Diagnosis of infections with *Leishmania infantum* using PCR–ELISA

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SUMMARY

On the basis of partial amplification of a cloned fragment of kDNA of *Leishmania infantum* which is specific for this species, we developed a PCR–ELISA technique which avoids the problems associated with classical diagnostic techniques. This technique was tested on 33 *L. infantum* strains from 19 different zymodemes, which were recognized equally. It was also used on human and canine clinical samples. PCR–ELISA has a higher sensitivity than the other techniques used (IFAT, parasite cultures, optical microscopy of stained samples) and permits detection of a minimum of 0·1 promastigotes or 1 fg of genomic DNA. PCR–ELISA can be used to diagnose human cutaneous leishmaniasis using material obtained by scraping the lesion margin, and human visceral leishmaniasis in HIV(+) individuals and canine leishmaniasis with peripheral blood samples. The presence of *L. infantum* in dogs with low antibody titres with IFAT technique (20 and 40) was demonstrated indicating that seroprevalence data from epidemiological studies underestimate the true rates of infection.

Key words: *Leishmania infantum*, kinetoplast DNA, PCR–ELISA, human cutanean leishmaniasis, human visceral leishmaniasis, canine leishmaniasis.

INTRODUCTION

Leishmania infantum is the aetiological agent of leishmaniasis in Spain where it is responsible for both the cutaneous (HCL) and the visceral forms (HVL). Those include cases of coinfection with HIV, with important clinical, diagnostic, chemotherapeutic, epidemiological and economic complications (WHO, 1996). It is also responsible for canine leishmaniasis (CaL) which has been the focus of a number of studies in Spain to determine seroprevalence rates for *Leishmania*. These normally range from 5 to 10% but rise to above 20% in some areas (Martínez-Cruz *et al*. 1990; Fisa *et al*. 1992; Nieto *et al*. 1992; Acedo-Sánchez *et al*. 1996; Morillas *et al*. 1996). A similar situation has been reported for other southern European countries such as Portugal, France and Italy (Lanotte *et al*. 1978; Abranches *et al*. 1983; Bettini & Gradoni, 1986; Jambou *et al*. 1986; Semiao Santos *et al*. 1995). One very interesting aspect of the epidemiological studies of CaL is the high percentage of dogs with a titre of anti-*Leishmania* antibodies lower than the positivity threshold (titres in indirect fluorescent antibody test, IFAT, 80 to 160), which could be due to (1) infection is in the pre-patent period, (2) infection is in the remission period or (3) cross-reaction (Fisa *et al*. 1992).

For the diagnosis of HCL, direct detection of the parasite in skin samples is required (Giemsa's staining and/or cultures). The two main drawbacks of these techniques are the low number of parasites usually present in these samples and the invasive nature of a sampling technique such as biopsy. In immunocompromised subjects the humoral response to *Leishmania* infection is often negative (42·2%) (WHO, 1996) which limits the diagnostic value of serological techniques. Parasitological diagnosis in bone-marrow aspirates is the most commonly used technique and is among the most sensitive techniques (98%) compared to blood, normal skin, gastrointestinal tract, liver, spleen, pleural liquid and lymph nodes (WHO, 1996). However, this requires an invasive sampling method which is not advisable in individuals generally debilitated by HIV infection.

PCR-based methods for detecting *Leishmania* species in clinical samples have been developed which amplify rRNA and miniexon genes, kinetoplast DNA (kDNA) and repeated nuclear DNA

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sequences (Quiao, Miles & Wilson, 1995; Delgado *et al*. 1996; Ramos *et al*. 1996; Noyes *et al*. 1998). These methods are of variable specificity; some can detect all *Leishmania* species while other methods identify the infecting *Leishmania* parasite to the species level. These techniques mostly have a high sensitivity although some, such as PCR with a subsequent Southern blot hybridization (Uliana *et al*. 1994; Laskay *et al*. 1995; Andresen *et al*. 1996), are laborious and time consuming, and in practice are not very useful as routine diagnostic techniques. On the other hand, the diagnostic value of some of these PCR-based methods has not been confirmed on a sufficient number of parasite strains and clinical samples (Piarroux *et al*. 1993, 1995; Quiao *et al*. 1995).

