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Detection of *Leishmania infantum* by PCR, serology and cellular immune response in a cohort study of Brazilian dogs

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SUMMARY

The sensitivity and specificity of PCR, serology (ELISA) and lymphoproliferative response to *Leishmania* antigen for the detection of *Leishmania infantum* infection were evaluated in a cohort of 126 dogs exposed to natural infection in Brazil. For PCR, *Leishmania* DNA from bone-marrow was amplified with both minicircle and ribosomal primers. The infection status and time of infection of each dog were estimated from longitudinal data. The sensitivity of PCR in parasite-positive samples was 98%. However, the overall sensitivity of PCR in post-infection samples, from dogs with confirmed infection, was only 68%. The sensitivity of PCR varied during the course of infection, being highest (78–88%) 0–135 days post-infection and declining to around 50% after 300 days. The sensitivity of PCR also varied between dogs, and was highest in sick dogs. The sensitivity of serology was similar in parasite-positive (84%), PCR-positive (86%) and post-infection (88%) samples. The sensitivity of serology varied during the course of infection, being lowest at the time of infection and high (93–100%) thereafter. Problems in determining the specificity of serology are discussed. The sensitivity and specificity of cellular responsiveness were low. These data suggest that PCR is most useful in detecting active or symptomatic infection, and that serology can be a more sensitive technique for the detection of all infected dogs.

Key words: *Leishmania infantum*, PCR, diagnosis, lymphoproliferation, Brazil, dogs.

INTRODUCTION

Visceral leishmaniasis, caused by *Leishmania infantum* (=*L. chagasi*, Mauricio *et al*. 1999), is a zoonotic disease found in Latin America, Europe, Asia and Africa (Ashford, Desjeux & Deraadt, 1992). In most of this range, the domestic dog is the main reservoir. The prevalence and incidence of canine infection are important epidemiological parameters, the estimation of which depends on the reliable identification of infected dogs (Dye *et al*. 1992). Parasitological examination, by microscopy, culture or hamster-inoculation of biopsy material, provides an unambiguous means of detecting infection, but is known to be insensitive (Schnur & Jacobson, 1987). Serological methods are likely to be more sensitive, and a wide variety of possible serological techniques and antigens have been evaluated. However, serology may be less specific due to cross-reactions with other infections, and the choice of a suitable cut-off may not be obvious (Dye *et al*. 1992). Moreover, seroprevalence may underestimate the true prevalence of infection, since there is known to be a significant period between infection and seroconversion (Abranches *et al*. 1991; Dye, Vidor & Dereure, 1993; Quinnell *et al*. 1997), and it is possible that a significant fraction of infected dogs may never seroconvert (Dye *et al*. 1993; Pinelli *et al*. 1994), or may become seronegative.

Recently, a number of studies have investigated the use of PCR for detecting *L. infantum*. A variety of PCR primers have been developed, and PCR has been confirmed to be a sensitive technique for the detection of symptomatic or parasitologically proven infections (Ashford *et al*. 1995; Mathis & Deplazes, 1995; Berrahal *et al*. 1996; Reale *et al*. 1999; Roura, Sanchez & Ferrer, 1999). However, whether PCR is sensitive enough to detect all infected dogs is unknown: although PCR detects infection in a proportion of seronegative dogs from endemic areas, a proportion of seropositive dogs are negative by

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PCR (Ashford *et al*. 1995; Berrahal *et al*. 1996). PCR has, to date, only been used on cross-sectional or selected samples from small numbers of dogs. There have been no longitudinal studies with PCR, and no reports of variation in sensitivity with host age. Consequently, the interpretation of the available cross-sectional data is difficult. In particular, it is not known whether the failure of PCR in a proportion of seropositives represents lack of sensitivity of PCR, recovery from infection with loss of parasites but not antibodies, or serological cross-reactions.

