Value of two PCR methods for the diagnosis of canine visceral leishmaniasis and the detection of asymptomatic carriers

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SUMMARY

The value of 2 PCR methods, targeting genomic and kinetoplast minicircle DNA respectively, was investigated for both diagnosis and prevalence studies of canine visceral leishmaniasis (CVL). The first method (R) was 5000-fold less sensitive than the second (method KRV). Both were tested for diagnosis of CVL in 44 sick dogs with confirmed disease using different biological samples. Method R was highly efficient when using invasive samples, but the use of method KRV proved necessary for a 100% sensitive diagnosis using peripheral blood. This method was applied to peripheral blood and skin samples in 263 dogs during a mass survey in the Cévennes focus. PCR was compared to serology and all results were analysed according to clinical status. The 'CVL-infection' prevalence was found to be 79·8% by PCR compared with 29·6% by serology: 89·4% of symptomatic and 65·2% of asymptomatic dogs harboured parasites in peripheral blood. This study confirms the high prevalence of asymptomatic carriers of *Leishmania*. In total, for the diagnosis of CVL in sick dogs, method R is recommended in view of its 100% positive predictive value (compared with 30% for method KRV). A strategy best adapted for prevalence surveys might combine serology and highly sensitive PCR on peripheral blood.

Key words: PCR, visceral leishmaniasis, dogs, prevalence, diagnosis, serology.

INTRODUCTION

Visceral leishmaniasis is an anthropozoonosis of almost worldwide distribution caused by the protozoan parasite *Leishmania*. In the mammalian reservoir, *Leishmania* are obligate intracellular parasites and live in cells of the mononuclear phagocyte lineage (Dedet *et al*. 1999). In the Mediterranean Basin, the human disease is caused by *L. infantum*, and dogs are the major reservoir of the infection. The seroprevalence in dogs varies between 10 and 30% depending on the region (Lanotte *et al*. 1979; Harith *et al*. 1986; Reed *et al*. 1990; Bernardina *et al*. 1997). The animal may or may not develop the disease after an extremely variable lapse of time, from a few months to several years, after parasite inoculation (Lanotte *et al*. 1979; Vidor *et al*. 1991). Clinical signs appear progressively and combine visceral and skin disease. In addition to cases of clinical disease, there are asymptomatic (aΣic) carriers (reviewed by Oliveira, Santoro & Sadigursky, 1993). Epidemiological studies using classical serology (IFAT) suggested that about half of the infected dogs showed no clinical signs (Lanotte *et al*. 1979; Gradoni *et al*. 1980; Mancianti *et al*. 1986; Abranches *et al*. 1991). This was further supported by direct detection of the parasite (by *in vitro* culture or microscopical examination) in a proportion of these aΣic dogs (Cardoso *et al*. 1998; Deplazes *et al*. 1998; Reale *et al*. 1999), together with the fact that a number of them could infect sandflies (Molina *et al*. 1994). Moreover, experimentally infected dogs may not develop the disease, and thus remain aΣic and seronegative, and the parasite may not be found (Pinelli *et al*. 1994; Campino *et al*. 2000). Seronegative 'healthy' carriers might be either resistant dogs or early/latent infections, or else dogs which have recovered and become negative. The status of aΣic dogs is therefore difficult to determine with certainty. Prevalence studies using PCR (Berrahal *et al*. 1996; Reale *et al*. 1999; Solano-Gallego *et al*. 2001) demonstrate that the proportion of aΣic carriers in a dog population is underestimated if non-molecular detection methods are used. As in other infectious diseases, one may then distinguish between 'CVL-disease' and 'CVL-infection' (Dye, Vidor & Dereure, 1993; Gradoni, 1999), the former corresponding to the presence of clinical signs, and the latter including both symptomatic (Σic) and aΣic dogs.

Clinically, the disease is often vague, signs may be very non-specific (Slappendel, 1988), and a large

