Evaluation of PCR as a Diagnostic Mass-Screening Tool To Detect *Leishmania* (Viannia) spp. in Domestic Dogs (*Canis familiaris*)

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Several studies have suggested that the PCR could be used in epidemiological mass-screening surveys to detect *Leishmania* (Viannia) spp. infection in human and animal hosts. Dogs from an area of *Leishmania braziliensis* and *Leishmania peruviana* endemicity were screened for American cutaneous leishmaniasis (ACL) infection by established PCR-based and enzyme-linked immunosorbent antibody test (ELISA) protocols. PCR detected *Leishmania* (Viannia) infection in a total of 90 of 1,066 (8.4%) dogs: 32 of 368 (8.7%), 65 of 769 (8.5%), and 7 of 42 (16.7%) dogs were PCR positive by testing of whole blood,uffy coat, and bone marrow aspirates, respectively. ELISA detected infection in 221 of 1,059 (20.9%) tested dogs. The high prevalence of *Leishmania* (Viannia) detected by PCR and ELISA in both asymptomatic (7.5 and 19.2%, respectively) and symptomatic (32 and 62.5%, respectively) dogs is further circumstantial evidence for their suspected role as reservoir hosts of ACL. However, the low sensitivity of PCR (31%) compared to ELISA (81%) indicates that PCR cannot be used for mass screening of samples in ACL epidemiological studies. Unless more-sensitive PCR protocols were to be developed, its use should be restricted to the diagnosis of active (canine and human) cases and to the parasitological monitoring of patients after chemotherapy.

*Leishmania* (Viannia) braziliensis and *Leishmania* (Viannia) peruviana cause American cutaneous leishmaniasis (ACL), a zoonosis transmitted to humans by the bite of phlebotomine sandfly vectors (13). Originally sylvatic, the transmission cycle of ACL has now adapted to the domestic environment due to deforestation and urbanization. Because several studies have reported high ACL infection rates in domestic dogs, there is a growing belief that dogs are reservoir hosts not only of zoonotic visceral leishmaniasis but also of ACL (30).

When carrying out epidemiological surveys to consider putative canine leishmaniasis control strategies, sensitive and specific tests for identifying ACL-infected dogs are paramount. Serological tests are the standard tools for identifying *Leishmania*-infected dogs during epidemiological mass-screening surveys because clinical and parasitological diagnoses (e.g., biopsy smears, parasite culture) are typically insensitive and because ACL infections in dogs are frequently asymptomatic (30). However, difficulties associated with the interpretation of cross-sectional seroprevalence data are that (i) serology is prone to nonspecific cross-reactions (false positives, e.g., to *Trypanosoma cruzi* or *Leishmania infantum* infection) (37), (ii) there may be a delay between infection and seroconversion (11, 28), (iii) a fraction of infected dogs may never seroconvert (e.g., due to innate resistance) (11), and (iv) seroconversion may not be permanent (e.g., due to development of humoral or cell-mediated immune response) (11, 28). Thus, sensitivity and specificity of serological tests can vary considerably and may underestimate the true prevalence and incidence of disease and, hence, the scale of the control problem.

It has been suggested that PCR-based methods for *Leishmania* diagnosis may provide the “gold standard” for determining the presence and identity of leishmanial infections not only for diagnosing active cases but also for monitoring parasitological cure of patients after chemotherapy and as a mass-screening tool to detect *Leishmania* infections in vertebrate hosts or sandfly vectors (35). Various PCR protocols have been developed for the detection of ACL-causing *Leishmania* in humans by using either purified DNA (from cultured parasites) (9) or clinical specimens (including lesion and scar biopsy specimens [4, 6, 9, 17, 21, 27, 29, 33, 34, 40] or blood [10, 12, 14, 15, 26]) and have consistently been proven to be more sensitive than other diagnostic methods, including in vitro culture of biopsy specimens, biopsy smears, and hamster inoculation. However, at present only one large (>50 samples tested) study has been reported where PCR was used as a diagnostic mass-screening tool to detect ACL in putative animal reservoirs (20). *Leishmania* (Viannia) parasites were detected in skin aspirates or biopsy specimens from 15 of 276 (5.4%) dogs, 4 of 153 (2.6%) *Akodon* spp., 2 of 72 (2.8%) *Didelphis albiventris*, 1 of 499 (0.2%) *Phyllosticta andinum*, 0 of 178 *Mus musculus*, 0 of 8 *Oryzomys* spp., and 0 of 8 *Rattus rattus*. A second diagnostic test for comparative data on infection rates was not carried out, and hence, no conclusions about the PCR assay’s sensitivity and specificity can be made.

In the work presented here the utility of PCR as a diagnostic mass-screening tool in epidemiological studies was evaluated. Specifically, we assessed the reproducibility of established PCR protocols on canine blood and bone marrow and compared their sensitivity and specificity to an enzyme-linked immu-
nosoraborn assay (ELISA) for detecting *Leishmania (Viannia)* spp. infection in dogs.