In the present paper we describe a PCR–ELISA technique which can overcome the problems of the traditional diagnostic techniques, i.e. to (1) diagnose cases of HCL using less invasive sampling techniques than biopsy, (2) diagnose cases of HVL in HIV(+) individuals using peripheral blood samples instead of bone-marrow and (3) correctly meaning anti-*Leishmania* titres lower than the positivity threshold of CaL in dogs.

MATERIALS AND METHODS

L. infantum *kDNA target and development of primers*

A 196 bp *Eco*RI/*Hae*III fragment diagnostic for *L. infantum* derived from a minicircle region that exhibits interspecies variation was used (Gramiccia *et al*. 1992, GenBank accession no. S49390). Sequence data were analysed and sets of primers for PCR were designed using the GCG Wisconsin Package software. Primers were synthesized by Pharmacia Biotech.

Strains and DNA preparation

A total of 33 Spanish strains of *L. infantum* from 19 different zymodemes that had been previously characterized by isoenzyme electrophoresis was used (Martín-Sánchez *et al*. 1996, 1999) (Table 1). Other reference strains used belong to the species *L. donovani* (1 strain), *L. major* (1), *L. tropica* (1), *L. mexicana* (1) and *Trypanosoma cruzi* (2).

Promastigotes were maintained in EMTM (WHO, 1989) and the parasites were grown to stationary phase. Genomic DNA was prepared by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation (Sambrook, Fritsch & Maniatis, 1989). Human, dog and sandfly (*Phlebotomus perniciosus*) DNA were used as negative controls in the PCR assay.

PCR amplification

Primers 9 (forward primer: 5′-CAAAAGTCCCC-ACCAATCCC-3′) and 83 (reverse primer: 5′-AAACCCTGGTCTGGAGGCTTAG-3′) were used to amplify a 75 bp fragment contained within the 196 bp fragment. Reactions were carried out in 50 mM KCl, 10 mM Tris–HCl, pH 8·3, 1·5 mM $MgCl_2$, 200 μM dNTPs, 12·5 pM each primer, 5–10 ng DNA or 0·5 μl samples, and 0·6 U *Taq* polymerase in a final volume of 25 μl. Each reaction was first denatured at 94 °C for 3 min and then cycled 40 times in a Progene Techne Thermal Cycler. Cycles were: 94 °C, 30 sec; 60 °C, 30 sec; 72 °C, 30 sec, with a final extension at 72 °C during 2 min. Amplified products were analysed by electrophoresis in 3·0 % agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Southern hybridization and DNA sequencing

Transfer of DNA from agarose gels to nylon membranes was performed as described by Sambrook *et al*. (1989). Labelling of DNA with digoxigenin hybridizations were carried out as described by Martín-Sánchez *et al*. (1998) using genomic DNA of one of the *L. infantum* strains (Table 1) and the amplified fragment of 121 bp as probes.

PCR products were purified by agarose gel fractionation and binding to glass milk. Sequencing was done using each of the PCR primers and the ABI PRISM™ BigDye Cycle Sequencing Ready Reaction kit.

PCR–ELISA

The amplification reaction was carried out using identical conditions to those used for PCR amplification except for the use of 2·5 μl of PCR labelling mix (Boehringer Mannheim) instead of the dNTP mixture. For ELISA detection of PCR products, we used the PCR–ELISA kit (DIG Detection) of Boehringer Mannheim, following the manufacturer's instructions. The oligonucleotide probe was selected from the 75 bp fragment without the primers necessary for its amplification. The oligonucleotide 5′-CCA AAC AGG GCA AAA ACC-3′ of Tm = 53·7 and %G+C = 50 was synthesized, purified and modified in position 5′ with biotin by Roche Diagnostics, LTD. Hybridization is carried out at 50 °C for 3 h agitation and washings carried out 3 times of 5 min duration each time. After incubation with the substrate ($ABTS^R$), 40 min at room temperature with gentle shaking, absorbance values of plates were read at 405 and 492 nm (patent request number: P9901804).