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number of dogs present only a few clinical signs. Therefore, parasitological or immunological tests are required, both for diagnosis of the disease and for parasite detection in epidemiological studies. One of the most widely used methods to assess CVL prevalence is the classical serology. In clinically ill dogs, with parasitologically proven infection, the sensitivity of serology is good ($\geqslant 70\,\%$) but insufficient as it does not allow the diagnosis in a significant proportion of these dogs (Harith *et al*. 1986; Slappendel, 1988; Reed *et al*. 1990; Dye *et al*. 1993; Bernadina *et al*. 1997; Ozensoy *et al*. 1998; Solano-Gallego *et al*. 2001). Moreover, serological methods are inefficient at detecting aΣic carriers, and therefore underestimate the true rates of *Leishmania* infection (Slappendel, 1988; Vidor *et al*. 1991; Martin-Sanchez *et al*. 2001). The detection of *Leishmania*-specific cellular immune responses, using a lymphoproliferation assay or the leishmanin skin test, may be more sensitive (Cabral *et al*. 1998; Cardoso *et al*. 1998), but it may lack specificity (Quinnell *et al*. 2001); more importantly, and conversely to serology, it is particularly useful for aΣic carriers, as the cellular immune response is often defective in clinically ill dogs (Pinelli *et al*. 1994). ELISA techniques (Bernadina *et al*. 1997; Solano-Gallego *et al*. 2001; Quinnell *et al*. 2001), and even more so the Western blot (Neogy *et al*. 1992; Berrahal *et al*. 1996), seem to enhance significantly the sensitivity of serology. Nevertheless, this still detects only a humoral response to previous contact with the pathogen. Proof of the presence of the parasite in the host requires direct detection methods, classically by lymph node or bone marrow microscopical examination and culture. However, the latter have proved insufficiently sensitive, detecting overall at best 30 % and 60 %, respectively, of proven infections (Lanotte *et al*. 1974; Schnur & Jacobson, 1987; Ashford *et al*. 1995; Cardoso *et al*. 1998; Roura, Sanchez & Ferrer, 1999). Xenodiagnosis may be sensitive (Molina *et al*. 1994; Travi *et al*. 2001) but is not feasible in field studies. Over the recent years, the polymerase chain reaction (PCR) has brought a considerable improvement in CVL diagnosis, detecting 89–100 % of symptomatic or parasitologically confirmed infections (Mathis & Deplazes, 1995; Berrahal *et al*. 1996; Roura *et al*. 1999; Reale *et al*. 1999; Solano-Gallego *et al*. 2001). In human VL, PCR was shown to perform even better, with a sensitivity rate equal or close to 100 % (Mathis & Deplazes, 1995; Adhya *et al*. 1995; Costa *et al*. 1996; Lachaud *et al*. 2000). In both CVL and human VL, some PCR assays used 'low-invasive' samples, such as peripheral blood, with no or only a slight reduction in sensitivity (Adhya *et al*. 1995; Costa *et al*. 1996; Reale *et al*. 1999; Lachaud *et al*. 2000; Martin-Sanchez *et al*. 2001; Pizzuto *et al*. 2001). PCR also proved an interesting tool for CVL prevalence surveys due to its high sensitivity and the detection of aΣic carriers (Ashford *et al*. 1995; Berrahal *et al*. 1996; Reale *et al*. 1999; Solano-Gallego *et al*. 2001). However, the general picture of the real improvement brought by molecular methods remains confusing, as most studies used different PCR assays of varying sensitivities on different biological samples. The infection status of asymptomatic or PCR-negative seropositive dogs is yet unclear (Pinelli *et al*. 1994; Quinnell *et al*. 2001). Here, we report on a field survey of CVL on a cohort of dogs living outdoors in an endemic area in the south of France. In a previous study, we compared 6 PCR methods for the diagnosis of CVL (Lachaud *et al*. 2002). Here, in order to develop an efficient diagnostic test for the disease, the 2 PCR methods considered as the most 'robust' of the 6 were tested with blood, bone marrow, lymph node, skin, and dermal scrapings from polyΣic dogs. Then, the more sensitive method was used in a mass survey of 263 dogs using skin biopsies (SBs) and peripheral blood (PB) samples. The changes that our results may bring to the diagnosis of 'CVL-disease' and to the epidemiology of 'CVL-infection' are discussed; we then propose to adapt the strategies for field studies of CVL in the view of these and other molecular data.

MATERIALS AND METHODS

Study area and animal population

The study was carried out in areas endemic for CVL in the south of France. In total 307 dogs were sampled. The majority of dogs (263) was sampled during mass surveys carried out over a period of 3 months (February–April 1997), in the southern part of the Cévennes focus (Hérault and Gard departments), in a variety of localities covering about 700 km^2: these dogs were hunting dogs, living in outdoors kennels, and of different breeds and ages (from 8 months to 12 years, mean = 3 years old). Dogs treated for leishmaniasis were not included in this study. Two more dogs with CVL, confirmed by direct examination of bone marrow, had to be sacrificed. Also, in the Alpes-Maritimes area, 24 polyΣic dogs were sampled in veterinary clinics. Oligosymptomatic (oligoΣic) dogs were defined by the presence of one of the following signs: lymphadenopathy, skin involvement, epistaxis, or onychogriphosis; and polyΣic dogs by the presence of > 2 of these signs that may be accompanied by weight loss or cachexia. Six polyΣic dogs with CVL confirmed by direct examination of bone marrow were used as positive controls. Finally, a group of 18 dogs from Corrèze (France), a non-endemic area, were included as negative controls.

Serology

The serological diagnosis used antigens of *L. infantum* prepared from a reference strain from the

Centre National de Référence sur les Leishmanioses (MHOM/FR/78/LEM75). Two techniques were used: indirect immunofluorescent antibody test (cut-off value = 1/40) and counter immunoelectrophoresis (cut-off value = 1 line). The antigen preparation and serological analysis have been described previously (Lanotte *et al*. 1974; Alvar *et al*. 1994).

