Tangier [5]. Further subsequent cases have been reported in Taounate, Al Hoceima, Chefchaouen, and Ouezzane provinces [6], in Sefrou and Zouagha Moulay Yacoub [7], in Khemisset province [8], and in the province

of Nador [9]. *Leishmania infantum* zymodeme MON-1 is the unique causative agent of CanL in the northern slopes of the Rif mountains [6] and *L. infantum* MON-24 was identified in dogs from the Pre-Rif area [10]. In the Rif region specifically, dog seroprevalence ranges between ten and 41% [9] and *L. infantum* strains are highly virulent [8, 9].

In northern slope of High Atlas Moroccan mountains, Dereure et al. [11] reported the presence of dogs infected with *L. tropica* (MON-102 and MON-113). Additionally, Guessous-Idrissi et al. [12] reported a case of dog with visceral leishmaniasis caused by *L. tropica* in Taounate province. More recently, in Al Hoceima province, *L. tropica* MON-279 was identified in dogs with clinical signs of CanL [13].

To date, only sporadic cases of human visceral leishmaniasis are reported in southern Morocco [14, 15]. In the present study we report the results of clinical and serological

TABLE 1: Species composition of the sand fly fauna in study area.

Province	Species composition	References
Al Haouz	<i>Phlebotomus papatasi</i>	[33, 36, 37, 45]
	<i>P. sergenti</i>	
	<i>P. perniciosus</i> *	
	<i>P. longicuspis</i>	
	<i>P. alexandri</i>	
	<i>Sergentomyia fallax</i>	
	<i>S. minuta</i>	
Azilal	<i>S. dreyfussi</i>	[36, 37, 46]
	<i>Phlebotomus papatasi</i>	
	<i>P. sergenti</i>	
	<i>P. perniciosus</i> *	
	<i>P. longicuspis</i>	
Chichaoua	<i>Sergentomyia fallax</i>	[34, 36, 37]
	<i>S. minuta</i>	
	<i>S. dreyfussi</i>	
	<i>Phlebotomus perniciosus</i> *	
	<i>P. sergenti</i>	
	<i>P. longicuspis</i>	
	<i>P. ariasi</i>	
	<i>P. papatasi</i>	
Marrakech	<i>P. alexandri</i>	[36, 37, 47, 48]
	<i>P. mariae</i>	
	<i>Sergentomyia fallax</i>	
	<i>S. minuta</i>	
	<i>S. dreyfussi</i>	

* As atypical form [36–39].

surveys on CanL in southern Morocco, an area traditionally considered to be free from CanL in spite of the omnipresence of dogs and phlebotomine sand flies. Our investigations were carried out in four provinces of Morocco: Al Haouz, Azilal, Chichaoua, and Marrakech, where species composition of the sand fly fauna was also established (Table 1).

2. Materials and Methods

2.1. Sampling. An epidemiological survey was carried out in 27 localities (Figure 1) in four provinces of southern Morocco: Al Haouz (31°22'N, 7°51'W), Azilal (31°58'N, 6°34'W), Chichaoua (31°32'N, 8°45'W), and Marrakech (31°36'N, 8°02'W).

Two hundred and ten dogs in 2004–2006 and 33 dogs in 2007 were studied. An individual form was completed for each dog: date, locality name and environment, dog age, sex, type (domestic or feral), and *Leishmania* specific clinical signs. The detail of sampling is shown in Table 2.

Because of practical, economic, and security constraints, it was not possible in 2004–2006 to take blood samples from

FIGURE 1: Moroccan study area provinces: (1) Chichaoua; (2) Marrakech; (3) Al Haouz; (4) Azilal.

stray and feral dogs with unknown rabies infection status. In 2007, after clinical examination, blood was collected by jugular vein puncture and 33 sera were stored at -80°C for serological tests.

2.2. Serological Assays. For serological analyses, the 33 sera were processed using two ELISA techniques: a homemade ELISA test and IDVET commercial kit and the results were confirmed by two western blot (homemade and LDBIO commercial kit) techniques.

2.2.1. Preparation of the *L. Infantum* Antigen. Antigens were produced from *Leishmania infantum* promastigotes grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma), L-glutamine (Gibco), and penicillin-streptomycin (Gibco). Log phase promastigotes were harvested by centrifugation (14000 g for 15 min at 4°C) and washed three times in sterile phosphate buffered saline (PBS). Cells were counted and adjusted to a concentration of 3.5×10^8 promastigotes/mL and, as previously described [16], and lysed by boiling for 5 min in sample buffer (0.5 M Tris-HCl, pH 6.8, 0.01 M EDTA, 5% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol, and 0.0125% bromophenol blue). Protein concentration (1.5 mg/mL) was determined by the bicinchoninic acid method (Pierce) and antigen was stored at -80°C .

