

CANINE LEISHMANIASIS DUE TO *LEISHMANIA INFANTUM* MON-1 IN NORTHERN MOROCCO

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Summary :

A seroprevalence study of canine leishmaniasis was carried out in five provinces in northern Morocco: Taounate, Al Hoceima, Zouagha Moulay Yacoub, Chefchaouen and Ouezzane. 55 localities have been concerned and a total of 1,013 dogs were screened, which represents almost 100 % of the canine census. Of the screened dogs: 87 showed antibody titer ≥ 100 when tested by IFAT (seroprevalence of 8.6 %) and were distributed in 83 asymptomatics (without clinical symptoms) and four symptomatics (with one or several symptoms of leishmaniasis) with important variations according to the locality. Relative frequency of asymptomatic dogs was observed (8.2 %), and the seroprevalence increased in middle altitude (500 m < altitude < 1,000 m) and high altitude ($\geq 1,000$ m). Parasites isolated from dogs were identified as *L. infantum* MON-1 by isoenzyme profile and RsaI digestion.

KEY WORDS : Canine leishmaniasis, seroprevalence, fluorescence antibody test, northern Morocco.

Résumé :

LEISHMANIOSE CANINE DUE À *LEISHMANIA INFANTUM* MON-1 AU NORD DU MAROC
Une étude de la séroprévalence de la leishmaniose canine a été réalisée dans cinq provinces au nord du Maroc : Zouagha My Yacoub, Taounate, Al Hoceima, Chefchaouen et Ouezzane. 55 localités ont été étudiées et un total de 1013 chiens a été testé. La sérologie a été effectuée par immunofluorescence indirecte et un titre supérieur ou égal à 100 était considéré comme positif. Parmi les chiens testés, 87 ont montré un taux d'anticorps supérieur ou égal à 100, d'où une séroprévalence de 8,6 %. On a aussi remarqué une variation de séropositivité selon les localités même les plus proches et situées à la même altitude. De plus, nous avons noté la fréquence relative des chiens séropositifs asymptomatiques, 83 chiens soit une fréquence de 8,2 %. Une variation de la séroprévalence en fonction de l'altitude a été constatée, en effet plus l'altitude est élevée plus la séroprévalence est importante. A partir des chiens infectés, on a pu identifier *L. infantum* MON-1 par typages isoenzymatique et moléculaire.

MOTS CLÉS : Leishmaniose canine, séroprévalence, test d'immunofluorescence, nord du Maroc.

INTRODUCTION

The leishmaniasis parasites are obligatory intracellular protozoans which cause human cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL). The distribution of the disease is widespread in intertropical area with high degree in North Africa, Europe and Asia where they constitute an important public health problem (Ashford *et al.*, 1992; Neoumine, 1996). Visceral leishmaniasis is a zoonosis and an anthroponozis with worldwide distribution. Canine visceral leishmaniasis caused by *Leishmania infantum* is endemic throughout the mediterranean basin. Since Nicolle & Comte (1908) first discovered canine leishmaniasis, parasites have been isolated from dogs in most of the countries around the Mediterranean sea, where the prevalence of infection has fluctuated

since the second world war (Bettini & Gradoni, 1986), and dogs are important reservoir hosts of *L. infantum*. In Morocco, the first case of human visceral leishmaniasis (Kala-azar) was recognized by Klippel & Monier-Vinard (1922). Natural canine leishmaniasis was first reported by Jaume (1932). Since this first discovery, there has been no evaluation of the extent of visceral leishmaniasis and very limited information on the epidemiology of the disease has been available. Research on canine leishmaniasis has been widely neglected although it is generally understood that dogs serve as a constant source of infection for the vectors, phlebotomine insects (Kirms *et al.*, 1987).

In this decade, an increasing number of human visceral leishmaniasis has been observed in Morocco: 216 cases going from 1957 to 1989 against 138 in five years going from 1990 to 1994. This infection reached zones which have been until now unharmed and other zones where only cutaneous leishmaniasis was detected (Maaroufi *et al.*, 1995). Indeed, to consider the real importance of dog's infection, a seroprevalence study was carried out in northern Morocco in five provinces: Zouagha My Yacoub, Taounate, Al Hoceima, Chefchaouen and Ouezzane.