Samples for diagnosis by PCR

Two types of tissues were sampled in all dogs analysed: peripheral blood (PB) and skin (skin biopsy, SB). In a varying proportion of cases, the bone marrow (BM), lymph node (LN), conjunctiva, as well as dermal scrapings (DS) were also sampled. Each dog was sampled with a different single-use instrument (in particular, needles for vein puncture and punches for SBs). PB samples were collected in EDTA-coated tubes (4·5 ml). Both skin samples (SBs and DSs) were collected from the internal side of the ear in areas without lesions (hereafter termed 'healthy' skin). SBs were performed with a 5 mm diameter punch. Each biopsy, weighing approximately 25 mg, was immediately deposited in 500 μl of physiological saline (NaCl 0·9 %) at +4 °C, and transferred to another tube after 8–24 h. The remaining blood-containing lavage fluid (SBLF) was considered as a distinct sample and processed separately. For DSs, the 'healthy' skin was superficially incised by using a sterile scalpel, a small amount of material was scraped out, and 20 μl of the exudate were collected with a 200 μl pipette. LN samples were obtained by fine-needle aspiration of palpable popliteal LNs. BM aspirates ($\sim$ 0·5–1 ml) were taken from the sternum and collected in EDTA-coated tubes. All samples were transported to the laboratory at ambient temperature, which never exceeded 25 °C. They were then stored at +4 °C until processed, which took place in the following 24 h.

DNA isolation

Several methods were used for DNA isolation after empirical testing, in order to achieve maximal sensitivity for each type of sample (Lachaud *et al*. 2001). PB was prepared for PCR from the buffy coat. Thus 300 μl of buffy coat were collected after centrifugation of 4·5 ml of blood (10 min at 1300 ***g***), incubated for 12 h at 56 °C in 2 volumes of proteinase K lysis buffer (0·5 % Tween 20, 0·5 % Nonidet-P40, 10 mM NaOH, 10 mM Tris, pH 7·2, 640 mg/ml proteinase K), and then boiled for 10 min. A phenol–chloroform extraction was performed on 450 μl of this lysate, followed by ethanol precipitation and resuspension in 150 μl of sterile distilled water. BM, LN and DS samples were prepared according to the same method except that the whole sample was processed and all volumes of reagents were adapted to the sample volume. For SBs, in view of a high rate of PCR inhibitions encountered with the 'classical' phenol–chloroform method, the 'DNeasy tissue' kit (Qiagen®) was used, according to the supplier's instructions, except that proteinase K concentration was doubled and the final elution volume was 200 μl. As SBLFs contained a relatively small amount of blood, they were DNA-extracted by a simple heat-detergent method after red cells lysis, as previously described for amniotic fluids (Hohlfeld *et al*. 1994).

PCR amplification

The amplification DNA targets were the gene coding for ribosomal RNA 18S ($\sim$ 24-fold repeated sequence) (Martinez-Calvillo *et al*. 2001) and the conserved region of the kinetoplast DNA minicircle (several 1000's-fold repeated sequence) (Sheline & Ray, 1989). The primers used were 5′-GGTTCCTTTCCTGATTTACG-3′ (R221) and 5′-GGCCGGTAAAGGCCGAATAG-3′ (R332), producing a 603-bp fragment upon amplification (Van Eys *et al*. 1992), for the former, and 5′-CTTTTCTGGTCCCGCGGGTAGG-3′ (RV1) and 5′-CCACCTGGCCTATTTTACACCA-3′ (RV2), producing a 145-bp fragment, for the latter (Ravel *et al*. 1995; LeFichoux *et al*. 1999). PCR conditions were thoroughly optimized using PB as a substrate as described elsewhere (Lachaud *et al*. 2001). The optimized conditions were the following for R221–R332 (method R): 5 μl of 10× buffer (250 mM Tris–HCl, pH 9·3, 500 mM KCl, 10 mM β-mercaptoethanol), 0·6 mg/ml of bovine serum albumin, 200 μM of dNTPs, 2·5 mM of $MgCl_2$, 50 pmol of each primer, 3 U of *Taq* DNA polymerase (Goldstar, Eurogentec®) for a total reaction volume of 50 μl including 5 μl of sample DNA. For the RV1–RV2 primers (method KRV) the conditions were identical except the $MgCl_2$ was at 3 mM and the *Taq* polymerase at 1·5 U. The 'hot-start' technique was used to increase specificity (Dynawax, Eurogentec®). Reactions were cycled in an MJResearch® thermal cycler using the following conditions: 94 °C for 4 min, 40 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 90 s, followed by 72 °C for 10 min for method R. For method KRV, reactions were cycled in a Biometra® thermal cycler in a different laboratory using the same conditions except an annealing temperature at 59 °C (Lachaud *et al*. 2002). Each sample was tested at least in quadruplicate. In addition, for each sample, 1 'internal' positive control tube, for detecting PCR inhibitions, was included. The latter consisted of adding highly diluted purified DNA extracted from cultivated *L. infantum* promastigotes (0·5 μl, representing only 0·1 parasite) to the 5 μl of dog sample DNA. Dilution (1/5th to 1/10th) and re-extraction of the sample DNA were tried when the PCR was inhibited. The