The sensitivity of any diagnostic technique is likely to change both during the course of infection, and between dogs. Early in infection, in the period before seroconversion or development of symptoms, parasitology or PCR may be most sensitive. As infection progresses, variation between dogs in their ability to control infection will become apparent. A variable proportion of dogs infected with *L. infantum* become clinically sick, with high antibody titres, high parasite loads and suppressed cellular immune responses (Pinelli *et al*. 1994). In contrast, asymptomatic dogs may have strong cellular responses but have lost (or never developed) detectable antibodies or parasites (Pinelli *et al*. 1994). These results suggest that there may be no single test which reliably identifies infection in all dogs, and that current or previous infection in resistant dogs may be best detected by assays of cellular immune response, such as leishmanin skin tests or lymphoproliferation. Field studies have confirmed that cellular immune responses can be detected in a proportion of seronegative animals, and not in a proportion of seropositive animals (Cabral, O'Grady & Alexander, 1992; Cabral *et al*. 1998; Cardoso *et al*. 1998). However, as for serology, there may be no clear cut-off for cellular responsiveness, and the sensitivity and specificity of cellular responses have not been widely tested.

We have carried out a longitudinal study of 126 sentinel dogs exposed to natural infection in an endemic area of Brazil. Cellular immune responses (lymphoproliferative response to crude *L. infantum* antigen), serology (ELISA) and detection of parasite DNA in bone-marrow biopsies by PCR were performed on most dogs every 2 months. Serological data from this study have been used to estimate the incidence rate and basic reproduction number of *L. infantum* in dogs (Quinnell *et al*. 1997). Here we present results from both cross-sectional and longitudinal analyses of the PCR, lymphoproliferative and serological data. The aims of the present study were (1) to assess the relative sensitivity and specificity of each technique in detecting *L. infantum* infection in dogs, (2) to assess the variation in sensitivity of each technique with time post-infection (p.i.), and (3) to assess the sensitivity of each technique in asymptomatic and symptomatic infected dogs.

MATERIALS AND METHODS

Study design

We worked in 24 villages in the municipality of Salvaterra, Marajó Island, Pará State, Brazil (48° 03′ W, 00° 46′ S). Details of the study site and study design are described elsewhere (Quinnell, Dye & Shaw, 1992; Courtenay *et al*. 1994; Quinnell *et al*. 1997). Briefly, we studied 99 uninfected dogs from the city of Belém, and 27 young, serologically negative animals born in the study area. These 126 dogs were given to households in the endemic area to be kept as pets, and sampled a maximum of 13 times at a mean interval of 67·3 days (range 58–80 days). The study ran from April 1993 to July 1995, with dogs being placed in the field at 8 time-points in groups of 5–37 dogs. Negative control (uninfected) diagnostic samples comprised the first sample from the 126 study dogs, samples from additional dogs from Belém, and sera only from 85 dogs attending a veterinary clinic in Utrecht, Netherlands. Positive control samples were samples taken from study dogs at any time when *Leishmania* parasites were found in their bone marrow biopsy by culture or microscopy.

Sampling

Dogs were anaesthetized with a mixture of Medetomidine hydrocholoride (Domitor®) and Ketamine (Vetelar®). Then 20 ml of blood were taken by venepuncture from the jugular vein. Bone-marrow was aspirated from the iliac crest with a 16 × 25 mm Klima needle (Veterinary Instruments, Newcastle) into a 20 ml syringe containing 0·5 % EDTA. The sample was divided between triplicate tubes for PCR analysis, 2 sterile Difco blood-agar slopes (sampling rounds 1–9) and used to make 1–4 thin smears.

Antigen preparation

Sonicated Brazilian *L. infantum* (MHOM/BR/74/PP75) promastigotes were prepared as previously described (Quinnell *et al*. 1997).