Clinical samples and DNA preparation

Human samples were obtained from the following cases. (1) Three leishmaniasis asymptomatic patients

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Table 1. *Leishmania infantum* isolates used in this study and zymodeme data

Strains International code	Zymodeme	Type of lesion
MHOM/ES/85/DP152	MON183 = GR4	HVL
MHOM/ES/80/DP74	MON24 = GR5	HCL
MCAN/ES/90/DP202	MON105 = GR16	CaL (cutaneous)
MCAN/ES/90/DP204	MON1 = GR1	CaL (visceral)
MHOM/ES/90/DP121	MON28 = GR10	HCL
IPER/ES/90/DP169	MON199 var NP = GR11	—
IPER/ES/90/DP170	MON199 = GR12	—
IPER/ES/90/DP173	MON183 var NP = GR13	—
IPER/ES/90/DP175	MON198 var G6PD = GR14	—
IPER/ES/90/DP191	MON29 = GR6	—
IPER/ES/90/DP192	MON77 var MDH and GPI = GR8	—
MHOM/ES/90/DP208	MON24 = GR5	HVL-HIV+
IPER/ES/91/DP275	MON186 = GR7	—
IPER/ES/91/DP277	MON33 = GR3	—
IPER/ES/91/DP279	MON190 = GR15	—
IPER/ES/91/DP296	MON77 var MDH = GR2	—
MHOM/ES/91/DP289	MON80 = GR20	HCL
MHOM/ES/91/DP302	MON1 = GR1	HVL-HIV+
MHOM/ES/92/DP418	MON34 = GR18	HVL-HIV+
MHOM/ES/92/DP419	MON183 = GR4	HVL
MHOM/ES/92/DP421	MON1 = GR1	HCL
MHOM/ES/91/DP422	MON24 = GR5	HVL-HIV+
MHOM/ES/91/DP423	MON24 = GR5	HVL
MHOM/ES/93/DP424	MON183 = GR4	HVL-HIV+
MHOM/ES/93/DP435	MON34 = GR18	HVL-HIV+
MHOM/ES/93/DP444	MON24 var NP = GR19	HVL-HIV+
MCAN/ES/96/DP502	MON199 var NP = GR11	CaL (cutaneous)
MCAN/ES/96/DP503	MON199 = GR12	CaL (visceral)
MCAN/ES/96/DP505	MON199 = GR12	CaL (visceral)
MCAN/ES/96/DP506	MON199 var NP = GR11	CaL (visceral)
MCAN/ES/96/DP510	MON199 = GR12	CaL (visceral)
MCAN/ES/96/DP511	MON199 = GR12	CaL (visceral)
IPER/ES/98/DP514	MON183 = GR4	—

coinfected with *Leishmania*-HIV. The presence of *Leishmania* in the bone-marrow was confirmed by optical microscopy of the Giemsa's-stained smears. PCR–ELISA was performed on samples of peripheral blood. (2) Seven cases of suspected HCL, 3 children under 6 years old and 4 adults. Skin samples were obtained by biopsy in 4 of these cases, in the other 3 cases they were obtained by scraping the edges of the lesion. (3) Two healthy adults from whom peripheral blood samples were used.

A total of 70 canine samples were obtained from 31 dogs who had been previously destined for euthanasia by the Society for the Protection of Animals and Plants of 'Francisco de Asis' in Granada: 21 bone-marrow samples, 28 samples of peripheral blood, 18 samples of popliteal lymph node aspirate and 3 skin samples (biopsies of lesion margin).

DNA was prepared according to the technique described by D'Oliveira *et al*. (1995) followed by phenol/chloroform extraction and ethanol precipitation. With peripheral blood samples, buffy coat was carried out on an initial amount of 1–1·5 ml and the resulting product was divided into 2 equal parts for culture and DNA preparation.

Diagnostic techniques used as a reference

Simultaneously to and independent of PCR–ELISA, the following techniques for diagnosis of leishmaniasis were applied to the different clinical samples. (1) Human samples, (a) peripheral blood: culture; (b) skin, optical microscopy of samples stained with Geimsa's stain and cultured. (2) Canine samples, (a) bone-marrow, lymph node and skin: culture; (b) peripheral blood, culture and IFAT.

Parasite cultures were grown in EMTM medium (WHO, 1989) and kept for over 1 month before negative results were recorded. Positive cultures were mass cultured and the isolates were characterized by isoenzyme electrophoresis (Martín-Sánchez *et al*. 1994). The IFAT technique was carried out according to the description by Acedo *et al*. (1996).