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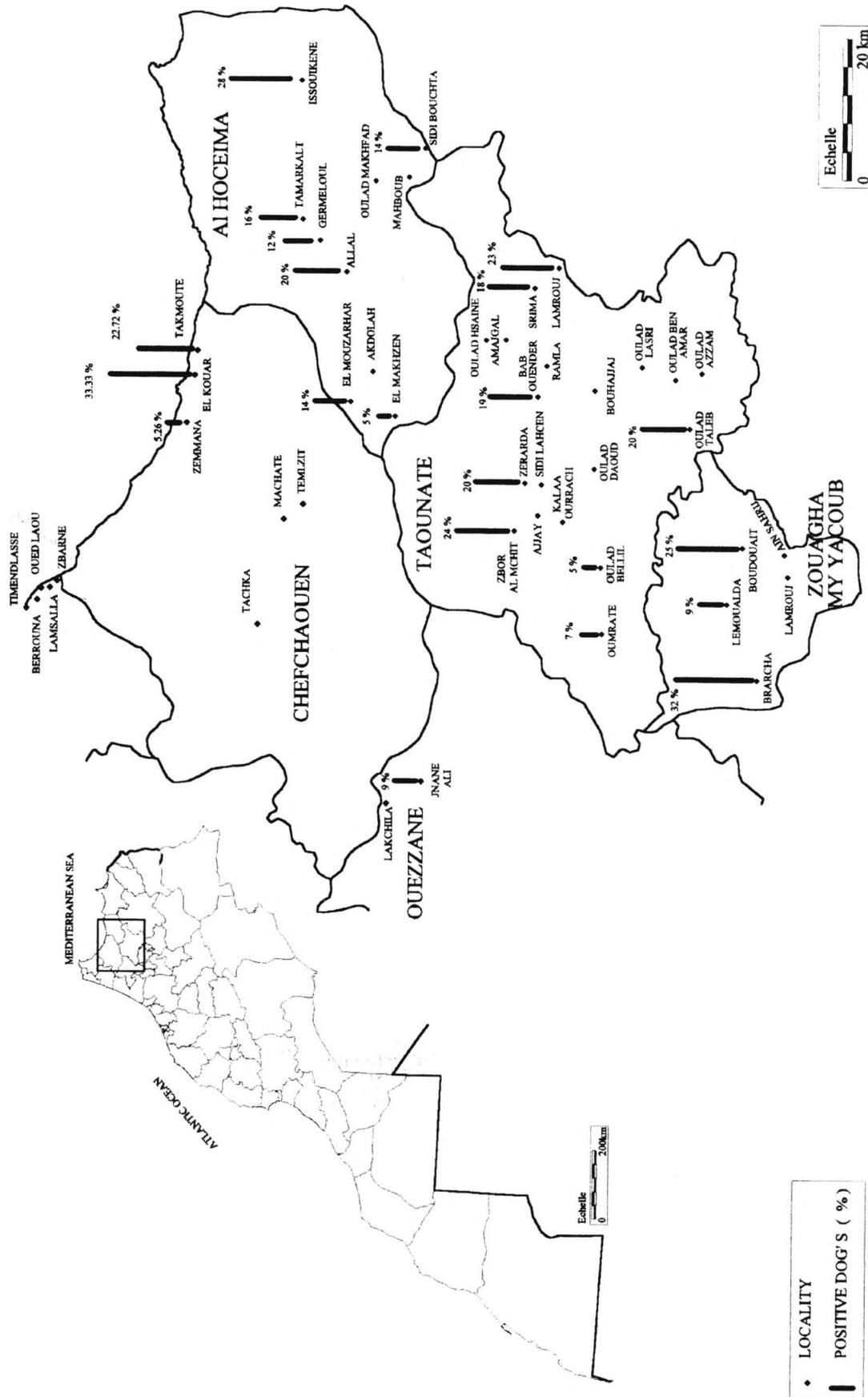


Fig. 1. – Results of canine leishmaniasis in five provinces in northern Morocco.

MATERIALS AND METHODS

GEOGRAPHICAL AREA

The study was carried out in five provinces in northern Morocco (Rif region): Zouagha My Yacoub, Taounate, Al Hoceima, Chefchaouen and Ouezzane.

It is a mountainous region with variable relief and therefore, it is common to find a wide range of vegetation and bioclimatic variation. Our study concerned 55 localities (Fig. 1) where the altitude vary from 0 m to 1,500 m and in which two bioclimatic zones appear: the semi-aride zone comprises altitudes between 300-600 m with moderate winter and precipitations between 400-800 mm (throughout Zouagha Moulay Yacoub to Taounate), and 900-1,000 mm in Taounate. The sub-humide zone include two substages: moderate (Taounate) and fresh (Ketama: province of Al Hoceima) with precipitations between 1,000-1,400 mm per year, and Bab Berred (province of Chefchaouen) (altitude between 900-1,200 m with rainfall of 1,300 mm per year). From Ketama to Targhist (province of Al Hoceima), the rainfall average was 400-500 mm per year (Targhist 450 mm) and reach 330 mm at Al Hoceima in the sea level (Fig. 1).

The vegetation was also variable with cork oak, thuya and holm oak.