DNA extraction was controlled by measurement of the optical density at 260 nm in a GeneQuantII spectrophotometer (Pharmacia®). Finally, 3 negative control tubes receiving 10 μl of H_2O instead of DNA were included in each test to detect any DNA contamination. Carry-over contaminations by amplicons were avoided by using radical physical separation (of rooms, materials and personnel) as well as decontamination (e.g. UV-exposition of rooms, consumables and materials, bleaching of all materials and surfaces) procedures. For method KRV, the PCR and the demonstration of PCR products were performed in distinct hospital buildings.

Determination of PCR sensitivity thresholds

After optimization, both PCR methods were repeatedly tested using (i) promastigote purified DNA and (ii) seeded PB samples, both prepared at various decreasing concentrations as described (Lachaud *et al*. 2001). Seeding blood with extremely low parasite concentrations would be an unreliable procedure. Therefore, in order to reach parasite concentrations < 1 parasite/ml of blood, the (1 parasite/ml) seeded sample DNA was serially diluted with negative control dog blood DNA (Lachaud *et al*. 2002).

PCR product analysis and hybridization

Reaction products were visualized under UV light after electrophoresis of 20 μl of the reaction solution in a 2% agarose gel. All gels were then Southern blotted and hybridized with an α ^{32}P-labelled PCR product from our reference *L. infantum* strain with the aim of improving specificity and sensitivity.

Cloning and sequencing

PCR products were gel-purified by electro-elution and cloned into the pGEMT plasmid vector (Promega®). Nucleotide sequences were determined in both directions from double-stranded DNA using dye-primer technology in a Vistra (Amersham®) automated sequencer. Raw sequences were submitted to BLASTN searches in the GenBank™ database.

RESULTS

Sensitivity and specificity of two PCR methods in negative and positive controls using different types of samples

Two PCR methods targetting nuclear genomic (method R) or kinetoplast minicircle (method KRV) DNA respectively were used with the aim of establishing their relative value in diagnosis and parasite detection. Prior to this dog study, the sensitivities of both methods were experimentally determined after repeated testing using purified promastigote DNA as well as seeded PB samples. The 'absolute' sensitivities were 0·05 and 0·0001 parasites/PCR tube for method R and method KRV respectively. In seeded PB samples, method R detected 5 parasites/ml and method KRV 0·001 parasites/ml of blood (Lachaud *et al*. 2002). Moreover, in the present study 5 PCR products from each method were cloned, sequenced, and matched against the EMBL nucleotide sequence database: their sequence matched a consensus sequence of the *L. infantum* ribosomal DNA or kinetoplast minicircle conserved region, for method R or KRV respectively.

Both methods were tested here in 6 polyΣic dogs with CVL confirmed by BM direct examination that served as positive controls. Two of them had to be sacrificed and were 'multi-sampled': in both, the BM, PB, popliteal LNs and 12 SBs performed in distinct sites of 'healthy' skin (forelegs, hindlegs, abdomen, internal side of the ears, nose, as well as conjunctiva) were found to be PCR-positive with both methods. In the 4 other dogs, the BM, LN, PB and SB samples were all positive with method KRV, but method R was negative in 1 PB and 1 SB sample and inhibited in another SB sample. On the other hand, the 18 negative control dogs were negative by serology and by both PCR methods.

Detection of Leishmania *in polysymptomatic dogs using two PCR methods*

Forty-four dogs with several associated signs typical of CVL as well as positive specific serology were tested; 24 were recruited in veterinary clinics in the Alpes-Maritimes and 20 in a field survey in the Languedoc. The PCR tests were performed with DNA extracted from 44 PB, 22 BM, 22 LN aspirate, 44 SB and 18 DS samples. Both types of skin samples were taken from areas without any lesions. Both PCR methods were highly sensitive, but method KRV proved clearly more sensitive than method R. The sensitivity of the former was close to 100% with all samples (except SBLFs, see below), including PB (Table 1). Method R was almost as sensitive (95·4%) as KRV with BM and LN samples, but less so with the other samples. All PCRs positive with method R were found positive with method KRV but not vice-versa. The sensitivity obtained using SBs versus DSs appeared similar, about 98% with the best method KRV (see footnote Table 1). With the aim of determining if the positivity of SBs was 'strictly cutaneous' or due to contamination with circulating blood, we performed washes of the SBs immediately after sampling (see Materials and Methods section). The resulting blood-containing SBLFs were found to be as frequently positive as PB and SB samples with both methods (non-significant difference, exact test, $P = 0{\cdot}087$), suggesting that the skin is indeed 'intrinsically' infected. Method KRV

