

USE OF PCR, IFAT AND *IN VITRO* CULTURE IN THE DETECTION OF *LEISHMANIA INFANTUM* INFECTION IN DOGS AND EVALUATION OF THE PREVALENCE OF CANINE LEISHMANIASIS IN A LOW ENDEMIC AREA IN TUNISIA

CHARGUI N.*, HAOUAS N.*, GORCHI M.*, LAHMAR S.**, GUESMI M.**, BEN ABDELHAFIDH A.***, MEZHOUD H.* & BABBA H.*

Summary:

The aim of this study was to assess the use of parasitological, serological and molecular methods for the detection of *Leishmania* infection in blood of 67 dogs and to investigate the prevalence of canine leishmaniasis (CanL) in Kairouan (central Tunisia), an area known to be of reduced endemicity and has not been studied since 1973. Veterinarians clinically examined all dogs, and the titer of anti-*Leishmania* antibodies was determined by indirect immune-fluorescence antibody test. The presence of *Leishmania* was performed by PCR and *in vitro* culture. IFAT was positive in 12 % of dogs and promastigote form of the parasite was isolated by *in vitro* culture from only 4.5 % of them. However, DNA of *Leishmania* was detected by PCR in 20.9 % of dogs. PCR was more sensitive than IFAT ($p = 0.004$) and *in vitro* culture ($p < 10^{-5}$). A prevalence of 21 % was found in Kairouan, which is significant high ($p < 10^{-3}$) when compared to that of thirty years ago. This state is in correlation with the increase in other Mediterranean countries. Furthermore, 50 % of positive dogs were asymptomatic. Preventive measures must be taken against these dogs as for symptomatic ones since their role in the transmission of the infection to vectors has been proven.

KEY WORDS : canine leishmaniasis, prevalence, Tunisia, serology, PCR, *in vitro* culture.

Résumé : APPLICATION DE LA PCR, DE L'IFI ET DE LA CULTURE *IN VITRO* DANS LA DÉTECTION DE L'INFECTION PAR *LEISHMANIA INFANTUM* CHEZ LE CHIEN ET ESTIMATION DE LA PRÉVALENCE DE LA LEISHMANIOSE CANINE DANS UNE RÉGION DE FAIBLE ENDÉMICITÉ EN TUNISIE

Le but de cette étude était, d'une part, d'évaluer les techniques de PCR, d'immunofluorescence indirecte (IFI) et la culture *in vitro* dans le diagnostic de l'infection par *Leishmania infantum* dans le sang de 67 chiens et, d'autre part, d'estimer la prévalence de la leishmaniose canine (LCan) à Kairouan, une région d'endémicité réduite en Tunisie et non étudiée depuis 1973. Les chiens de l'étude ont été cliniquement examinés par les vétérinaires pour des signes évoquant la LCan. La présence de *Leishmania* a été évaluée par PCR et par culture *in vitro*. L'ADN de *Leishmania* a été détecté par PCR chez 20.9 % des chiens alors que la forme promastigote des *Leishmania* a été isolée par culture chez 4.5 %. Les anticorps anti-leishmaniens ont été détectés par IFI chez 12 % des chiens. La technique PCR était plus sensible que l'IFI ($p = 0,004$) et la culture *in vitro* ($p < 10^{-5}$). Une prévalence de la LCan de 21 % a été retenue à Kairouan qui est significativement plus importante ($p < 10^{-3}$) que celle estimée dans d'autres travaux. En outre, 50 % de chiens positifs étaient asymptomatiques et prouvés être capable de transmettre le parasite aux vecteurs. Ainsi, des mesures préventives doivent être prises contre aussi bien les chiens symptomatiques que les chiens asymptomatiques.

MOTS CLÉS : leishmaniose canine, prévalence, Tunisie, IFI, PCR, culture *in vitro*.

Canine infection with *Leishmania infantum* is important as a cause of disease in dogs and as a reservoir for human leishmaniasis. Accurate and rapid detection of canine leishmaniasis (CanL) is of great importance to prevent transmission. Clinical diagnosis is difficult, due to variable symptomatology. Furthermore, infected dogs can be asymptomatic and develop the disease in many months or years (Dedet, 1999; Ferrer, 1999). Infected dogs have an important role in the transmission of the disease, mainly by symptomatic dogs even with healthy skin, and also by asymptomatic ones (Rioux *et al.*, 1979; Alvar *et al.*,