SAMPLING

Between January and November 1995, with assistance of local, civil and health authorities, gatherings of dogs were requested in provinces of Taounate, Al Hoceima, Zouagha My Yacoub, Chefchaouen and Ouezzane. Kept dogs per province and positive dogs were represented in Table II. After muzzling dogs, blood was extracted from the jugular vein and serum was separated then stored in a -20°C freezer for IFAT testing. Furthermore, dogs presenting symptoms of leishmaniasis (weight loss, ulcerated lesions, skin desquamation, loss of skin hair, an increase in nail length, etc.) were aspirated in the lymph nodes for NNN culture and PCR. Previously, interviews with dog owners provided information about the number of kept dogs, their name, age, presence or absence of clinical symptoms and the name and adress of these owners.

Punctures of lymphatic nodes were used to make preparations on slides which were stained with Giemsa and examined by standard light microscopy for amastigotes searching and cultures.

IFAT

The immunofluorescence assay (IFAT) was carried out as described by Pinelli *et al.* (1994) using as antigen promastigotes of *Leishmania infantum* (MHOM/TN/

80/IPT-1) MON-1. Dog's sera were tested at a series of 2-fold dilutions from 1/50 to 1/12,800, and titers ≥ 100 were considered positive. Detection of anti-*Leishmania* antibodies in canine sera was assayed using an FITC-conjugated anti-dog IgG.

PCR AND ISOENZYME CHARACTERIZATION

We used PCR for the detection and identification (PCR-RFLP) of *Leishmania* parasites using primers from the small sub-unit ribosomal gene (Van Eys *et al.*, 1992). Briefly, DNA of parasites was extracted out of the lysed samples (lymphatic node puncture) with phenol/chloroform and ethanol precipitated (Sambrook *et al.*, 1989). The amplified product of 650 basepair was digested with 10 units RsaI, for PCR-RFLP, and 20 μl of samples were visualized by 2 % agarose gel electrophoresis.

The isolated strains were characterized by isoenzyme electrophoresis in starch gel according to Moreno *et al.* (1986) using a panel of 15 enzymes: MDH, ME, G6PD, 6PGD, ICD, NP1 and NP2, PGM, DIA, GOT1 and GOT2, MPI, GPI, FH, GLUD. In each case ((PCR-RFLP) and isoenzyme characterization), results were compared to WHO reference strains of *L. infantum* (MHOM/TN/80/IPT-1), *L. tropica* (MHOM/SU/74/K27), and *L. major* (MHOM/SU/73/5ASKH).

RESULTS

A total of 1,013 canine sera were collected in our investigation. They were screened by IFAT test and sera with titers ≥ 100 were considered positive according to Rioux *et al.*, 1986 and Davoust *et al.*, 1994. Dogs with titer 50 were considered doubtful and will be followed serologically.

Among examined sera, 87 out of 1,013 were positive with antibody titers between 100 and 12,800 and were distributed in two groups:

- 83 asymptomatics (without any clinical symptoms),
- four dogs with one or several symptoms of leishmaniasis.

876 sera were negative, 26 out of 876 were symptomatics (parasitic infections with similar symptoms to those of canine leishmaniasis) and 50 were doubtful with titer 50 (Table I).

This serology showed variation between areas (Table II) which were 7.8%, 14.7%, 13.2%, 5% and 2.2% respectively in Taounate, Al Hoceima, Zouagha Moulay Yacoub, Chefchaouen and Ouezzane. Inside each province, important differences in serology were noted: 0 to 32% in Zouagha My Yacoub, 0 to 24% in Taounate, 0 to 28% in Al Hoceima and 0 to 33,33% in Chefchaouen.

Clinical evaluation	Titers			Total
	< 50	50	≥ 100	
Without symptoms	850	48	83	981
With symptoms	26	2	4	32
Total	876 (86.5 %)	50 (4.9 %)	87 (8.6 %)	1,013 (100 %)

Table I. – Comparison of clinical evaluation with titers of canine sera.

Province	Kept dogs	Positive dogs
Taounate	488	38 (7.8 %)
Al Hoceima	170	25 (14.7 %)
Zouagha Moulay Yacoub	91	12 (13.2 %)
Chefchaouen	218	11 (5 %)
Ouezzane	46	1 (2.2 %)

Table II. – Results of canine leishmaniasis in the five provinces.

Nivel = 0/m	500 m		
	Altitude ≤ 500 m	< Altitude < 1,000 m	Altitude ≥ 1,000 m
Total dogs/+ 125/8 (6 %)	Total dogs/+ 502/40 (8 %)	Total dogs/+ 229/24 (10.5 %)	Total dogs/+ 157/15 (9.5 %)

+ : Positive.

Table III. – Variation of seroprevalence with altitude.

Indeed, in the locality of Brarcha (Fig. 1) where 32 % of dogs were seropositive, two cases of human leishmaniasis were reported in 1995. The same were observed in other localities as Issouikene, Bab Ouender and Allal (Fig. 1). Furthermore, there are some districts where neither human leishmaniasis nor positive dogs were reported. Seroprevalence of canine leishmaniasis was relatively elevated in high (≥ 1,000 m) and middle altitude (500 m < altitude < 1,000 m) than in low altitude (≤ 500 m) and nivel = 0 (Table III).