RESULTS

Analysis of PCR amplification products

Amplification of 5–10 ng of genomic DNA of 3 strains of *L. infantum* (DP74, DP152 and DP204)

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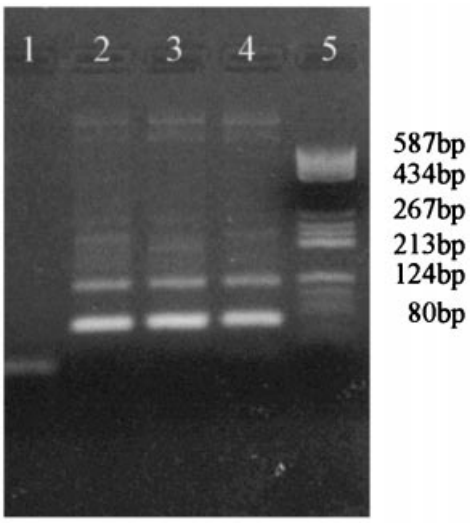

Fig. 1. PCR amplification products generated using genomic DNA of 3 strains of *Leishmania infantum*. Lane 1, PCR negative control; Lane 2, DP74 strain; Lane 3, DP152 strain; Lane 4, DP204 strain; Lane 5, DNA molecular weight marker V of Boehringer Mannheim.

generated 2 main products of 75 and 121 bp (Fig. 1) determined by DNA sequencing. The 75 bp fragment had 98·7% identity with the fragment theoretically designed using the probe of Gramiccia *et al.* (1992) (only one of 75 bp was different). The sequence of the 75 bp fragment was contained within the 121 bp fragment (Fig. 2). The amplification of this band of higher size is probably generated by the presence in the *L. infantum* kDNA minicircle, upstream of the primer 9 annealing site, of a region with high homology to the 3′ end of the primer 9. Since the annealing temperature used in PCR experiment was higher than Tm of the primer 9 we suggest that at least a large primer proportion is present upstream of the 75 bp minicircle fragment. These same bands were amplified when the remaining 30 strains of *L. infantum* (Table 1) were used. For some strains, other bands corresponding to fragments larger than 121 bp were amplified. All the amplification products of the 33 strains of *L. infantum* hybridized with the 2 probes used (genomic DNA and the 121 bp fragment) (data not shown). Amplification of 5–50 ng of genomic DNA of the other *Leishmania* species, including *L. donovani*, and *T. cruzi*, did not generate any product. Amplification was also negative when we used host DNA (man, dog, sandfly) free from *L. infantum* DNA (data not shown).

Sensitivity of the PCR assay

Serial dilutions of 10 ng *L. infantum* genomic DNA were subjected to PCR and the PCR products were analysed in agarose gel containing ethidium bromide. The assay was positive, with amplification of the 75 and 121 bp fragments up to dilution $1/10^3$, which is equivalent to the detection of approximately 10 pg of total DNA. Lower amounts of input DNA gave negative results. With Southern hybridization it was possible to detect 1 fg of genomic DNA (dilution $1/10^7$) (Fig. 3). Also, raw extracts prepared from suspensions of promastigotes in culture were used for PCR, and the detection limit was 50–100 promastigotes (Fig. 4).

ELISA detection of PCR products

This was designed to solve the two problems posed by detection by electrophoresis of PCR products i.e. (1) to increase the sensitivity and (2) amplification of fragments of higher molecular weight than the two main products of 75 and 121 bp.

The mean absorbance value (A_{405}–A_{492}) of the PCR control blank (PCR without DNA) was 0·04, and PCR with only host DNA (human or canine) was 0·1. The absorbance values obtained for *L. donovani*, *L. tropica*, *L. mexicana* and *L. major* were lower than 0·5 and for *T. cruzi* was below 0·2. For the 33 strains of *L. infantum* studied this was always greater than 3·0 (Fig. 5).

