DOGS *L. INFANTUM* INFECTION FROM AN ENDEMIC REGION OF THE NORTH OF TUNISIA: A PROSPECTIVE STUDY

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RESUME

Une cohorte de 917 chiens a été suivie entre 1994 et 1995 dans un foyer de leishmaniose viscérale au nord de la Tunisie. Cette étude a permis d'étudier la démographie de la population des chiens, l'importance de la leishmaniose canine (LC) ainsi que les déterminants de la séropositivité et de la mortalité des chiens. La population des chiens était stable en fonction du temps avec 231 chiens nouvellement recrutés et 218 chiens défaillants par an. La prévalence de la séropositivité était respectivement de 18% et 22.3% en 1994 et 1995 et 90% des chiens étaient asymptomatiques. Parmi 525 chiens négatifs en 1994 et réexaminés en 1995, 78 ont eu une séroconversion témoignant d'une incidence cumulée de 14.74%. D'autre part, 23.47% (27/115) des chiens séropositifs sont devenus négatifs en 1995. L'âge, la présence de symptômes et la densité des chiens étaient indépendamment associés avec la séropositivité. Ces résultats démontrent la difficulté des stratégies de contrôle de la leishmaniose viscérale visant la population des chiens.

Mots clés: *Leishmaniose canine, sérologie, démographie, surveillance, L. infantum, contrôle.*

SUMMARY

A follow-up study of 917 dogs was undertaken between 1994 and 1995 in the focus of visceral leishmaniasis in northern Tunisia. It permitted to assess the demography of the dog population, the importance of canine leishmaniasis (CL) and the determinants of seropositivity and mortality of dogs. Canine population was stable through time with an input of 231 dogs and an output of 218 dogs per year. The prevalence of seropositivity was 18% and 22.3% in 1994 and 1995 respectively and 90% of dogs were asymptomatic. Among 525 negative dogs in 1994 and reassessed in 1995, 78 seroconverted revealing an annual cumulative incidence of 14.74 %. On the other hand, 23.47 % (27/115) of seropositive dogs became negative in 1995. Age, presence of symptoms and density of dogs were independently associated with CL seropositivity. These results demonstrate the difficulty of control strategies of visceral leishmaniasis targeting the dog population.

Key words: *Canine leishmaniasis, serology, demography, follow-up, L. infantum, control.*

INTRODUCTION

Since Nicolle and Compte (1908) first discovered canine leishmaniasis at the Pasteur Institute of Tunis, Leishmania parasites have been isolated from dogs in most of the countries around the mediterranean. Many surveys were carried out in order to estimate the importance of the infection among dogs [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11]. The incrimination of the dog as a reservoir of human visceral leishmaniasis (VL) in Tunisia, was confirmed by the isolation of *L.infantum* Zymodeme MON1 in humans and dogs [12, 13]. Most of canine leishmaniasis (CL) surveys were previously undertaken retrospectively, revealing a seropositivity prevalence ranging widely from 5 to 37% [3, 5, 6, 8, 9, 10, 14, 15, 16]. This retrospective approach, failed to accurately quantify the turn-over of the dog population as well as the evolution of anti-leishmanial antibodies during the course of the infection [17]. Zoonotic visceral leishmaniasis (ZVL) was considered, from a public health perspective, as one of the most important parasitic emerging diseases [18], and control programs based on selective elimination of positive dogs were undertaken, without much success, in different places in the world [19, 20, 21]. This failure would be caused by a lack of understanding of the dynamics of the canine population, importance of re-newel of the dog population in a given VL focus, reasons of input and out put and infectious status of dogs immigrating to or emigrating from a geographic area. These informations are very important for the assessment of degree of mixing between different categories of dogs, risks of CL transmission and parasites dispersal in space. Indeed, field experience suggested that canine population and its infectiousness to sand flies are unstable over time and space and further research to address these issues are still required for the implementation and successof CL control strategies. The present study was undertaken prospectively in Northern Tunisia in order: i) to evaluate the turn-over of the dog population as well as the evolution of serological markers of the canine infection; ii) to estimate the incidence and prevalence of CL; iii) and to explore potential risk factors associated with the infection and mortality of dogs. This information would be very important for the better understanding of the dynamics of the dog reservoir of visceral leishmaniasis and the evaluation of the relevance and feasibility of ZVL control strategy based on selective elimination of positive dogs.

MATERIALS AND METHODS

Type of study and target population

A prospective study was undertaken in three "imadat" (the basic administrative unit) located in the focus of visceral leishmaniasis of Medjez El Bab in northern Tunisia. It involved all the owned dog population that was censussed and included for the follow-up since 1994. The first was urban: Medjez El Bab Ouest (MBO) containing 522 dwellings and 2942 inhabitants, and the remaining were rural: Khniguet Eddhene (KD) with 166 dwellings and 1415 inhabitants, and Bir Elleuch (BE) containing 189 dwellings and 1222 inhabitants.