Among ten dogs ponctionated, seven dogs were positive by PCR, six strains of *Leishmania* were isolated by culture method, of which one was positive through direct examination. RsaI digestion of the amplified product of the seven samples gave profile similar to *L. infantum*.

The six isolates (MCAN/MAR/95/IPM96, MCAN/MAR/95/IPM1285, MCAN/MAR/95/IPM263, MCAN/MAR/95/IPM77, MCAN/MAR/95/IPMG1, MCAN/MAR/95/IPMBR33) were identified as *L. infantum* MON-1 by isoenzyme analysis.

DISCUSSION

Seroprevalence study of canine leishmaniasis in northern Morocco in provinces of Zouagha My Yacoub, Taounate, Al Hoceima, Chefchaouen and Ouezzane have shown seropositivity variation between localities with an average of 8.6 %. Similar studies of canine leishmaniasis were led in other countries around the mediterranean sea: Ben Said *et al.* (1992) reported a rate of 6.03 % in Enfidha (central region of Tunisia). In France, prospective studies of canine leishmaniasis carried out in the department of Alpes-Maritimes between 1983 and 1993 have shown variations of 0 to 24 % of positive dogs with an average of 10 % (Marty *et al.*, 1994). Prevalence of canine leishmaniasis in northern Israel at wadi Hamam was evaluated at 5 % (Jaffe *et al.*, 1988). Also in Spain, Amela *et al.*, (1995) reported a prevalence of 5.25 % of canine leishmaniasis in the Madrid autonomous region and another dog's prevalence of 5.1 % was reported in Castellon (Arnedo *et al.*, 1994).

The proportion of dogs with antibody titers below the cut-off titer (100) was 4.9 % (Table I). Since our regions are endemic, this percentage could be caused by several factors: initial stages, latent forms of the diseases; or non specific cross reactions. The development of cellular immunity in dogs may be the origin of latent form of the disease as has been described by Cabral *et al.*, 1992; Pinelli *et al.*, 1994. The high proportion of dogs with antibodies ≥ 100 that are asymptomatics may be caused at an initial stage of the disease (Abranches *et al.*, 1991), but their epidemiological importance is greater because they are potentially infectious for sand flies (Alvar *et al.*, 1994).

This study has also demonstrated that a small number of dogs was sufficient for transmission of the disease, contrary to other studies which postulated that the transmission is significant for infecting phlebotomes if animal's proportion was considerably high (> 20 %) (Chable-Santos *et al.*, 1995). This is the case of two localities: Sidi Bouchta and El Mouzarhar where one reported positive among seven dogs and two cases of human visceral leishmaniasis were enregistred in 1995. In some areas, it appears that infected dogs have an impact on the epidemiology of human leishmaniasis. Evidence for this is provided by a coincidence of elevated pourcentage of canine leishmaniasis with cases of human leishmaniasis in localities as Issouikene, Boudouait, Ouled taleb and Brarcha where four human visceral leishmaniasis were enregistred in 1994. The same thing was observed in Pakistan where a study has demonstrated a relation between canine disease and human visceral leishmaniasis (Rab *et al.*, 1995). In addition, seroprevalence of canine leishmaniasis was relatively elevated in high (≥ 1,000m) (9.5 %) and

middle altitude (500 m < altitude < 1,000 m) (10.5 %) than in low altitude (\leq 500 m) (8 %) and nivel = 0 (6 %) (Table III).

Using a combination of molecular method (PCR-RFLP) and isoenzyme electrophoresis, six isolates were identified (four from symptomatic dogs and two from asymptomatic dogs) as *L. infantum* MON-1 from liqueur of lymphatic nodes punctures of infected dogs. Our results confirm those of other studies, which previously reported the existence of the species *L. infantum* MON-1 in the mediterranean basin (Bettini & Gradoni, 1986). The same species is responsible of human visceral leishmaniasis in Morocco (Guessous-Idrissi *et al.*, 1997 & Maaroufi *et al.*, 1995) and the reservoir hosts are dogs. In view of this results, a control strategy seems necessary for fighting against the reservoir of visceral leishmaniasis in regions of Al Hoceima and Zouagha Moulay Yacoub where the incidence of the disease is relatively high. The more effective and feasible strategy for this is the prevention of the disease in dogs in order to interrupt the domestic cycle of zoonotic visceral leishmaniasis by developing a vaccine that protect dogs from developing parasitemia or cutaneous infection, and from becoming reservoir hosts for the parasite, but since the vaccine does not exist, the only reasonable strategy is identifying and killing infected dogs as it was proposed by Tesh, 1995.

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