Cellular immune responses

Twenty ml of venous blood were defibrinated and triplicate 2 ml serum samples were taken after centrifugation. The remaining blood was diluted 1 : 1 in sterile PBS and peripheral blood mononuclear cells separated on Histopaque 1077 (Sigma) (800 ***g***, 30 min). Mononuclear cells were washed twice in wash medium (RPMI 1640 Dutch modification, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM Na pyruvate and 25 μM 2-mercaptoethanol), counted in 0·4 % trypan blue and resuspended at 10^6 cells/ml in wash medium + 10 % FCS. Cells were cultured in quintuplicate in 96-well

plates at 2×10^5 cells/well (200 μl/well) and incubated at 37 °C, in 5% CO_2 in air with and without sonicated *L. infantum* promastigotes (10^5 parasite equivalents/well). DNA synthesis was assessed by adding 1 μCi/well of methyl[^{3}H]thymidine (DuPont) after 6 days, incubating overnight (16 h) and harvesting onto glass-fibre filters. Radioactivity was counted in a β-scintillation counter, and results expressed as a stimulation index (SI): the ratio of the geometric mean cpm of wells plus antigen to the geometric mean cpm of unstimulated wells. The frequency distribution of stimulation indices was unimodal, with no distinction between non-responders and responders; an arbitrary cut-off of SI > 5 was chosen to define positives. A total of 420 samples was tested for lymphoproliferation.

ELISA

Anti-*L. infantum* IgG was detected by ELISA as previously described (Quinnell *et al*. 1997). Test sera were titrated from 1/50 to 1/800, and antibody concentrations were expressed in arbitrary units, relative to a highly positive control serum titrated on each plate. Two cut-offs were chosen to define seropositives: (i) the best cut-off estimated from the frequency distribution of antibody titres (=2253 units/ml) (Quinnell *et al*. 1997), and (ii) the mean + 3 S.D. of the negative control samples (=4006 units/ml). For longitudinal analysis, the timing of seroconversion was determined from both the frequency distribution of seronegative samples and longitudinal changes in antibody titre (Quinnell *et al*. 1997). A total of 756 samples was tested by ELISA.

Parasitology

Examination of cultures and smears was by standard techniques (Quinnell *et al*. 1997).

DNA extraction

Bone-marrow biopsies were collected into 200 μl of NET buffer (15 mM Tris–HCl, pH 8·3, 1 mM EDTA, 150 mM NaCl) containing 1% SDS, incubated at 65 °C for 2 h and stored at −20 °C. Samples were digested overnight at 56 °C with 1 mg/ml proteinase K. DNA was extracted using phenol–chloroform and resuspended in 20 μl of TE buffer (10 mM Tris–HCl, pH 8·0, 1 mM EDTA), 1 μl of which was run on a 1% agarose gel to confirm the integrity of the DNA.

PCR

Two sets of primers, amplifying different targets, were used. Primers DBY and AJS31, with probe B4Rsa, amplify and detect a 805-bp minicircle and are specific for parasites of the *L. donovani* group (Smyth *et al*. 1992; Scrimgeour *et al*. 1998). Primers R221 and R332 amplify a 604-bp segment of the small subunit ribosomal RNA gene of all *Leishmania* species (van Eys *et al*. 1992); an internal oligonucleotide (R331) was used as a probe. DNA samples (1 μl) were amplified in 50 mM NH_4Cl, 10 mM Tris–HCl, pH 8·3, 1·5 mM $MgCl_2$, 0·2 mM dNTPs, 20 pmol of each primer and 1 unit of *Taq* polymerase (Bioline) in a total volume of 25 μl. For primers DBY/AJS31, samples were initially denatured at 96 °C for 5 min, followed by 35 cycles of 95 °C (30 sec)–67 °C (1 min)–72 °C (1 min), and finally 72 °C (10 min). For primers R221/R332, 35 cycles of 94 °C (75 sec)–60 °C (1 min)–72 °C (2 min) were performed. Positive (*L. infantum* lysate) and negative controls (water) were run in each set of PCR reactions. Samples of 5 μl of each PCR reaction were analysed by electrophoresis on 2% agarose gels and transferred to nylon membranes. Probes were labelled with digoxygenin and processed according to the manufacturer's instructions (Boehringer–Mannheim). In total 439 samples were tested by PCR; all samples were amplified with primers AJS31/DBY. Samples that were strongly positive by gel electrophoresis and hybridization were not amplified again; samples which were either negative or weakly positive only after hybridization were also amplified with primers R221/R332. Where the results of the two PCRs differed, each was repeated. Positive samples were defined as those that were positive in both reactions, and negative samples as consistently negative in both reactions. Thirty-one samples which were positive in only 1 reaction were discarded. Of 38 samples which had no visible DNA by agarose gel electrophoresis 32 were negative in both PCR reactions and were excluded from analysis, although these samples were positive by PCR for the dog GAPD gene (data not shown). The total number of samples included in the analysis was thus 376.