The detection limit of the PCR–ELISA assay performed with serial dilutions of 10 ng of *L. infantum* genomic DNA (DP74 strain) was 1 fg (dilution $1/10^7$), and when this was determined using raw promastigote extract it corresponded to 0·1 promastigotes. In all cases, the absorbance readings (A_{405}–A_{492}) were always greater than 1·0. This corresponds to a rise in the sensitivity of PCR–ELISA in comparison with PCR detection of the amplified product by electrophoresis of 100–1000 times that obtained with PCR and ethidium bromide. Amplification of the mixtures with different proportions of raw extract of 1 promastigote, or *L. infantum* genomic DNA, and host DNA (human or canine) did not bring about changes in the absorbance levels compared to when *L. infantum* DNA was amplified in the absence of host DNA (Fig. 5: h and i).

Clinical samples : comparison of PCR–ELISA with other diagnostic techniques applied to HCL, HVL and CaL

The results obtained with the different samples of blood, bone-marrow, lymph node material and human or canine skin, with each of the diagnostic techniques used, are compiled in Tables 2 and 3. The reading was considered as positive when absorbance (A_{405}–A_{492}) was $\geqslant 1$.

Human clinical samples (*Table 2*). The 7 patients with a clinical picture suggestive of HCL were positive with PCR–ELISA. Patient no. 1 had been diagnosed with HCL several years previously and had received specific treatment for this consisting of infiltration into the lesion of Glucantime and systemic administration of Allopurinol, without achieving complete

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seq75          ----------------------------------------------------------------------------------------CAAAAGTCCCC
seq121         AAAAGTCCCCACCAATCCCACCAAAGCAGCCCGTACCCGTGAATTGA - - CAAAAGTCCCC
seqgramiccia   ----------------------------------------------------------------------------------AATTCAACCAAAAGTCCCC
                                                                                                  ********************

seq75          ACCAATCCCACCAAACAGGGCAAAAACCCCAAAATGAAGAAACTAAGCCTCCAGACCAGG
seq121         ACCAATCCCACCAAACAGGGCAAAAACCCCAAAATGAAGAAACTAAGCCTCCAGACCAGG
seqgramiccia   ACCAATCCCACCAAACAGGGCAAAAACCCCAAAATGAGGAAACTAAGCCTCCAGACCAGG
               ************************************************************  ************************************

seq75          GTTT----------------------------------------------------------------------------------------------------
seq121         GTTT----------------------------------------------------------------------------------------------------
seqgramiccia   GTTTCGAGCCATCCCAGCCCAGGGGAGCCAATTTCCGGCCGACTGAGCATCGGGCACGAG
               ******