2.2.2. WB Analysis

Homemade Western Blot Kit. SDS-polyacrylamide gel electrophoresis was performed on a 15% polyacrylamide gel with a Mini-Protean II apparatus (Biorad) and 200 μg of antigen was used per gel width with molecular mass proteins standards (standard low range, BioRad) as previously described [17]. Gels were run at 50 V for 3 hours at room temperature.

TABLE 2: Origin and nature of studied dog populations.

Date	Provinces	Localities	Male	Female	Total	Age range*	Feral dogs	Domestic dogs	Veterinary follow-up	Suspected dogs	Dogs without clinical signs
2004–2006	Marrakech	Semlalia	4	2	6	2–4 y	6	0	0	0	6
		Landfill site	43	17	60	18 m–4 y	60	0	0	3	57
		Tadchirt	2	0	2	3–5 y	0	2	0	0	2
		Aoudad	6	0	6	1–6 y	0	6	0	0	6
		Belbakkar	1	0	1	8 y	1	0	0	1	0
		Choufliidi	1	0	1	1 y	0	1	0	0	1
		Amerchich	2	0	2	2–3 y	2	0	0	0	2
		Douar Laaker	1	1	2	20 m–3 y	2	0	0	0	2
		Sansou	2	0	2	20 m–2 y	0	2	0	2	0
		Guenoun	2	0	2	2.5–3 y	1	1	0	1	1
	Al Haouz	Ghmate	22	5	27	4 m–6 y	10	17	2	10	17
		Ourika	2	3	5	2 m–6 y	3	2	1	2	3
		Zinit	3	0	3	3–8 y	0	3	0	0	3
		Ait Moussa	4	0	4	2–3 y	4	0	0	0	4
		Timsal	1	0	1	3 y	0	1	0	0	1
	Chichaoua	Ait Ishaq	1	2	3	3–7 y	0	3	0	1	2
		Ikhruben	6	1	7	2–16 y	1	6	0	2	5
		Tanant	7	1	8	8 m–2 y	2	6	1	1	7
		Foum Jemaa	6	3	9	18 m–4 y	8	1	0	9	0
		Ait Tagalla	5	2	7	8 m–2 y	1	6	0	0	7
2007	Azilal	Ait tkhjet	3	0	3	6 m–5 y	0	3	1	0	3
		Ait Chwarit	1	0	1	2 m	0	1	1	0	1
		Ait Malek	4	0	4	6 m–2 y	0	4	0	0	4
		Tigit	3	2	5	4 m–3.5 y	2	3	1	2	3
		Imadahen	11	3	14	2 m–11 y	6	8	0	4	10
	Makoussa	Makoussa	13	2	15	5 m–10 y	3	12	0	5	10
		Zelguen	6	4	10	8 m–2 y	1	9	0	1	9
		Landfill site	5	5	10	6 m–5 y	8	2	1	1	9
	Al Haouz	Ghmate	9	4	13	6 m–3 y	8	5	4	1	12
		Ourika	6	4	10	5 m–7 y	8	2	2	2	8
	Total (%)		182 (74.9)	61 (25.1)	243 (100)	—	137 (56.4)	106 (43.6)	14 (5.8)	48 (19.8)	195 (80.2)

* m: month; y: year.

After electrophoresis, the proteins were transferred onto a nitrocellulose sheet (Hybond ECL, Amersham Biosciences). The sheets were blocked in PBS containing 5% skimmed milk and 0.2% Tween 20, overnight at 4°C, and then washed five times. The dry membranes were cut and kept refrigerated.

For immunoblotting, two dilutions of sera in PBS, 0.5% skimmed milk and 0.2% Tween 20, were used with total antigen 1/25 and 1/100. The nitrocellulose individual strips were incubated for 3 hours with sera, washed five times in PBS 0.2% Tween 20, and incubated in a 1/300 dilution of alkaline phosphatase-conjugated rabbit anti-canine IgG (PARIS Anticorps, Compiègne, France) for 2 hours. After five washes, the bound proteins were revealed with nitro blue tetrazolium (NBT) and bromo-chloro-indolyl phosphate (BCIP) as substrates. The reaction was stopped with distilled water. Positive and negative controls were included in each procedure. The interpretation was based on the presence of the antibodies to 14 and 16 kDa antigens, which are common to all *Leishmania* species [18].

Commercial Western Blot Kit. Leishmania LDBIO kit (ref. LES-WB12G, Lyon, France) was used according to the manufacturer's specifications.