Clinical examination

At most sampling times (n = 561), dogs were scored for 6 typical signs of canine visceral leishmaniasis: alopecia, dermatitis, chancres, conjunctivitis, onychogryphosis (excessive nail growth), and lymphadenopathy (enlarged popliteal lymph nodes). Each sign was scored on a semi-quantitative scale from 0 (absent) to 3 (severe), and these scores were added together to give an overall clinical score. Dogs with a total score of 0–2 were arbitrarily classed as asymptomatic, with a score of 3–6 as oligosymptomatic, and with a score of 7–18 as polysymptomatic. Dogs which developed very severe disease were euthanized with sodium pentobarbitone (Euthanol®).

Statistical analysis

Cellular response (SI) was analysed by regression of log-transformed data. The relationships between

time p.i., clinical status and the proportion of samples positive by each technique was analysed by multiple logistic regression. Robust standard errors on clustered data were calculated to control for non-independence of data due to repeated sampling from each dog. Analysis was carried out using Stata 6.0 (StataCorp, 1999).

RESULTS

Sensitivity and specificity of techniques in positive and negative controls

PCR was highly sensitive in positive control samples, detecting 40/41 (97·6%) of samples in which parasites were confirmed by culture or direct examination (Table 1). The sensitivity of serology was lower (80–84%), and that of cellular responsiveness only 64·6%. The specificities of PCR and serology were high, 98–100%, but 16·7% of samples from negative control (uninfected) dogs had a positive cellular response.

Cross-sectional comparison of techniques

The sensitivity of serology to detect PCR positives was 86·0% with the lower cut-off and 77·7% with the higher cut-off (Table 1). Irrespective of the choice of cut-off, a high proportion (37–43%) of PCR negatives were seropositive. PCR gave a positive result in only 59·0% of samples from seropositive dogs, while 15·0% (22/147) of samples from seronegative dogs were PCR positive. There was a positive cellular response in 70·0% of PCR positives and 72·0% of seropositives, while 58·7% of PCR negatives and 29·2% of seronegatives had a positive cellular response.

The interpretation of the data in Table 1 is difficult: as an example, it is not clear if the 43% of PCR negative samples which were positive by serology are false-positives e.g. cross-reactions, are from previously infected dogs which have lost parasites but not antibodies, or reflect a lack of sensitivity of the PCR technique. However, using the longitudinal data, samples can be more reliably classified as uninfected or infected.

Longitudinal classification of dogs

Of the 126 study dogs, infection was confirmed in 72 dogs by PCR or parasitological examination, and in 1 additional dog by xenodiagnosis (Courtenay, 1998). Sixty-two of these dogs were observed to seroconvert; all of the other 11 dogs either died or left the study within 135 days of infection. Twenty-eight samples from 26 dogs were PCR or parasite positive but seronegative: 16 of these came from dogs which later seroconverted (after 67–135 days), and 12 came from the 11 dogs which died or left the study. Thus all infected dogs apparently seroconverted, and all PCR-positive, seronegative samples came from dogs early in infection, before actual or expected seroconversion.

An additional 13 dogs seroconverted but did not have infection confirmed by parasitology or PCR. These dogs may represent serological cross-reactions, or resistant dogs with very low parasite loads. Cellular responses were measured in 10 of these dogs: all had positive cellular responses after seroconversion, when their mean stimulation index did not differ from that of the 62 dogs with confirmed infection ($F_{1,66} = 0{\cdot}32$, $P = 0{\cdot}57$), which suggests that they had been infected. Finally, 40 dogs showed no evidence of infection by either serology or PCR, most of which died early in the study. These dogs had significantly lower cellular responses than dogs with confirmed or suspected infection ($F_{1,85} = 49{\cdot}3$, $P < 0{\cdot}001$), and there was no evidence for an increase in their cellular responsiveness through time ($F_{1,14} = 0{\cdot}36$, $P = 0{\cdot}56$).