Table 1. Comparison of two PCR methods for the diagnosis of CVL in 44 polysymptomatic seropositive dogs using different biological samples

Dog biological samples (total number)	PCR results*	
	Method R	Method KRV
Blood (44)	36 pos (81·8 %) 8 neg	44 pos (100 %)
Bone marrow (22)	21 pos (95·4 %) 1 neg	22 pos (100 %)
Lymph node (22)	21 pos (95·4 %) 1 neg	22 pos (100 %)
Skin biopsy (44)	32 pos (82 %)† 5 inh 7 neg	42 pos (97·6 %)† 1 inh 1 neg
Dermal scraping (18)	11 pos (73·3 %)† 3 inh 4 neg	18 pos (100 %)‡
SBLF (22)	17 pos (80·9 %)† 1 inh 4 neg	20 pos (90·9 %) 2 neg

* pos, positive; neg, negative; inh, inhibited PCR.
† Inhibited PCRs were removed for calculation of percentages of positivity.
‡ SBs were positive for these 18 dogs.

Table 2. Comparison of serology and PCR results for the detection of 'CVL-infection' in 66 symptomatic dogs during the prevalence mass survey

PCR results*	Positive serology	Negative serology	Total
Blood+ Skin+	39 (59 %)	10 (15·3 %)	49 (74·3 %)
Blood+ Skin−	3 (4·5 %)	7 (10·7 %)	10 (15·2 %)
Blood− Skin+	0	1 (1·5 %)	1 (1·5 %)
Blood− Skin−	4 (6 %)	2 (3 %)	6 (9 %)
Total	46 (69·5 %)	20 (30·5 %)	66

* Percentages out of total number of symptomatic dogs.

was also found more 'efficient' than method R, in the sense that PCR inhibitions, observed essentially with SB and DS samples, were significantly more frequent with method R ($9/172 = 5{\cdot}2\,\%$) than with method KRV ($1/172 = 0{\cdot}6\,\%$) (Mc Nemar's test, $P < 0{\cdot}01$). As in our previous studies (Lachaud *et al.* 2000, 2001), Southern analysis using a specific DNA probe did not improve the sensitivity of any methods; in contrast, it was useful in 2 % of cases for improving the specificity of method KRV with PB samples, where artefactual bands were observed. Using these conditions, the 'technical' specificity was 100 % with both methods.

Prevalence mass surveys

Mass surveys were carried out over a period of 3 months on dogs living in outdoor kennels. All dogs present on site were tested, but dogs treated for leishmaniasis were discarded from this study. Because BM and LN sampling are more cumbersome in field studies, only PB and SBs were collected. Moreover, as method R could not detect 100 % of polyΣic dogs (Table 1), we mainly used KRV for this part of the study (see Discussion section). In total 253 dogs were included in the PCR analysis: 10 dogs were discarded due to PCR inhibitions observed with one of both samples. They were grouped according to the presence of clinical signs and serological results.

Sixty-six dogs (26 %), termed 'Σic', presented with at least 1 clinical sign suggestive of CVL. The detailed PCR results are shown in Table 2. In total, 91 % (60) of them were positive by PCR with at least 1 of both samples, compared with 69·5 % by specific serology.

Overall, 187 dogs (74 %) were aΣic. The detailed PCR results are shown in Table 3. Only 15·6 % (29) of them had a positive serology but 75·9 % (142) were PCR-positive with at least 1 of both samples: 65·2 % with PB and 37·4 % with SBs. For these dogs, the PCR thus appeared more sensitive on PB than on

Table 3. Comparison of serology and PCR results for the detection of 'CVL-infection' in 187 asymptomatic dogs during the prevalence mass survey

PCR results*	Positive serology	Negative serology	Total
Blood+ Skin+	10 (5·3 %)	54 (28·9 %)	64 (34·2 %)
Blood+ Skin−	11 (5·9 %)	47 (25·2 %)	58 (31·1 %)
Blood− Skin+	4 (2·2 %)	16 (8·4 %)	20 (10·6 %)
Blood− Skin−	4 (2·2 %)	41 (21·9 %)	45 (24·1 %)
Total	29 (15·6 %)	158 (84·4 %)	187

* Percentages out of total number of asymptomatic dogs.