1994; Dye, 1996). In Tunisia, CanL is due to *L. infantum* (Kinetoplastida: Trypanosomatidae) and transmitted by the bite of infected females of *Pblebotomus (Larroussius) perniciosus*. Few studies have investigated CanL in Tunisia (Dedet *et al.*, 1973; Ben Said *et al.*, 1992; Chargui *et al.*, 2007), consequently, there has been a lack of information about CanL in the region of Kairouan since 1973. Furthermore, all previous studies have been performed by serological methods. Several studies suggesting that the rate of infection is higher than the figures found by serological investigations (Lachaud *et al.*, 2002; Leontides *et al.*, 2002; Oliva *et al.*, 2006). Several molecular methods had been developed for the detection of *Leishmania* (Van Eys *et al.*, 1992; Ashford *et al.*, 1995; Spanakos *et al.*, 2002). Though PCR was already used in the diagnosis of human leishmaniasis in Tunisia (Chargui *et al.*, 2005), this is the first study to investigate the prevalence of CanL with molecular methods compared to serological and parasitological ones.

* Laboratoire de Parasitologie-Mycologie, Faculté de Pharmacie, 99UR/08-05, 1, rue Avicenne, Monastir, Tunisia.

** Service de Parasitologie, École Nationale de Médecine Vétérinaire, 2020 Sidi Thabet, Tunisia.

*** Service de Production Animale, El Alaâ-CRDA Kairouan, Tunisia.

Correspondence: Pr Babba Hamouda.

Tel.: +216 73 44 86 66 – Fax: +216 73 46 18 30.

E-mail: hamouda.babba@gnet.tn

MATERIAL AND METHODS

CANINE POPULATIONS

67 household dogs, but left outdoors in the day, were randomly collected between June and July 2005. All dogs tested lived in a rural district of Kairouan (ElAlaa) known as a reduced endemic zone in central Tunisia (Dedet *et al.*, 1973). This region is belonging to the arid bioclimatic stage. In a recent study, a codominance of subgenus *Larroussius* and *P. papatasi* species of *Phebotomus* vector was observed in central Tunisia (Ghrab *et al.*, 2006). Human visceral leishmaniasis is known to be detected occasionally in the central Tunisia (Ayadi *et al.*, 1991; Besbes *et al.*, 1994). However, an important number of cases emerged recently in this region (Ben Salah *et al.*, 2000). Tested dogs were examined for clinical signs of the disease, including dermatological lesions, ocular changes, lost of weight, apathy, lymph node and spleen enlargement. Venous blood samples were taken from the foreleg. After blood centrifugation, sera were used in serological test and buffy coats were tested by PCR and *in vitro* culture.

SEROLOGICAL STUDY

Serum samples were analyzed for antibodies to *Leishmania* spp. by the indirect immunofluorescence antibody test (IFAT). IFAT was performed using a standard described previously protocol (Chargui *et al.*, 2007). In brief, cell culture-derived promastigote forms of *Leishmania infantum* MON-1 MHOM/FR/78/LEM75 served as antigen. Sera were screened at dilutions of 1:20 to 1:2560. An anti-immunoglobuline canine (anti IgG dog) coupled to Fluoline was used as conjugate. The threshold dilution for a positive test was 1:80. The high specificity of this test for screening the prevalence of CVL has been already confirmed in our previous study (Chargui *et al.*, 2007).

IN VITRO CULTIVATION

Buffy coats were inoculated on blood agar base containing 10 % rabbit blood and Gentamicin 40 mg/ml. Cultures were examined at weekly intervals for one month. Isoenzymatic identification was performed in our laboratory for positive cultures according to Rioux *et al.* (1990).

PCR

PCR reactions were performed as described previously (Chargui *et al.*, 2007). Briefly, DNA extraction was carried out on buffy coat samples in lysis buffer (0.5 % Tween 20, 0.5 % Nonidet P40, 10 mM NaOH, 100 mM Tris HCl pH 7.6) and of Proteinase K (900 µg/ml). The

samples were incubated at 56°C for two to 12 hours followed by phenol/chloroform extraction and ethanol precipitation. The PCR with primers LEI70R (5'-CGCGGTGCTGGACACAGGGTA-3') and LEI70L (5'-CGCAACCTCGGTTCGGTGTG-3'), which targets a region of the SSU rRNA gene, was performed as described by Spanakos *et al.* (2002) and previously found to be more sensitive than PCR with R332/221 primers in the detection of *Leishmania* (Chargui *et al.*, 2007). 10 µl of purified DNA sample or control was used for PCR reactions in a final volume of 50 µl containing, 1XPCR buffer, 1.5 mM MgCl₂, 0.2 µM of each deoxynucleotide, 1.25 U of Taq DNA polymerase and 50 pmol of each primer. Reactions were cycled in an MJ Research thermocycler model PT-100 using the following conditions: an initial denaturation step at 94°C for 3 min, 40 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 1.5 min, and a final elongation step at 72°C for 10 min.