2.2.3. Enzyme-Linked Immunosorbent Assay (ELISA)

Homemade ELISA Kit. Quantification of antibodies by ELISA was performed according to following procedure adapted from Mary et al. and Correia da Costa et al. [17, 19].

96-well microtiter plates were coated with the previously described antigen diluted at four different concentrations (5, 10, 20, and 50 µg/mL) in PBS, pH 7.2 in a volume of 100 µL. Plates were then incubated for 2 hours at 37°C and overnight at 4°C.

Sera were serially diluted from 1/40 to 1/1280 in PBS, 0.5% skimmed milk, and 0.2% Tween 20 (PBS-T) and incubated on microplates previously coated for 1 hour at 37°C. After three washes, a labelled anti-dog IgG alkaline phosphatase conjugate (PARIS Anticorps, Compiègne, France), diluted to 1/1000 in PBS-T, was added per well and incubated for 1 hour at 37°C. Enzymatic activity was revealed by hydrolysis of *para*-nitrophenyl phosphate for 15 min at 37°C and the reaction stopped by adding K₂HPO₄ 2 M. Plates were read at 405 nm in an ELISA reader (Multiscan ThermoFischer).

Commercial ELISA Kit. The ID Screen Leishmaniasis Indirect (ref. LEISHS-2P, IDVET, Montpellier, France) kit was used according to the manufacturer's specifications.

2.3. Statistical Analysis. Data was analysed by crosstab and logistic regression.

3. Results

3.1. Characterisation of the Dog Population. A total of 243 dogs were clinically examined: 74.9% males and 43.6% domestic type (pets) versus 56.4% stray dogs. Only 5.8% dogs

FIGURE 2: Frequency of each clinical sign displayed by all symptomatic and suspected dogs: (a) looking old; (b) cachexia; (c) anorexia; (d) weakening; (e) dry exfoliative dermatitis and/or periorbital alopecia; (f) ulcers; (g) onychogryphosis; (h) noise at walk; (i) epistaxis; (j) ocular signs (keratoconjunctivitis and uveitis); (k) lymphadenopathy; (l) splenomegaly.

FIGURE 3: Number (Nbr) of clinical signs displayed by all symptomatic and suspected dogs.

were under veterinary follow-up and 19.8% were clinically suspected (Table 2).

In this study, we investigated twelve commonly described clinical cutaneous and visceral CanL signs, as reported in the scientific literature. Gállego [20] confirmed that the most frequent signs are skin abnormalities (dry exfoliative dermatitis, ulcers, periorbital alopecia, and onychogryphosis), but ocular signs (keratoconjunctivitis and uveitis) and lymphadenomegaly are also common. According to Solano-Gallego and Baneth [21], the main clinical signs associated with CanL are skin lesions, lymphadenomegaly, splenomegaly, ocular abnormalities, onychogryphosis, weight loss, and general poor body condition. Carré et al. [22] classified these symptoms to general, visceral, and mucocutaneous signs.

In our results, alteration of general aspect (9.1%) and skin abnormalities (8.2%) were the most common clinical signs, while ocular (1.2%) and visceral (1.6%) signs were very discrete (Figure 2).

Figure 3 shows the association of clinical signs. Most of the infected dogs were oligosymptomatic: 37.5% of symptomatic dogs presented only one sign, 27.1% two signs, 22.9% three signs, and 12.5% four or more clinical signs.

3.2. Serological Results. Firstly, concerning the validation of the homemade western blot protocol, better intensity and

TABLE 3: Seroprevalence of canine leishmaniasis infection in southern Morocco: comparison of commercial and homemade ELISA and western blot tests.

Localities	Dogs clinically suspected (%)	Seropositive dogs (%)			
		ELISA	Western blot		
		Homemade kit	IDVET kit	Homemade kit	LDBIO kit
Landfill site	1/10 (10)	70 (7/10)	(5/10) 50	80 (8/10)	80 (8/10)
Ghmate	1/13 (7.7)	92.3 (12/13)	(7/13) 53.8	92.3 (12/13)	100 (13/13)
Ourika	2/10 (20)	80 (8/10)	(2/10) 20	60 (6/10)	80 (8/10)
Total	4/33 (12.1)	81.8 (27/33)	42.4 (14/33)	78.8 (26/33)	87.8 (29/33)
95% confidence interval	[0.97; 23.2]	[68.6; 94.9]	[25.5; 59.2]	[64.8; 92.7]	[82.1; 93.5]

TABLE 4: Concordance percentage between the different serological kits.