Table 4. Prevalence of 'CVL-infection' found in the mass survey in 66 symptomatic (Σic) and 187 asymptomatic (aΣic) dogs, grouped according to serology, PCR and tissue sampled

	Σic dogs	aΣic dogs	Total
PCR blood+	89·4 % (59/66)	65·2 % (122/187)	71·5 % (181/253)
PCR skin+	75·7 % (50/66)	44·9 % (84/187)	52·9 % (134/253)
PCR SBLF+	85·7 % (48/56)	44·5 % (73/164)	55 % (121/220)
PCR+ (total)	90·9 % (60/66)	75·9 % (142/187)	79·8 % (202/253)
Serology+ (total)	69·7 % (46/66)	15·5 % (29/187)	29·6 % (75/253)
PCR+ and/or serology+	97 % (64/66)	78 % (146/187)	83 % (210/253)
Serology− among PCR+	30 % (18/60)	82·4 % (117/142)	66·8 % (135/202)
PCR− among serology+	8·7 % (4/46)	13·8 % (4/29)	10·6 % (8/75)
PCR+ among serology−	90 % (18/20)	74 % (117/158)	75·8 % (135/178)

SBs (McNemar's test, $P < 10^{-4}$). It is noteworthy, however, that the parasite was detected only in the skin in 11 % of these dogs, most of them being serologically negative (Table 3). About half (55 %) of the SBLFs were found PCR-positive (not shown). The PB of the corresponding dogs was also found positive in all of these cases, but the SB in only 30 % of them; this (i) confirms that the SBLF results reflect the presence of blood contained in the SB sample, (ii) shows no correlation between SBLF and SB PCR-positivity. For negative SBLFs, 54 % and 45 % of the corresponding PB and SB samples, respectively, were found positive; this reflects a lack of sensitivity of PCR on SBLFs, probably due to the small blood cell amounts analysed.

In total, our PCR assay detected *Leishmania* in 71·5 % (181) and 52·9 % (134) of all the dogs analysed in the circulating blood and skin respectively (Table 4). The prevalence of 'CVL-infection' in our field study could be estimated at 79·8 % from the PCR data, compared with 29·6 % from serological data. It rose to 83 % only when adding both PCR and serological data.

DISCUSSION

This study aimed to (i) set up a diagnostic tool with a 100 % sensitivity in clinically ill dogs; (ii) estimate the prevalence of dogs truly carrying the parasite ('CVL-infection') as opposed to seropositive dogs in an endemic focus in Southern France; (iii) try to define strategies for detection of CVL that would use modern molecular tools and still be best adapted to field surveys. The search for a 'gold standard' in CVL detection (Dye *et al*. 1993), i.e. an ideal test that 'would identify all infected dogs and no uninfected dogs' (Quinnell *et al*. 2001), has been stimulated by the advent of highly sensitive methods such as PCR. It is complicated by the fact that aΣic carriers represent a significant proportion of the dog population in endemic areas, the epidemiological consequences of which remain unclear. Yet, there is no single 100 % specific and sensitive detection test for both 'CVL-disease' or 'CVL-infection'. Molecular methods have proved more efficient and sensitive than conventional ones for the diagnosis of the disease, both in humans and dogs (Adhya *et al*. 1995; Mathis & Deplazes, 1995; Costa *et al*. 1996; Berrahal *et al*. 1996; Reale *et al*. 1999; Lachaud *et al*. 2000; Pizzuto *et al*. 2001). In contrast, although it was reported to be a sensitive tool for the detection of aΣic carriers (Berrahal *et al*. 1996; Reale *et al*. 1999; Solano-Gallego *et al*. 2001), the superiority of PCR in epidemiological surveys remains subject to debate (Quinnell *et al*. 2001). This may be because few studies tested large numbers of dogs, or else because most of them used different PCR methods and types of samples. Indeed, like for serology (Mancianti *et al*. 1986; Berrahal *et al*. 1996), large variations in sensitivities can be observed among different PCR methods, and the choice of the primers and of the tissue to be sampled are of utmost importance in the

test performances (see below). Here, the 2 PCR methods that we validated in comparison with serology and clinical status, proved of different value according to the tissue examined and whether they were applied for diagnosis or prevalence studies.

Value of PCR in CVL diagnosis (*polyΣic dogs*)

For CVL diagnosis in sick dogs, all types of samples can generally be considered, as the dog owner is ready to accept invasive procedures. We tested 2 PCR methods exhibiting widely different sensitivity thresholds in polyΣic dogs with positive specific serology. The 'theoretical' sensitivity of both methods was 5 parasites/ml and 0·001 parasites/ml of blood for method R and KRV respectively. It must be stressed that these sensitivity thresholds still imply that at least 25 parasites for method R and 1 parasite for method KRV be present in our blood sample (5 ml), thereby considerably lowering the 'practical' sensitivity of method KRV. Method KRV proved 100% and method R 96% sensitive with 48 samples from 6 positive control dogs with confirmed *Leishmania* infection. When applied to a larger number of polyΣic dogs, only method KRV kept its level of sensitivity (100%). Method R was not sensitive enough to detect the parasite in all sick dogs: the sensitivity was maintained at 95·4% with invasive samples only, and on the whole was 81·8%. This highlights the need for a highly sensitive method to get an efficient diagnosis of CVL. Here, a primer pair targetting a highly repetitive sequence (such as minicircle kinetoplast DNA) appears necessary. It is noteworthy, however, that only 1 PCR assay was reported as highly sensitive as KRV (Reithinger *et al*. 2000), and that other kinetoplastic primer pairs may show a lower sensitivity than the one used in this study (Ashford *et al*. 1995; Reithinger *et al*. 2000; Martin-Sanchez *et al*. 2001). To our knowledge, there is only 1 other published comparison of PCR methods for leishmaniasis, which also stressed the differences of sensitivity among PCR assays (Reithinger *et al*. 2000). The determination of the 'theoretical' and 'practical' sensitivity of the PCR assay used is therefore a prerequisite to any PCR study.