To control the quality of DNA extraction as well as the presence of possible reaction inhibitors, an internal control was used for each sample. It consisted of an amplification of the acidic ribosomal phosphoprotein fragment of housekeeping canine gene with PO3 and PO5 primers amplifying a 469 pb PCR product (Ashford *et al.*, 1995). If the inhibition control was negative, a five-fold dilution of the DNA was applied for the subsequent PCR. Carryover contaminations were prevented by using separate rooms and materials for each step of the PCR (DNA extraction, preparation of PCR mixture and electrophoresis of PCR products), as well as decontamination procedures by UV exposure of consumables and materials and bleaching of materials and surfaces.

STATISTICAL ANALYSIS

Epi Info™ 6.0 (CDC, Atlanta, GA, USA) was used for data statistical analysis by the χ^2 test for the comparison between PCR, IFAT, and *in vitro* culture in the detection of CanL.

RESULTS

67 dogs were chosen randomly in El Alaa (Kairouan). All but one dog were mongrel and the other dog is Alsatian. The dog age ranges between one and 11 years with a mean of 3.4 years (47 males, 20 females). Some studied dogs presented signs evoking CanL (24 symptomatic dogs), while others did not (43 asymptomatic dogs). Major signs were lymph node enlargement in 16 dogs; loss of weight was observed in ten dogs, dermatological changes in nine, and onychogriphosis in four. The results of different methods used for the detection of CanL (*in vitro* culture, IFAT,

IFAT	<i>In vitro</i>		N. of dogs		
	culture	PCR	Symptomatic	Asymptomatic	Total
+	+	+	3	0	3
+	-	+	3	2	5
-	+	+	0	0	0
+	+	-	0	0	0
-	-	+	2	6	8
+	-	-	0	0	0
-	+	-	0	0	0
-	-	-	14	32	46
Total			24	43	67

Table I. – Clinical status, serology, *in vitro* culture, and PCR results in a canine population living in an area of Kairouan.

and PCR) performed on blood were given in Table I. Only, three dogs were found to be positive by all methods, five dogs were positive by IFAT and PCR, while eight dogs were positive only by PCR. Altogether, 16 dogs (20.9 % of the cases) were positive by PCR. Among the samples negative by PCR, one case of PCR inhibition was detected and was resolved by five-fold dilution and was negative. Eight dogs (11.9 %) were positive by IFAT with dilutions between 1/80 and 1/2560. Only three strains (4.5 %) were isolated by *in vitro* culture and were identified by isoenzymatic method as *Leishmania infantum* MON-1. Within positive cases, eight dogs (50 %) were symptomatic with two or more signs evoking CanL (loss of weight, onychogriphosis, lymph node, and spleen enlargement). The other infected dogs (50 %) were asymptomatic. The *Leishmania* DNA was detected in eight of symptomatic (33.3 %) and eight asymptomatic ones (18.6 %). Anti-*Leishmania* antibodies were detected in six of symptomatic (25 %) and two asymptomatic (4.6 %) dogs. *Leishmania* strains were isolated from three symptomatic dogs (4.5 %) by *in vitro* culture.

Since there is no gold standard for the biological detection of CanL in the presence of a hypersensitive method such as PCR (Lachaud *et al.*, 2002; Chargui *et al.*, 2005), the sensitivity of each technique was defined using the total number of samples positive with at least one method (Table II). Thus, the sensitivities of PCR, IFAT, and *in vitro* cultivation would be 100 %, 50 % and 18.75 %, respectively. PCR was found more sensitive than IFAT ($p = 0.004$) and *in vitro* culture ($p <$

10^{-5}). Moreover, infection was detected by one or more of the methods used in 33.3 % of symptomatic dogs and in 18.6 % of asymptomatic dogs.

DISCUSSION

DIAGNOSIS OF CANINE LEISHMANIASIS IN PERIPHERAL BLOOD

Molecular (PCR), serological (IFAT), and parasitological (*in vitro* culture) methods were used in the present study to detect *Leishmania* infection in the blood of 67 dogs. In this study, we have chosen the peripheral blood to detect canine leishmaniasis. A based diagnosis test on blood is advantageous because samples can be obtained less invasively from patient (human and dog), relatively easy to process and it is the most used tissues in mass screening epidemiological studies. Other samples can yield excellent sensitivity (lymph node, bone marrow, skin or conjunctive biopsies) (Lachaud *et al.*, 2002; Reale *et al.*, 1999). However, they are impractical outside of a veterinary centre and cannot be used in field mass surveys. Moreover, the PCR chosen amplified genomic DNA fragment (PCR gDNA) and not a kinetoplastidic DNA (PCR kDNA) because of the artefacts which can be observed with PCR kDNA and rarely with PCR gDNA and because of the low positive predictive value of the PCR kDNA (Lachaud *et al.*, 2002).