Thus we have 2 estimates from longitudinal data of the total number of infected dogs in the study: 73 dogs had confirmed infection, and 86 dogs had confirmed infection or serologically suspected infection. We estimated the time of infection as the earliest date on which either parasites were detected (by PCR, culture or direct examination), or seroconversion occurred (Quinnell *et al*. 1997). Samples from each dog were then classified as uninfected (pre-infection or never infected) or post-infection and the sensitivity and specificity of the techniques estimated using these two groups of samples. The true date of infection will be earlier than our estimated date, and thus the 'uninfected' samples will include some samples taken from infected dogs soon after infection.

Longitudinal comparison of techniques

If we consider only dogs with confirmed infections as infected (i.e. suspected infections as uninfected), the sensitivity of PCR to detect post-infection samples was only 68·0% (Table 2). In contrast, the sensitivity of serology was 87·5% with the lower cut-off, and 78·4% with the higher cut-off. The specificity of serology for identifying confirmed infections was 84·5% and 88·9% for the 2 cut-offs. The sensitivity of cellular responsiveness was 68·8%, and the specificity only 54·3%.

Considering infected dogs to include both confirmed or suspected infection, the sensitivity of PCR in post-infection samples was, as expected, lower, 60·6%. The sensitivity of serology was similar to before: 88·3% for the lower cut-off, and 77·7% for the higher cut-off.

Variation in sensitivity during the course of infection

Considering only dogs with confirmed infections, the sensitivity of PCR was highest shortly after

Table 1. The proportion of samples from different groups of dogs that were positive for *Leishmania infantum* by PCR, serology (using 2 cut-offs) and cellular immune response

	Percentage of samples positive (no. positive/total) by			
Type of sample	PCR	Serology >2253	Serology >4006	Cellular response
Parasite positive	97·6 % (40/41)	84·4 % (54/64)	79·7 % (51/64)	64·6 % (31/48)
Negative control	0 % (0/5)	2·3 % (7/308)	0·3 % (1/308)	16·7 % (5/30)
PCR positive	—	86·0 % (135/157)	77·7 % (122/157)	70·0 % (77/110)
PCR negative	—	42·9 % (94/219)	36·5 % (80/219)	58·7 % (74/126)
Seropositive*	59·0 % (135/229)	—	86·5 % (315/364)	72·0 % (221/307)
Seronegative	15·0 % (22/147)	—	—	29·2 % (33/113)

* >2253 arbitrary units/ml of IgG.

Table 2. The proportion of samples that were positive for *Leishmania infantum* by PCR, serology and cellular immune response, according to the infection status of dogs

(Dogs were classified longitudinally as developing confirmed infections, suspected infections, or never infected. Samples from these dogs were grouped as post-infection, or uninfected (pre-infection or never infected).)

	Percentage of samples positive (no. positive/total) by			
Type of sample	PCR	Serology >2253	Serology >4006	Cellular response
Post-infection samples from confirmed infections (suspected infection classed as uninfected)				
Post-infection	68·0 % (157/231)	87·5 % (300/343)	78·4 % (269/343)	68·8 % (185/269)
Uninfected	—	15·5 % (64/413)	11·1 % (46/413)	45·7 % (69/151)
Post-infection samples from confirmed and suspected infections				
Post-infection	60·6 % (157/259)	88·3 % (356/403)	77·7 % (313/403)	71·2 % (230/323)
Uninfected	—	2·3 % (8/353)	0·6 % (2/353)	24·7 % (24/97)