We also wanted to determine which type of sample was best for PCR diagnosis of 'CVL-disease'. With both methods used, the samples giving the highest sensitivity were the BM and LN. The comparison of results with both methods suggests that the parasite load is higher in BM and LN than in the skin or PB. This correlates well with the generally shared opinion and the suggestions by a few authors that LN aspirates are the best sample for the detection of 'CVL-disease', both by non-molecular and molecular methods (Lanotte *et al*. 1974; Ozensoy *et al*. 1998; Reale *et al*. 1999). On the other hand, in these polyΣic dogs, the sensitivity obtained with one given method was similar whether we used PB or SBs. But method KRV appeared significantly more sensitive than R particularly with these two samples (exact test, $P = 0{\cdot}0001$ and 0·002 for PB and SBs respectively): thus, with the former, almost all sick dogs harboured the parasite in both PB and 'healthy' skin. PB appears reliable for diagnosing CVL in Σic dogs. Nevertheless, the extreme sensitivity of method KRV may be problematic: indeed, considering both sets of data, the positive predictive value for 'CVL-disease' is 30% with KRV compared with 100% with R. The use of less sensitive PCR methods may, therefore, be considered as more appropriate for CVL diagnosis than the most sensitive ones (see below); to obtain a highly sensitive diagnosis with the former, the examination of BM and LN aspirates is recommended when PB is found negative, e.g. here in 18% of the cases.

PCR in CVL prevalence mass surveys

Since method R could not detect all clinically ill dogs, we used method KRV for the prevalence study. The sensitivity of PCR in Σic dogs here (90·9%) was not 100% as in the first part of the study. This can be explained in 2 ways: (i) oligoΣic, and not just polyΣic, dogs were included here and these might have a lower parasite load, undetectable by our method; and/or (ii) the symptoms observed may not be related to leishmaniasis – e.g. for the 2 dogs with negative PCR and serology. On the other hand, CVL prevalence mass surveys come up against the problem of aΣic parasite carriers. In our survey, 74% of dogs presented as aΣic and 75·9% of them were found to harbour the parasite by PCR. In contrast, the seroprevalence was only 15·5% in these dogs. If we had lowered the cut-off value for IFAT to 1/20 like some authors (Cabral *et al*. 1998), the seroprevalence would have been 19·2% in these aΣics.

The diagnosis of CVL in PCR-positive aΣic dogs with positive serology is usually not put in doubt by veterinarians, but those aΣics with positive PCR and negative serology may be questioned as false positives. Here, a large majority (84·5%) of aΣics were seronegative. Nevertheless, 74% of these seronegative aΣics were PCR-positive with our most sensitive method. Several points argue for the latter being true positives. (i) The 'technical' specificity of our PCR assay was 100% in the conditions used here: indeed, there was no evidence for DNA contamination, as all negative control tubes remained negative during the whole course of this study ($\sim$ 300 tubes for method KRV and 2400 tubes for method R which is used in routine human VL diagnosis). Moreover, DNA hybridization was used systematically to test for the presence of spurious amplification products which could be mistaken as positives. Hybridization-positive PCR products amplified in these dogs were

cloned and sequenced, and their sequence matched a consensus sequence of the *L. infantum* kinetoplast minicircle conserved region. (ii) The specificity against other microorganisms was also 100 % (Ravel *et al*. 1995). (iii) A group of 18 negative control dogs were all found PCR negative. (iv) Finally, 38 positive PB samples from these seronegative aΣic cases were DNA-extracted twice at different time-intervals and all extractions were found positive (not shown). We are, therefore, confident that the aΣic PCR-positive dogs detected here may be considered as authentic 'healthy' carriers. They represented 56 % of the dogs surveyed and would have remained undetected using conventional methods. Other authors have also reported PCR-positive seronegative aΣic dogs, some of them with a microscopically confirmed infection (Martin-Sanchez *et al*. 2001; Reale *et al*. 1999; Solano-Gallego *et al*. 2001). These findings are consistent with longitudinal studies which indicated that a proportion of dogs never sero-convert and the fact that protective immunity in CVL is essentially cell-mediated (Cabral *et al*. 1998; Campino *et al*. 2000; Pinelli *et al*. 1994).