The sensitivity of PCR was found higher than that of IFAT ($p = 0.004$) and *in vitro* culture ($p < 10^{-5}$), which is in agreement with results found by other studies (Leontides *et al.*, 2002; Oliva *et al.*, 2006). The low sensitivity of *in vitro* culture observed in our study is in correlation with what is found in some studies (Berahal *et al.*, 1996; Fisa *et al.*, 2001). However, other studies found sensitivity as high as that of PCR in dog blood (Iniesta *et al.*, 2002) or in human blood (Kaouach *et al.*, 2008). This difference can be due to the method used and the local conditions of *in vitro* culture. Altogether, canine leishmaniasis was observed in 16 dogs (20.9 %) using one or more methods. Within positive cases, 50 % were asymptomatic. Other studies have detected infection in asymptomatic dogs by serology

	IFAT (%)	Culture (%)	PCR (%)	Total (%)
Symptomatic dogs (24)	6 (25)	3 (12,5)	8 (33,3)	8 (33,3)
Asymptomatic dogs (43)	2 (4,6)	0 (0)	8 (18,6)	8 (18,6)
Total (67)	8	3	16	16
%	11.9 %	4.5 %	20.9 %	20.9 %
Sensitivity ^a	50 %	18.75 %	100 %	-

^a: sensitivities were calculated as the number of positive cases of one method per total number of positive cases by at least one method.

Table II. – Sensitivity of different methods used in the detection of *Leishmania*.

and/or PCR (Lachaud *et al.*, 2002; Oliva *et al.*, 2006; Moreira *et al.*, 2007).

The role of asymptomatic dogs in the transmission of the infection has been already confirmed (Dye, 1996). These dogs can develop the disease in many months or years (Dedet, 1999; Ferrer, 1999). Thus, to control the disease preventive measures should be taken against all infected dogs including symptomatic and asymptomatic ones after being detected by high sensitive methods as PCR. Isolated strains corresponded to *Leishmania infantum* MON-1. This confirms again the role of the dog as the only reservoir of the visceral form of human leishmaniasis in Tunisia (Haouas *et al.*, 2007; Aoun *et al.*, 2003; Bouratbine *et al.*, 2005).

PREVALENCE AND STATE OF CANL IN KAIROUAN (CENTRAL TUNISIA)

The prevalence of CanL in Kairouan, known as a reduced endemic area in Tunisia, was assessed in this study by different methods and was estimated to be of 20.9 % using one or more of the method used, in correlation with the prevalence of CanL in the Mediterranean basin which ranges between 10 and 37 % (Dereure *et al.*, 1999). There are three zones within the Tunisian leishmaniasis focus according to Dedet *et al.* (1973): a strong endemicity zone corresponds to the sub-humid and the semi-arid stages (in the northern part of the country) with a prevalence of 5.6 %, a zone of reduced endemicity in the arid stage (in the central part of Tunisia) with a prevalence of 1.6 %, and a very low endemicity zone corresponding to saharan and the humid stage where the disease seems absent. In another more recent study (Ben Said *et al.*, 1992), the prevalence was found to have increased in an arid region (Sousse) to 6 %. In our previous study (Chargui *et al.*, 2007), we also found an increase in endemicity in another arid region (Sfax) to 6 %. However, in this study, we found that Kairouan, which is an arid region, supposed to be of very low endemicity 30 years ago (Dedet *et al.*, 1973), had a prevalence as high as 20.9 %. This result may be due to: *i*) the increase of the disease in this region which has not been studied since 1973 in correlation with the important number of cases of Human visceral leishmaniasis emerged recently (Ben Salah *et al.*, 2000), and suggesting for a spread of the disease in the centre of Tunisia (Ayadi *et al.*, 1991; Besbes *et al.*, 1994; Ben Salah *et al.*, 2000); *ii*) the prevalence was underestimated since all previous studies were conducted by serological methods, which, as we reported in this study, can not detect all infected dogs.

ACKNOWLEDGEMENTS

This study was carried out with the financial support of the Research Unit 99-UR/08-05. We thank the Regional Directors for Public Health and Cul-

ture. N. Chargui was the recipient of a grant from the Agence Universitaire de la Francophonie (AUF). We thank Dr Harrabi Imed (CHU of Farhat Hached, Sousse, Tunisia) for statistical analysis.

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Reçu le 30 mai 2008

Accepté le 10 décembre 2008