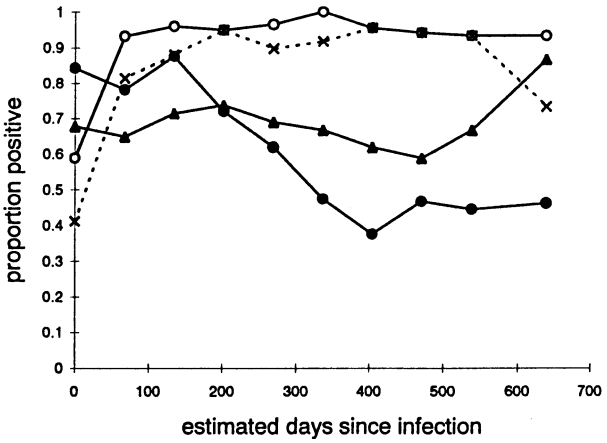

Fig. 1. Variation in the proportion of dogs with confirmed infection that were positive for *Leishmania infantum* by PCR (●), cellular responsiveness (▲), serology (>2253 units/ml of IgG) (○) and serology (>4006 units/ml of IgG) (×) with estimated time since infection.

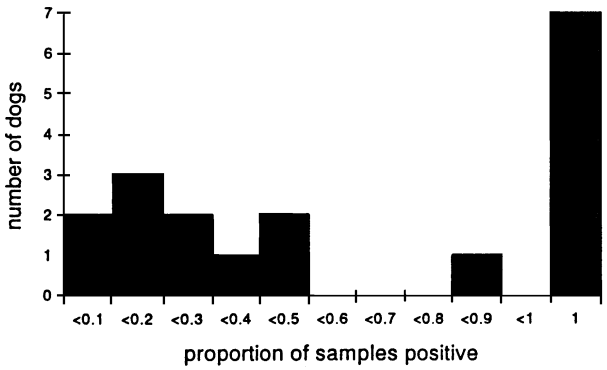

Fig. 2. Variation in the proportion of post-infection samples which were positive for *Leishmania infantum* by PCR. Only dogs with confirmed infection that were sampled 5 or more times post-infection were included.

infection (78–88 %), but then declined significantly to around 50 % ($P < 0{\cdot}001$) (Fig. 1). The sensitivity of serology was comparatively low at the time of infection (41–59 %), but high (93–100 %) thereafter, although there was no significant relationship between sensitivity and time since infection. The sensitivity of cellular responsiveness also did not vary with the time since infection.

Parasite detection rates by PCR differed between individual dogs. Eighteen of the dogs which developed a confirmed infection were sampled 5 or more times p.i. These dogs were positive by PCR on between 0 and 100 % of occasions (Fig. 2). There was no relationship between the number of times

Table 3. The proportion of post-infection samples from dogs with confirmed infections that were positive for *Leishmania infantum* by PCR, serology and cellular immune response, according to clinical condition at the time of sampling

(Differences between groups were tested by multiple logistic regression, controlling for time post-infection.)

	Percentage of samples positive (no. positive/total) by			
Type of sample	PCR	Serology >2253	Serology >4006	Cellular response
Asymptomatic	65·0% (91/140)	83·8% (176/210)	71·0% (149/210)	75·3% (113/150)
Oligosymptomatic	61·4% (27/44)	97·2% (70/72)*	93·1% (67/72)*	61·4% (43/70)*
Polysymptomatic	85·4% (35/41)**	94·1% (48/51)	94·1% (48/51)*	60·4% (29/48)

* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$ (*vs* asymptomatic group).

these dogs were sampled and the percentage of times they were PCR positive ($\chi^2 = 1{\cdot}8$, D.F. = 1, N.S.).

Variation in sensitivity according to clinical status

Serology, using either cut-off, was a highly sensitive method for detecting oligosymptomatic and polysymptomatic dogs post-infection, but was least sensitive in asymptomatics (Table 3). The sensitivity of PCR was high in polysymptomatic dogs, but relatively low in oligosymptomatic and asymptomatic dogs. In contrast, the sensitivity of cellular responsiveness was highest in asymptomatics and declined with increasing clinical score.

DISCUSSION

An ideal diagnostic test for canine visceral leishmaniasis would identify all infected dogs, and no uninfected dogs: a 'gold standard' for infection. In the absence of such a gold standard, we have examined the longitudinal course of infection in a cohort of initially uninfected dogs exposed to natural infection. The sensitivity and specificity of PCR, serology and cellular immunology were calculated for 2 groups of dogs: dogs which developed confirmed infections (positive by parasitology or PCR), and dogs which developed either confirmed infection or infection suspected from a positive antibody and cellular immune response.