When considering the type of sample examined, it is striking that 89·4 % of Σic and 65·2 % of aΣic dogs harboured circulating parasites in the PB. The latter percentage is high, although lower than the 80 % reported by Reale *et al*. (1999) in aΣics. The latter, however, included some dogs with enlarged LNs. When examining healthy skin (free of blood), we found a positive PCR in 75·7 % of Σic and 44·9 % of aΣic dogs. The latter figure agrees with Solano-Gallego *et al*. (2001) and the higher figure (64 %) reported by Berrahal *et al*. (1996) on 14 aΣic dogs is not statistically different (χ^2 test). On the whole, therefore, the PCR sensitivity in all dogs surveyed was much higher in PB than in 'healthy' skin, i.e. 71·5 % versus 52·9 % (McNemar's test, $P < 10^{-6}$). However, it is noteworthy that this difference between the tissues appears to decrease with the increase in clinical signs.

In our opinion, other sample types are less suited for mass surveys than PB or SBs. LN aspirates can yield an excellent sensitivity in Σic dogs (> 90 % by PCR) and present fewer technical problems for PCR (Reale *et al*. 1999; this study); but they are straightforward only in dogs presenting with an enlarged LN, and therefore in (according to our criteria) Σic dogs. BM examination was not more sensitive than LN in our study and, surprisingly, yielded a much lower PCR sensitivity in other studies (Solano-Gallego *et al*. 2001), even in areas of apparently higher endemicity (Ashford *et al*. 1995). In any case, BM aspiration is usually impractical in field mass surveys. Finally, another site, conjunctiva, has been biopsied in CVL surveys with variable success: 32 % (Solano-Gallego *et al*. 2001) and 94 % (Berrahal *et al*. 1996); here again, it is impractical outside of a veterinary centre.

What is/are the best modern strategy(ies) for CVL prevalence surveys?

The above data may help to define the best adapted strategy(ies) for the biological detection of CVL in mass surveys. Here, the KRV PCR on PB and SBs yielded excellent results. It permitted the prevalence of *L. infantum* infection in our region to be estimated at 79·8 % in a cohort of 253 dogs (versus 29·6 % by serology). This is consistent with other PCR reports in areas of comparable endemicity: 80 % (Berrahal *et al*. 1996), 79·5 % (Reale *et al*. 1999), 57 % (Solano-Gallego *et al*. 2001). It is noteworthy that we tested about one third of the present cohort with our least sensitive PCR method (R), and the estimated prevalence rate using this method might then have been 26 % only (our unpublished data).

PB appears as the most useful sample for this type of survey, and it allows serology to be performed concurrently. A PCR on LN aspirates may also be performed when the LN is easy to puncture with a needle. Healthy skin may be sampled if the circumstances permit, since, with our PCR assay, it allowed the detection of 8 % more infected dogs, essentially among aΣic dogs. The drawback of the SB is that it is relatively painful and bloody.

From our and others' data, a highly sensitive PCR appears as the method of choice to improve the biological detection of 'CVL-infection' in both Σic and aΣic dogs. Among other methods, direct examination and culture are not sensitive enough as well as being too cumbersome for large prevalence surveys. But there is a role for immunological tests: classical serology is cheap, and the ELISA technique might improve its sensitivity. If we tentatively consider parasite carriers as those detected by the most sensitive PCR method in one of the two tissues sampled (PB and/or SB), the serological screening in our study would have detected 33·5 % of parasite carriers. The PCR on PB would have pushed the total up to 90·9 % (57·4 % more), and then the PCR on SBs up to 100 %.

CVL may be considered as a good illustration of the considerable change that molecular tools can bring in diagnosis and epidemiology. For the diagnosis of 'CVL-disease', a highly sensitive PCR assay such as KRV is a double-edged tool in view of its low positive predictive value; it appears that both methods analysed here offer an improved but not yet ideal diagnostic tool. By contrast, in epidemiology, as already suggested by others (Ashford *et al*. 1995; Reale *et al*. 1999; Reithinger *et al*. 2000), a highly sensitive PCR assay may be considered as the possible 'gold standard' to define *Leishmania* infection. This disagrees with the views of Quinnell *et al*. (2001) who favoured serology, this divergence might be explained by better performances of our PCR assay and/or of their serological technique respectively, or else by differences in the immu-

nology and pathophysiology of CVL between their and our dog population, particularly in relation to nutritional status. Our and others' findings about the high proportion of aΣic carriers contrast with the 'classical' ideas of CVL endemicity, established using serology. In view of these findings, the role of aΣic dogs in the maintenance of leishmanial endemicity might be much more relevant than previously thought. However, (i) the long-term clinical evolution of aΣic dogs is yet unclear, as they might be at a pre-symptomatic stage; (ii) their role in transmission is probably lower than that of Σic dogs (Hasideber, Dye & Carpenter, 1992; Dye, 1996). Xenodiagnosis studies brought conflicting evidence on this matter (Rioux *et al*. 1972; Molina *et al*. 1994; Travi *et al*. 2001). A better understanding of the parasite dynamics in and among hosts in endemic foci might now come from large-scale and longitudinal studies combining quantitative PCR (for a better assessment of parasite loads) and xenodiagnosis in the field.

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