Data collection procedures

Data were collected by door to door visits to all the dwellings of the study area by the same vet and the health worker of the village who introduced the survey team to the local population. Information regarding age, sex, type of dog (stray, fixed), location (inside/outside the house) and its role (guardian/ linked to sheep) was obtained by interviewing the owner of the dog, who gave his consent to be involved in the study. After filling the questionnaire, every dog was clinically examined by the vet and a blood sample was collected by venepuncture of the forelimb. Aspiration of enlarged popliteal or supra-scapular lymph nodes was also performed at the same time for direct examination and culture of the parasite.

Serologic assays

- ***Indirect Immunofluorescence***

L.Infantum promastigote forms were used as antigen. They were prepared in glass slides and fixed with acetone. Dog sera were serially diluted from 1:50 to 1:6400 and distributed in different wells. After 30 minutes of incubation at 37°C, the slides were washed in PBS and incubated with fluoresceïne conjugated anti dog IgG antibodies. After 30 minutes of incubation, the slides were washed in PBS and examined with an immunofluorescence microscope at Pasteur Institute of Tunis, by the same technician.

- ***ELISA***

Leishmania infantum strain (MHOM/TN/80/IPT1) was used in this study. Promastigotes were grown at 26°C in RPMI 1640 containing 2mM glutamine, 100U/ml penicillin, 100mg/ml streptomycin and

10% of heat-inactivated fetal calf serum. Soluble leishmanial antigens (SLA) were prepared as following : promastigotes were washed four times in cold PBS and resuspended at 10^9 parasites/ml in 100mM tris-HCl, 1mM EDTA (pH: 8.0) with 50mg/ml leupeptin, 50mg/ml aprotinin and 1.6mM PMSF (all from Sigma, Saint Louis, MO). The suspensions were sonicated on ice by six times 10s pulses using sonic disrupter. Cell lysates were centrifuged at 27,000 g for 20min at 4°C and the supernatant collected and recentrifuged at 100.000g for 4h at 4°C. The obtained supernatant was dialysed against sterile PBS, filtered through a 0.2 mm filter (Millex GV, Millipore) and stored at -80°C. Immunoplates (Nunc, Rekilde, Danmark) were coated overnight at 4°C with SLA at a final concentration of 2 mg/ml in 0.1M carbonat/ bicarbonate buffer, pH 9.6. Active binding sites were then blocked by PBS containing 0.1% Tween 20 and 0.5% gelatin (PBS-T-G) for 1h at room temperature. Wells were washed with PBS-T and filled with 100 ml of serial dilutions of dog sera in PBS-T-G (1:100, 1:300 and 1:900). After 2h incubation at 37°C, wells were washed and 100ml of affinity purified goat anti-dog IgG antibodies conjugated with peroxydase (Sigma) diluted 1:2000 in PBS-T-G were added to plate and incubated for 1h at 37°C. After washing 100 ml of orthophenylene-diamine (0.7 mg/ml; Sigma) in 0.1M citrate buffer pH 5.0 containing 0.01% H_2O_2 were added. The absorbance of each sample was recorded at 492 nm in an automated ELISA reader (Titerteck Multiscan, Helsinki, Finland). The values obtained by ELISA with a given serum sample were compared with the mean value obtained with 30 negative control sera examined under the same conditions. The cut-off value for normal sera was defined as mean Optical Density (OD) + 2 Standard Deviation (SD).

Clinical criteria

Signs of CL such as depilation, cutaneous ulceration, onycogryphosis, enlarged lymph nodes (popliteal and suprascapular) and weight loss were recorded by the vet. Asymptomatic dogs were those that showed the absence of any clinical sign. Dogs with two or three signs and more, were classified as oligo and poly symptomatic respectively.

Criteria of positivity

A dog was considered positive by serology if both ELISA and IFAT tests were positive.

Statistical analysis

Data were stored and analysed using Oracle 7 and BMDP software. The age (≤ 3years, > 3years), sex, type of dog (unchained or chained), location (rural or urban), being symptomatic for canine leishmaniasis, function (guardian of the house/linked to sheep), and the density of dogs in the dwelling (≤ 3 dogs, >3 dogs) were explored as potential risk factors for the canine seropositivity (positive test for ELISA and IFAT) and mortality. Fisher's exact χ^2 test and crude Odds ratios were used to assess the statistical associations between the infection and mortality of the dog and potential risk factors. Stepwise logistic regression models were performed in order to evaluate the weight of the risk factors by assessing their adjusted odds ratios.