A number of studies have shown PCR to be a sensitive technique for diagnosis of clinical *L. infantum* infection in dogs, detecting 89–100% of symptomatic or parasitologically proven infections (Ashford *et al.* 1995; Mathis & Deplazes, 1995; Berrahal *et al.* 1996; Reale *et al.* 1999; Roura *et al.* 1999). Similarly, we found PCR to be very sensitive (97·6%) in detecting infection in samples where parasites could be detected by culture or direct examination. However, the sensitivity was much lower (68·0%) when all confirmed post-infection samples were examined, even though the PCR results were used to confirm infection in these dogs. If we consider post-infection samples from all dogs with either confirmed or suspected infection, the sensitivity of PCR was even lower, only 60·6%. Our estimated sensitivities in post-infection samples will be overestimates, since some samples from infected dogs taken early in infection will have been misclassified as uninfected. The proportion of samples misclassified will depend on the length of the prepatent period between infection and detectable infection: this was estimated as an average of 94 days for serological detection (Quinnell *et al.* 1997), and will be shorter for detection by PCR. This is relatively short compared to the total length of the study, and thus the proportion of samples misclassified is not likely to be have been large.

The sensitivity of PCR varied markedly with time post-infection. Very early in infection, the sensitivity will have been zero, rising to a peak of 78–88% at 0–135 days after observed infection. Thereafter, sensitivity declined to around 50%; this decline may have been due to loss of parasites and/or selective mortality of PCR positive dogs. PCR was a sensitive technique in only a proportion of infected dogs: the proportion of samples positive by PCR varied considerably between dogs. The sensitivity of PCR is likely to depend on the parasite load, and when parasites are abundant enough to be detected in culture, PCR is more successful. PCR is thus most sensitive in a 'susceptible' fraction of dogs, which have high parasite loads and clinical disease.

The sensitivity of serology was examined using two cut-offs: one estimated from the frequency distribution of antibody titres (2253 units/ml) (Quinnell *et al.* 1997), and one calculated from the titres of negative control animals (4006 units/ml). As expected, the former cut-off performs better, with a greater sensitivity in detecting confirmed infections. Using this cut-off, the sensitivity of serology to detect parasite positives, PCR positives or post-infection samples varied from 84 to 88%. Serology failed to detect 43 of 343 (12·5%) confirmed post-infection samples; 36 of these were from dogs early in infection (prior to seroconversion), and 7 from 2 dogs which

serorecovered. Since the true date of infection will have been earlier than that estimated, the actual number of pre-seroconversion, infected samples will be higher than estimated (and thus the true sensitivity will be lower). However, it is clear that, after this pre-seroconversion period, the sensitivity of serology to detect infected dogs was high. Two factors contribute to this high sensitivity. First, the rate of serorecovery in the study dogs was very low (Quinnell *et al*. 1997); despite the loss of detectable parasites through time, serology remained sensitive. Second, all infected dogs apparently seroconverted: all PCR or parasite positive samples in seronegatives were followed by either seroconversion or the dog leaving the study within 135 days, and dogs which were both seronegative and PCR negative did not develop positive cellular responses. This confirms the results from modelling of the serological data (Quinnell *et al*. 1997). In contrast, some European studies have suggested that a significant fraction of infected dogs may never seroconvert (Dye *et al*. 1993; Pinelli *et al*. 1994).

Unfortunately, in the absence of a gold standard for infection, we cannot be certain of the specificity of serology. The false-positive serology rate in negative control samples was only 2·3 %, even for the lower cut-off. However, 15·5 % of samples without confirmed infection were seropositive, a specificity of only 84·5 %. These samples came from 13 dogs which seroconverted but in whom parasites could not be confirmed. In view of the observed lack of sensitivity of PCR, and the high cellular responses in these dogs, it is likely that many or all of them represent 'resistant' infected dogs with very low parasite loads. Thus the true specificity is likely to be much higher than 84·5 %. In contrast, the specificity of serology in detecting active infection with high parasite load (i.e. PCR positives) is much lower, 57·1 %.