seq75          ----------------------------------------------------------------------------------------------------
seq121         ----------------------------------------------------------------------------------------------------
seqgramiccia   CGTTAGTCACCCTGGGAGTGCGTCCCTTTCGGTTCCTTGAAGCCACTCACCGGAGGG
```

Fig. 2. Alignment of the sequences of 75 bp, 121 bp and the 196 bp fragment of Gramiccia *et al*. (1992). (EMBL Nucleotide Sequence Database Accession Number: AJ272098 LIN272098 is not confidential). The primer 9 nucleotide sequence located in the 5′ end region of the 121 bp fragment is shown in bold.

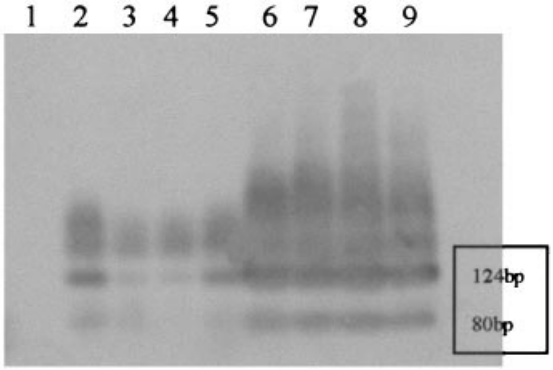

Fig. 3. Southern hybridization with the 121 bp DNA fragment as probe. Lane 1, PCR negative control; Lanes 2–9, *Leishmania infantum* DNA (10^{-7} to 10 ng).

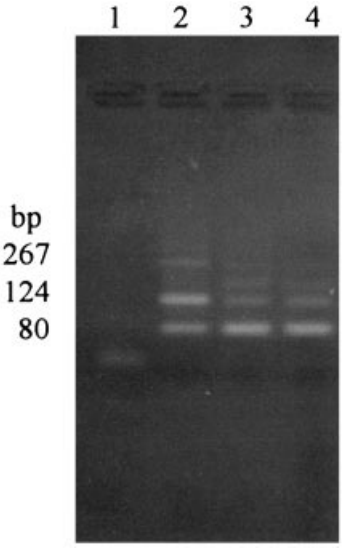

Fig. 4. PCR amplification products generated using raw extracts prepared from suspensions of promastigotes in culture. Lane 1, PCR negative control; Lane 2, 1000 promastigotes; Lane 3, 500 promastigotes; Lane 4, 100 promastigotes.

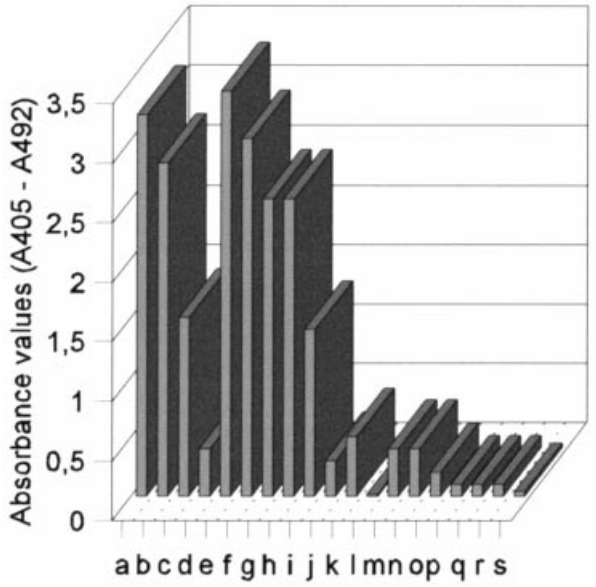

Fig. 5. Mean absorbance values (A_{405}–A_{492}) obtained with the PCR–ELISA; (a) 10 ng of genomic DNA of *Leishmania infantum*, (b) 0·01 ng of genomic DNA of *L. infantum*, (c) 1 fg of genomic DNA of *L. infantum*, (d) 0·1 fg of genomic DNA of *L. infantum*, (e) 1000 parasites of *L. infantum*, (f) 10 parasites of *L. infantum*, (g) 1 parasite of *L. infantum*, (h) 0·1 parasite of *L. infantum*, (i) mixtures with 0·1 parasite of *L. infantum* and host DNA (human and canine), (j) 0·01 parasite of *L. infantum*, (k) 10 ng of genomic DNA of *L. donovani*, (l) 10 ng of genomic DNA of *L. major*, (m) 10 ng of genomic DNA of *L. tropica*, (n) 10 ng of genomic DNA of *L. mexicana*, (o) 10 ng of genomic of DNA of *Trypanosoma cruzi*, (p) human DNA, (q) canine DNA, (r) phlebotomine DNA, (s) PCR negative control.

remission. The culture was negative with 6 skin samples and only positive with the skin sample obtained from patient no. 7. However, optical microscopy of Giemsa's-stained smears gave positive

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Table 2. Results obtained in the human clinical samples with each of the diagnostic techniques used

(O.M., optical microscopy of the Giemsa's stained smears; P, positive result; N, negative result; p. blood, peripheral blood; b. marrow, bone marrow.)

Individual number (Clinical picture, clinical samples)	Diagnostic techniques (Clinical samples)		
	PCR–ELISA	O.M.	Culture
1 (HCL, skin biopsy)	P	N	N
2 (HCL, scraping)	P	N	N
3 (HCL, scraping)	P	N	N
4 (HCL, skin biopsy)	P	P	N
5 (HCL, scraping)	P	N	N
6 (HCL, skin biopsy)	P	N	N
7 (HCL, skin biopsy)	P	P	P
8 (HVL-HIV+)	P (p. blood)	P (b. marrow)	N (p. blood)
9 (HVL-HIV+)	P (p. blood)	P (b. marrow)	N (p. blood)
10 (HVL-HIV+)	P (p. blood)	P (b. marrow)	N (p. blood)
11 (healthy adult)	N (p. blood)	N (p. blood)	N (p. blood)
12 (healthy adult)	N (p. blood)	N (p. blood)	N (p. blood)

Table 3. Results obtained in the canine clinical samples with each of the diagnostic technique used

(1) Dog with cutaneous lesions: skin material obtained by biopsy was positive with PCR–ELISA and culture. N.P., Not performed; P, positive result, N, negative result.)

Clinical samples		Peripheral blood		Bone marrow		Lymph node	
Dog number	IFAT titres	PCR–ELISA	Culture	PCR–ELISA	Culture	PCR–ELISA	Culture
1	0	N.P.	N.P.	N	N	N.P.	N.P.
2	0	N	N	N.P.	N.P.	N	N
3	80	P	N	P	N	P	P
4	40	P	N	P	N	P	N
5	80	P	N	P	N	P	N
6	> 320	P	N	P	P	P	N
7	> 320	P	N	P	P	N.P.	N.P.
8	80	P	N.P.	N.P.	N.P.	P	P
9	> 320	P	N.P.	N.P.	N.P.	P	P
10(1)	> 320	P	N.P.	N.P.	N.P.	P	P
11(1)	> 320	P	N.P.	N.P.	N.P.	P	P
12	> 320	P	N.P.	N.P.	N.P.	P	P
13	> 320	P	N.P.	N.P.	N.P.	P	P