RESULTS

Description of the study canine population

The canine population investigated came from the ZVL focus of Medjez El Bab located in the governorate of Beja in northern Tunisia and was composed of 917 dogs with owners. The first canine survey was conducted between June and September 1994 and the second one in 1995 during the same season. Table I shows some characteristics of the canine population in the study area. A predominance of male dogs, 60% of dogs are unchained guard dogs and the majority live in close contact with humans inside compound.

Table I: Characteristics of the canine population during the follow-up period In the study area.

	1994		1995		
	No	**%**	**No**	**%**	**p**
Sex					
Male	611	66.78	601	86.61	NS
Female	304	33.22	275	31.39	NS
Status					
Chained dog	374	40.79	451	51.60	<0.0001
Unchained dog	543	59.21	423	48.40	<0.0001
Location					
Inside the compound	841	92.97	825	96.04	0.004
Outside the compound	64	7.03	34	3.96	0.005
Role					
Guard of the house	823	90.44	805	92.42	NS
Guard of the sheep	71	7.80	34	7.23	NS
Other	16	1.76	3	0.34	0.004
Total	**917**		**876**		

Figure 1 illustrates the age distribution of the dogs in the different sectors of the study. Clinical examination of the dogs revealed that more than 90% were asymptomatic and only ~ 7 to 10% were symptomatic among dog population in both measurements ($p < 0.0001$) (Table II). Of the sera examined, 165 (18%) and 195 (22.3%) showed a positive reaction according to the ELISA and the IFAT (titre ≥1/50) simultaneously, in 1994 and 1995 respectively.
The second measurement undertaken in 1995, allowed to assess the importance of the turn-over of canine population. It indicated that, although the size of the population was almost stable over one year, more than 25 % of the dogs are not the same.

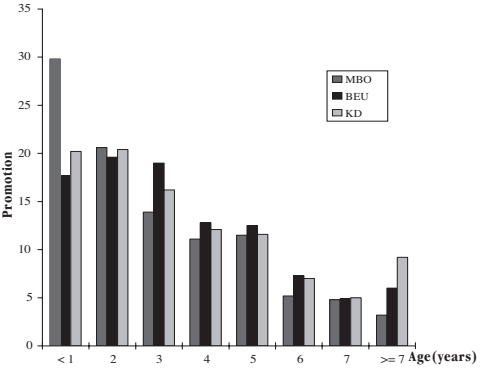

Figure 1. *Age structure of the canine population in the study area*

Table II: Clinical description and prevalence of the canine seropositivity during the follow-up period.

	1994		1995		
	No	**%**	**No**	**%**	**P**
Clinical description					
Asymptomatic	846	93.06	789	90.17	0.026
Oligo-symptomatic	47	5.17	69	7.88	0.020
Poly-symptomatic	16	1.76	17	1.94	NS
Seropositivity					
ELISA +	348	38.28	248	28.34	< 0.0001
IFAT +	168	18.48	195	22.28	0.046
ELISA and IFAT+	165	18.00	195	22.26	0.025

Indeed, as shown in table III, the output of dogs is mainly caused by death of various reasons including presumptive signs of leishmaniasis, while the input is caused by newborns and dogs introduced to the area from other sectors.
In addition, among 525 negative dogs by both techniques in 1994, 78 seroconverted to positives by IFAT and ELISA leading to a yearly cumulative incidence of 14.74%. Conversely, among 115 dogs found positive in 1994 and reassessed in 1995, 27 (23.47%) were negative for leishmanial antibodies (Table III).
Factors significantly associated with canine mortality and seropositivity are detailed in table IV, using bivariate analysis and multivariate regression models. The analysis of the association between canine leishmaniasis seropositivity and potential risk factors demonstrated that age (>3 years) and the presence of CL symptoms were the most consistently associated factors with the former ($p<0.001$), either in the bivariate analysis or in the stepwise logistic regression model. The high density of dogs present in the dwelling during the survey (presence of more than 3 dogs in the dwelling) was also significantly associated with this outcome. On the other hand, only the urban location and the presence of signs appeared to be risk factors for canine mortality while being linked to sheep was a protective one.

Table III : Turn-over of the canine population and reasons of input and output during the study period.

Input of new dogs		**Assumed cause of output of dogs**		
New born	155	Death		181
		* Canine leishmaniasis	99	
		* Accident	21	
		* Unknown reason	61	
Dogs brought from outside the focus	73	Given to families outside the focus		24
		Unspecified reason		13
Total	**228**			**218**

Table IV: Determinants of seropositivity and mortality in the study area during the follow-up period using logistic regression model.