Our results suggest that serology, where carefully defined, can be a more sensitive test than PCR to detect all *L. infantum*-infected animals, as opposed to clinical or parasite-positive dogs. Few other studies have investigated the relative performance of PCR and serology in asymptomatic dogs. In one such study, PCR detected 76 % of seropositive, asymptomatic dogs from N.E. Brazil, compared to 59 % in the current study (Ashford *et al*. 1995). A lower proportion (63 %) of PCR-positive dogs were seropositive than in the current study (86 %). These results suggest that neither technique identifies all infected animals; differences between the studies may reflect differences between the dog populations studied, or variations in relative sensitivity of techniques between studies. A number of variables may affect PCR sensitivity, such as target copy number, DNA extraction method, PCR protocol or detection method (Reithinger *et al*. 2000). Although a number of primers for the detection of canine leishmaniasis have been described, there have been few published reports of their relative sensitivities (Reithinger *et al*. 2000). The type of biopsy material will also be important: we biopsied the bone-marrow, where parasite loads are likely to be high, although it is possible that parasites may be easier to detect by PCR in spleen, lymph node or skin biopsies. Parasites can also be detected in peripheral blood, although the sensitivity of PCR on blood samples is lower than on other tissue samples (Smyth *et al*. 1992; Mathis & Deplazes, 1995; Osman *et al*. 1997; Reale *et al*. 1999).

In the current study, the sensitivity of lymphoproliferation was comparatively low, 68·8 % for samples from dogs with confirmed infections. Visceral leishmaniasis is associated with suppression of cellular immune responses in a proportion of infected dogs (Pinelli *et al*. 1994; Rhalem *et al*. 1999) and, as expected, sensitivity was lowest in sick dogs and parasite positive dogs. However, even in asymptomatic dogs the sensitivity of cellular responses was only 75·3 %. This low sensitivity partly reflects the relatively high cut-off used here, $SI > 5$, compared to $SI > 2{\cdot}5$ in other studies (Cabral *et al*. 1998). This choice of cut-off was determined by the low specificity of cellular responsiveness: a significant proportion of uninfected dogs showed positive cellular responses, even at the relatively high cut-off of $SI > 5$. Similarly, cellular responsiveness to *Leishmania* antigens has been reported in unexposed humans (Kemp, Hansen & Theander, 1992), although Cabral *et al*. (1998) found no cellular responses in 25 unexposed Scottish dogs. This may reflect the smaller range of potentially cross-reacting infections in a temperate compared to a tropical dog population. Studies of European dogs have found that a proportion of animals from endemic areas have positive cellular responses but are seronegative (Cabral *et al*. 1992, 1998; Cardoso *et al*. 1998), and longitudinal studies suggest that a proportion of dogs never seroconvert (Dye *et al*. 1993; Pinelli *et al*. 1994). In contrast, our data suggest that all infected dogs seroconvert, and thus that cellular responses will identify additional infected dogs only prior to seroconversion or in the small proportion of dogs which serorecovered. These differences may reflect differences between the dog populations studied: our study population in Amazonian Brazil had generally poor condition and low nutritional status, which is likely to both suppress cellular responses and increase susceptibility to high parasite loads and thus antibody responses.

In summary, our data provide the first comparative test of diagnostic methods for *L. infantum* infection in a naturally infected dog population followed through the course of infection. Many previous studies have reported tests with high sensitivities and specificities for the detection of clinical cases or parasite-positive dogs. Here, we also

consider the performance of tests at detecting all infected animals. This is more difficult, as a result of factors which include the relatively slow time-course of infection (i.e. significant pre-patent period), and differences in parasite loads and immune responses between resistant and susceptible dogs. However, our results show that serology, using a carefully defined cut-off, can be a highly sensitive technique for most of the course of infection. In contrast, PCR was less sensitive at detecting infected dogs, except early in infection, but provides a highly specific test.

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