		ELISA	
		Homemade	IDVET
WB	Homemade	88%	60.6%
	LDBIO	94%	54.6%
ELISA	Homemade versus IDVET		60.6%
WB	Homemade versus LDBIO		88%

readability of antigenic bands were found with sera diluted to 1/100 in PBS rather than dilution of 1/25.

Besides, homemade ELISA protocol showed better results when antigen coating concentration was 20 µg/mL in a volume of 100 µL per well (2 µg/well). For the next broader epidemiological studies on prevalence of canine leishmaniasis in all these preprospected areas, we recommend the use of a sera dilution of 1/100. A threshold at 0.24 OD was established after testing 30 negative control sera (mean + 2 SD).

The highest seroprevalence of leishmaniasis infection was found to be, respectively, 81.8% (95% confidence interval (CI): 68.6–94.9) and 87.8% (95% CI: 82.1–93.5), with the homemade ELISA test and LDBIO commercial western blot kit (Table 3).

With the exception of IDVET commercial ELISA kit, all tests revealed a very high prevalence of infection, around 80%. No significant difference (Fisher's exact test, $P > 0.5$) was found between western blot and ELISA results, probably because of high titer of anti-*Leishmania* antibodies within this population of dogs. Among the 29 seropositive dogs, only four were symptomatic (13.8%). As expected, all clinically suspected dogs were both ELISA and western blot seropositive.

The best concordance percentage (94%) was found between LDBIO commercial western blot kit and homemade ELISA test (Table 4). Only two samples out of 33 were negative with both ELISA tests but positive with the western blot kits. The concordance percent between the commercial IDVET and homemade ELISA tests was low (60.6%) where the homemade ELISA test was much more sensitive. However, the significant difference (Fisher's exact test, $P = 0.002$) in sensitivity between the two ELISA techniques may be due to the conservation of sera samples. The IDVET ELISA kit was the last test used when sera had already undergone

several cycles of freeze-thawing. We also cannot exclude a problem of batch or inappropriate conservation of IDVET kit reagents themselves.

Western blot was the most sensitive test and there was no signification difference (Fisher's exact test, $P > 0.5$) between the sensitivity of the commercial versus homemade western blot kits.

Using a crosstab analysis, the results of the Chi-square test showed no significant correlation between clinical signs of disease and seropositivity ($P > 0.05$ and Cramer's $V < 0.70$). Logistic regression was applied to study further possible correlations between the disease, on the one hand, and the locality, age, sex, and type of dogs (domestic or feral), on the other hand. Our results based on P value and Cox and Snell's and Nagelkerke's R -square measures showed no significant correlation between disease and locality (Sig = 0.205), age (Sig = 0.058), sex (Sig = 0.109), or type of dogs (Sig = 0.999).

4. Discussion

CanL constitutes a considerable veterinary challenge, as well as important public health problem, because infected dogs, ill or asymptomatic, act as reservoir hosts for the human disease: sand flies infected by a blood meal taken from infected dogs may, during a later blood meal, infect humans.

With the exception of sporadic cases of human visceral leishmaniasis [14, 15], southern Morocco is currently considered *L. infantum*- and CanL-free area. To provide a better understanding of *L. infantum* epidemiology in southern regions of Morocco, clinical and serological surveys were carried out to establish the presence of *L. infantum* in canine populations.

The western blot analysis is a valuable tool for epidemiological studies on visceral leishmaniasis especially when it is associated, for confirmation, with Elisa test. It provides a significant increase in the sensitivity, because the 14-kD and/or 16-kD antigens are recognized by most part of infected animals or patients (if not all) and show no cross-reactivity [17, 23].

We showed the presence of *L. infantum* in our study area with a high serological prevalence in dogs, around 80%. Therefore, southern Morocco might be highly endemic compared to the lower range seroprevalence (10 to 40%) usually reported in northern Morocco [8, 9] and in the Mediterranean area [24–26].

These unusual high seroprevalence results could be explained by different factors. Firstly, the previous multiple and successive stray dog culling campaigns have been more frequent and regular in the northern populated provinces, well known for leishmaniasis endemicity, rather than in south areas. Secondly, the previous leishmaniasis seroprevalence studies, carried out in the northern Morocco or in European countries around Mediterranean basin, concerned rather pet dogs with veterinary follow-up, while 56.4% (137/243) of animals in our study were stray dogs (Table 2), and among the 43.6% of domestic dogs with owners (pets), only 5.8% have been subjected to veterinary follow-up and possible treatment. Lastly, considering the small sampling size of this preliminary study, an unknown selection bias cannot be completely excluded.