Factor	Coefficient	Standard Error	Coeff./S.E	Adjusted Odds Ratio
Canine Seropositivity in 1994				
Age > 3 years	0.46705	0.09225	5.063	1.595
Symptomatic	0.44327	0.10200	4.348	1.558
Presence of more than 3 dogs	0.19341	0.09067	2.133	1.213
Canine Seropositivity in 1995				
Age > 3 years	0.49398	0.08845	5.585	1.639
Symptomatic	0.45777	0.09366	4.888	1.581
Presence of more than 3 dogs	0.21650	0.08656	2.501	1.242
Canine Mortality				
Urban location	0.55848	0.08271	6.753	1.748
Symptomatic	0.39213	0.09572	4.097	1.480
Linked to sheep	-0.44237	0.19610	-2.256	0.643

DISCUSSION

The prevalence of the canine seropositivity from the two measurements undertaken in 1994 and 1995 during a follow-up study, ranged from 18 to 22 %, without a significant difference between the urban and rural settings. It was in the range reported from other studies around the Mediterranean basin, although using different cut-off IFAT titres for positivity. For instance the IFAT threshold titre for a positive test was: 1/40 and 1/128 in other studies [4, 5, 6, 7, 8, 9, 10, 11, 13, 14, 15, 18, 19, 20, 22, 23].

For ELISA tests, some authors use the positive to negative ratio with a threshold varying from 2 to 3 and in other ones a constant cut-off point for positive cases was chosen [4, 6, 10]. A standardization of diagnostic techniques is highly recommended to allow comparability between the different studies.

These results are in agreement with Abranches et al.[3] and in contradiction with Jambou et al. [4], who found a higher prevalence in the peri-urban area. Abranches et al. [3] argued that the higher prevalence of the infection in the rural area could be explained by the zoophilic preferences of the vector. Surprisingly, no significant difference was detected with regard to the serological status according to the type of dog (unchained, chained dog). This finding suggests that both types of dogs are equivalently exposed to the infection, and that transmission occurs mainly inside and around the dwellings. A difference according to the sex was not found in the present study confirming previous findings [23, 24].

On the other hand, canine seropositivity was consistently associated to the age of the dog and the presence of clinical signs of the disease and to a less extent the high density of dogs in the dwelling during the two measurements undertaken in 1994 and in 1995 using multivariate analysis. Hasibeder et al.[25] demonstrated that the force of infection in dogs is determined by the contact rates between dogs and sand flies and the number of infectious flies per dog [26]. Unfortunately, no information regarding the density of the vector was collected within the present survey. The increasing prevalence with age, shown in figure 2, corroborates the results of previous studies and can be explained by a relatively long life span of the dogs exposed to infection [1, 24, 28]. Not surprisingly, the asymptote of the prevalence of the infection did not reach 100% among the aged dogs, because of the proportion of dogs that had regressed to the pool of susceptibles, significantly high in the present survey (23.4%) and the increased mortality related to the infection [10].

The follow-up study demonstrated that, although stable with time, the size of canine population is subject to an important turn over and that almost

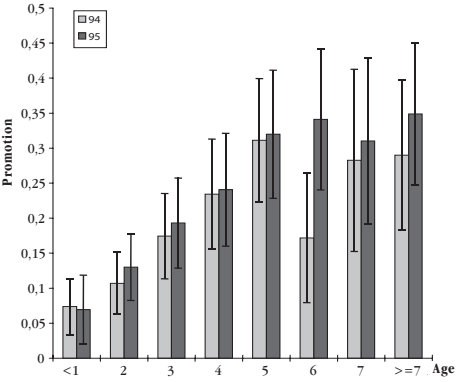

Figure 2. *Age prevalence of seropositivity in the canine population in 1994 and 1995.*

the total population would be completely renewed every 4 years. Furthermore, serologic markers of the infection are far from being constant over time. Indeed in one year period, almost 1 out of four positive dogs lost any trace of detectable antibodies by the ELISA and IFAT tests.

These findings clearly indicated the difficulty of any control strategy of visceral leishmaniasis targeting the dog reservoir in Tunisia and in similar countries for the following reasons: i) the rapid turn-over of the canine population which seems to be completely renewed every 4 years. This will significantly reduce the coverage of any control method targeting this population, ii) the serologic available tests are not able to identify the total infected dogs in a valid and accurate way [29, 30], iii) Given that we develop the most reproducible test for the detection of the infection, it is nearly impossible to reach the total canine population particularly in Mediterranean rural areas [31], iv) an important proportion of the dog population, impossible to identify a priori, would "recover" in a relatively short period [32], iv) unlike wild animals, dogs are usually needed by humans and the size of their population is usually regulated by humans' behaviour [32, 33, 34]. Therefore, reducing the size of the dog population by selective elimination of positives for instance, will result in the rapid replacement of the proportion reduced which might exacerbate CL by introducing a naïve canine population to the VL focus and increase the epidemic risk among the human host.

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