14	> 320	P	N.P.	N.P.	N.P.	P	P
15	> 320	P	N.P.	N.P.	N.P.	P	P
16	> 320	P	N.P.	N.P.	N.P.	P	P
17	20	P	N	P	N	N.P.	N.P.
18	40	P	N	P	N	P	N
19	0	N.P.	N.P.	N	N	N.P.	N.P.
20	40	P	N	P	N	P	N
21(1)	80	N.P.	N.P.	P	N	N.P.	N.P.
22	40	P	N	P	N	N.P.	N.P.
23	20	P	N	P	N	N.P.	N.P.
24	40	N	N	P	N	N.P.	N.P.
25	40	P	N	N	N	N.P.	N.P.
26	80	P	N	NP	N.P.	N.P.	N.P.
27	40	P	N	P	N	P	N
28	320	P	N	P	N	N.P.	N.P.
29	160	P	N	P	P	N.P.	N.P.
30	20	P	N	P	N	N.P.	N.P.
31	40	P	N	P	N	P	N

results in patients nos. 4 and 7 on whom a biopsy had been performed, and negative in the remaining patients.

The 3 samples of peripheral blood from the 3 cases of HIV-*Leishmania* co-infection and with a positive bone-marrow Giemsa's-stained smear using optical

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microscopy, were positive with PCR–ELISA and negative in culture and Giemsa's-stained smear. The 2 healthy adults were negative with PCR–ELISA and also with the other diagnostic techniques uses.

Canine clinical samples (*Table 3*). Sixteen dogs (3 with IFAT 0 and 13 with IFAT $\geqslant 80$), were confirmed as negative (the 3 former ones) or positive for all the diagnostic techniques performed (IFAT, culture and PCR–ELISA). Four dogs were negative in culture and positive with IFAT and PCR–ELISA. The 8 dogs with IFAT = 40 and 3 with IFAT = 20 were positive with PCR–ELISA and negative in culture. There was 100% agreement between the results obtained by PCR–ELISA using DNA from blood samples and DNA from lymph node material, and 88·2% between those obtained from blood and bone-marrow samples. The 3 skin samples analysed were positive in culture and with PCR–ELISA; the serology of the 3 animals was positive for CaL.

The parasites isolated from 10 of the 13 dogs with a positive culture were characterized by starch gel isoenzyme electrophoresis, and it was confirmed that they belonged to the species *L. infantum* and, more specifically, to 3 different zymodemes: GR-1 (MON-1), GR-11 (MON-199 var NP1) and GR-12 (MON-199) (Martín-Sánchez *et al*. 1999).

DISCUSSION

The PCR method described is based on the partial amplification of a cloned 196 bp fragment of *L. infantum* kDNA. When used as a probe in hybridization experiments with isotopic and non-isotopic systems this 196 bp fragment has been shown to be specific to *L. infantum* and to recognize the wide range of zymodemes of this species occurring around the Mediterranean basin (Gramiccia *et al*. 1992; Martín-Sánchez *et al*. 1998). The PCR was tested on 33 *L. infantum* strains belonging to 19 different zymodemes, and the amplified product was detected using 2 techniques, electrophoresis with ethidium bromide and ELISA, and the same results was obtained among strains. It is, therefore, very unlikely that false negatives would be produced because the target DNA would not be recognized in all *L. infantum* strains, as suggested by other authors. False negative PCR results were investigated by Piarroux *et al*. (1995) and it was demonstrated that the two main factors leading to such errors were the presence of inhibitory factors remaining in some samples after DNA preparation, and intra-specific polymorphism of the primer target DNA.

Sensitivity of the PCR–ELISA of *L. infantum* was 1 fg of genomic DNA. Because of this high sensitivity, this technique could be used to detect the presence of the parasite in samples of peripheral blood from dogs with CaL. *L. infantum* has rarely been detected in the circulating blood of the host (more specifically in the buffy coat), possibly because it is hardly ever practised with the exception of HIV(+) individuals who present a positivity of around 74·8% (WHO, 1996), Using culture techniques, Mathis & Deplazes (1995) detected *Leishmania* in leuco-concentrated blood of a dog with CaL but obtained negative results in another two. We also demonstrated the presence of *Leishmania* in leuco-concentrated blood from 2 dogs using cultures (Martín-Sánchez *et al*. 1998). However, we do not have data on the positivity rate in haemoculture.