Our results corroborate with Dereure et al. [27] who demonstrated the presence of an autochthonous canine visceral leishmaniasis cycle in the High and Anti-Atlas mountains of Morocco, not far from our study area.

Clinical diagnosis of leishmaniasis can be difficult due to the great variety of symptoms and should, therefore, be confirmed by parasitological, serological, or molecular methods [20]. We showed that all clinically suspected dogs (4/33) were Elisa and western blot seropositive. However, CanL usually shows a high prevalence of subclinical infection [21, 22], commonly explained by the long incubation time of this disease [28] and here supported by the high frequency of asymptomatic dogs (86.2%) we found.

Besides, few human visceral leishmaniasis cases reported in our study area suggest that the dynamic of these southern Moroccan foci of CanL is different from northern ones. This unusual pattern has sometimes been observed in Khemisset province (northern Morocco) where CanL does not sustain human visceral leishmaniasis [29]. CanL and human visceral leishmaniasis distribution can geographically overlap where the incidence of the former is much higher [30].

On the other hand, the distributions of *L. infantum* and *L. tropica* overlap in Morocco. The causative agents of cutaneous leishmaniasis in Sefrou province (northern Morocco) are attributed to both *L. tropica* and *L. infantum* by species-specific ITS1-PCR-RFLP assay [31]. Furthermore, several cases of canine visceral leishmaniasis caused by *L. tropica* have been reported in regions where canine visceral leishmaniasis is caused by *L. infantum* [32].

In our study area, Azilal, Chichaoua, and El Haouz provinces are simultaneously anthroponotic foci of *L. tropica* cutaneous leishmaniasis [33–35]. Previously, some zymodemes of *L. tropica* have been several times found in dogs in anthroponotic foci of *L. tropica* cutaneous leishmaniasis in Taounate and Al Hoceima northern provinces [12, 13] but also in south part (Azilal province) [11]. Consequently, in our study area, we cannot exclude the possibility of visceral leishmaniasis in dogs that could be due to some specific strains of *L. tropica*. Therefore, this study should be continued by the isolation and typing of the strains circulating in all southern CanL foci.

Moreover, concerning the vector, *L. infantum* in Morocco is known to be transmitted by two species of the subgenus

Larroussius, *Phlebotomus perniciosus* and *P. ariasi* [15]. However, our previous entomological investigations in this area (Table 1) showed the presence of three species of subgenus *Larroussius*: *P. ariasi*, atypical morph of *P. perniciosus* [36–39], and *P. longicuspis*.

The vectorial role of *P. ariasi* has been shown in northern Morocco [40], while the epidemiological roles of atypical morph of *P. perniciosus* and *P. longicuspis* are not yet elucidated. Furthermore, *P. papatasi* and some species of the genus *Sergentomyia*, largely spread throughout Morocco and our study area, might also be able to transmit *L. infantum* [41, 42]. Hence, it is essential to identify the still unknown sand fly species involved in the CanL transmission in these southern regions. It could help to understand the reason of low prevalence of human visceral leishmaniasis in these southern CanL foci.

In southern Algeria, human visceral leishmaniasis is known to be sporadic but during the last decade the number of cases has increased significantly whereas entomological investigations showed the absence of sand flies of subgenus *Larroussius* [43].

Regarding canine leishmaniasis control, it remains a difficult task in Morocco because of important stray and feral dog populations, low attendance level to veterinary clinics by the owners of domestic dogs, rare use of insecticide-impregnated dog collars, an absence of proven effective vaccines at affordable cost and chemotherapeutic agents having a limited efficacy for carrier dogs, and a high cost.

For control, the World Health Organization [44] recommends the treatment of human cases, insecticide vector control, and infected dog sacrifice for the control of canine visceral leishmaniasis. So, the elimination of infected or at least symptomatic stray and feral dogs is justified in Morocco. It is an operation integrated into a global strategy of control of canine zoonosis with high impact on public health (especially rabies, echinococcosis, and leishmaniasis), but it is an activity difficult to realize. This suggests the need for a strong collaboration between the veterinary and medical services, the authorities, and the local populations.

To conclude, we reported a high seroprevalence of CanL in southern Morocco, although it is considered, until now, to be free of this infection. Both of our homemade serological kits were validated, are cost-effective, and will be useful for further large-scale epidemiological studies on the animal reservoir of visceral leishmaniasis.

These preliminary results could be a helpful basis for further investigations in these CanL high prevalent foci of southern regions in order to identify the still unknown sand fly species involved in the transmission, to isolate and characterize the circulating *Leishmania* strains, and to understand better the reason of lower prevalence of human visceral leishmaniasis in the southern Morocco.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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