Human clinical samples. The results suggest that it is possible to diagnose HCL by performing PCR–ELISA on samples obtained by scraping the lesion margins. Using this technique the problems of the current diagnostic methods for HCL can be avoided: the scar resulting from the biopsy and the low sensitivity of cultures and stained samples because of the low parasitic load. Indeed, culture, as a diagnostic technique, had a very low sensitivity in our study (1 out of 7), mainly due to the small number of parasites present but also because of the difficulty of growing some dermotropic strains in culture (Gramiccia & Gradoni, 1989; Gramiccia, Gradoni & Pozio, 1987). This also applies to the use of optical microscopy on the Giemsa's-stained samples: only 2 of the 7 patients gave positive results.

Interestingly, with regard to patients with HIV–*Leishmania* coinfection and haemoculture negative, the sensitivity of PCR–ELISA with peripheral blood samples was the same as that achieved with optical microscopy of the bone-marrow Giemsa's-stained smears. The results are interesting due to the use in this case of a non-invasive method. López-Vélez *et al*. (1995), in a study on 25 HIV–*Leishmania* coinfected patients with clinical symptoms, found a 67% positivity in haemocultures. It is possible that the difference in the results obtained is because these authors used 10 ml of blood in comparison to the 1–1·5 ml that we used for both techniques (culture and DNA preparation for PCR–ELISA), a volume that could be insufficient to obtain a positive result in culture. On the other hand, all 3 individuals included in our study were asymptomatic for VL. A larger number of samples is required in order to confirm these results.

Canine clinical samples. IFAT is the technique usually used for CaL diagnosis, and it is considered to be the reference method by the WHO. In this technique, threshold titres of 80 or 160 determine the presence or absence of the disease (Abranches *et al*. 1983; Bettini & Gradoni, 1986; Acedo-Sánchez *et al*. 1996; Morillas *et al*. 1996). All the samples we

studied with titres equal or higher than these values gave positive results with PCR–ELISA. However, in our opinion the main problem concerns sera with titres below the threshold values (e.g. of 20 and 40). On several occasions, cultures have been used to isolate the parasite from dogs with titres of around 40 but in many others this has not occurred and cultures have proven to be of limited diagnostic value in these cases (Lanotte *et al*. 1978; Martínez-Cruz *et al*. 1990; Acedo-Sánchez *et al*. 1996). Moreover, cultures are often carried out on lymph node or bone-marrow samples, difficult to obtain, and involve a more traumatic invasion of host organs than simple extraction of blood samples. This is complicated further by the fact that cultures must be kept for at least 1 month to make a definitive negative diagnosis and this is both time consuming and costly.

Concordance between results by using PCR–ELISA on blood samples and those obtained when applying this same technique to other kinds of samples (bone-marrow and/or lymph node material) suggests the possibility of using the former for diagnosis of CaL, as occurs currently with HIV(+) individuals. Finally, it is noteworthy that the 3 dogs with titres of 20 were also positive with PCR–ELISA, whereas all those studied with a zero titre were negative with this technique.

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