**MATERIALS AND METHODS**

**Study site.** Dogs came from 18 villages in the Department of Huánuco, an area of *L. braziliensis* and *L. peruviana* endemicity in Peru (10°00' S, 76°15' W). All villages lie at an altitude of ca. 2,000 to 3,500 m above sea level, in a mountainous and relatively dry area. A prospective epidemiological study of 2,374 people by the Universidad Peruana Cayetano Heredia and the London School of Hygiene and Tropical Medicine from 1994 to 1998 showed that village cumulative ACL prevalence rates (as measured by a positive response to the Montenegro skin test [MST]) range from 10 to 56% (C. R. Davies and A. Llanos-Cuentas, unpublished data). Previous studies also showed that (i) there is domestic ACL transmission caused by *Lutzomyia (Hecylorhynchomyia) teatuli* (N. Roncal and C. R. Davies, unpublished data), (ii) householders often own one or more dogs, and (ii) there are ACL infections in dogs (31), with *L. peruviana* (n = 1) and *L. braziliensis*- *L. peruviana* hybrids (n = 10) isolated from 2% (11 of 563) of randomly sampled dogs (24). There is no *L. infantum* or *L. chagasi* transmission in the study area.

**Dog population and sampling.** Between 1997 and 1999, dogs were surveyed by house-to-house visits or in the village square during rabies vaccination campaigns carried out by the local Ministry of Health. None of the dogs belonged to a recognizable breed and all were guard or hunting dogs. Dogs were examined for clinical signs of ACL, i.e., cutaneous lesions or scars. Impression smears were made of dermal scrapings or lesion biopsy samples from dogs with active cutaneous lesions or scars. Impression smears were considered positive if the resulting impression smears were processed as described previously, i.e., as buffy coat samples made of dermal scrapings or lesion biopsy samples from dogs with active cutaneous lesions. Impression smears were made of dermal scrapings or lesion biopsy samples from dogs with active cutaneous lesions, Giemsa stained, and examined microscopically (light microscope, oil immersion, ×100 objective) for *Leishmania* amastigotes. Blood (2 to 10 mL) was taken from a total of 1,104 (553 in 1997, 259 in 1998, and 226 in 1999) dogs by venipuncture and processed as described previously, i.e., as buffy coat samples (BCS) and/or guanidine blood lysates (GBL); bone marrow samples (BMS) were taken from a subset of dogs (n = 42) (31). Some dogs (n = 138) were sampled in consecutive years; the epidemiological analysis of *Leishmania (Viannia)* spp. transmission dynamics in dogs is, however, beyond the scope of data presented here and will be reported elsewhere.

**PCR.** DNA was phenol-chloroform extracted from BCS, GBL, and BMS as described previously and amplified using B1 (5'-GGG GTT GTT GTA ATA TAG TGG-3') and B2 (5'-CTA ATT GTG CAC GGG GGA GG-3') *Leishmania (Viannia)*-specific kinetoplast DNA primers. The PCR conditions and assay setup have been described previously (31). Amplification products were analyzed by electrophoresis on 1.5% agarose gels in 1× TAE (40 mM Tris-HCl, 40 mM acetic acid, 1 mM EDTA [pH 8.3]) buffer before being hybridized to a *Leishmania (Viannia)*-specific, [γ-32P]-labeled B3 (5'-TTG AAC GGG GTT TCT GTA TG-3') probe (31). Using this protocol, we were able to amplify one parasite in 400 μL of blood.

To assess the reproducibility of the used PCR-hybridization (HYB) protocol, all field samples were tested twice; when diagnostic outcomes differed, the samples were tested a third time, with the outcome being definitive. The diagnostic outcome was defined as samples being either positive by PCR and/or HYB or negative by both PCR and HYB. GBL were classed into four different groups: (i) PCR positive (PCR* +*), if an amplification product could be seen on the agarose gel, (ii) PCR negative (PCR* −*), if an amplification product could not be seen on the gel; (iii) HYB positive (HYB* +*), if an amplification product hybridized to the B3 probe; and (iv) HYB negative (HYB* −*), if an amplification product did not hybridize to the B3 probe. BCS and BMS were classed as either positive (PCR-HYB* +*), or negative (PCR-HYB* −*), by PCR-HYB, as the prior distinction as done for GBL could not be made due to the frequency of PCR artifacts (nonspecific bands) on the agarose gel.

**ELISA.** The ELISA protocol used has been described in detail previously (32), with 10th log-phase *L. braziliensis* promastigotes (MHOM/BR/76/M29031)/microtiter well used as the antigen. Serum samples were added at dilutions of 1/100, 1/400, and 1/800, and affinity-purified rabbit anti-dog immunoglobulin G (Sigma, Poole, United Kingdom) was added at a dilution of 1/1,500. The ELISA protocol was standardized as described before, with sample readings expressed as anti-*Leishmania* antibody units (LAU)/milliliter (32).

**Negative and positive controls.** Three groups of uninfected dog sera were used as negative controls for all diagnostic tests. The sera came from (i) dogs of various ages and breeds which had attended a veterinary clinic in Lima, Peru (n = 18); (ii) mongrel dogs from Belém, Brazil (n = 13); and (iii) dogs of various ages and breeds which had attended a veterinary clinic in Cambridge, United Kingdom (n = 13). Though *Leishmania (Viannia)* has been isolated from dogs both in Peru and Brazil, Lima and Belém are not areas of *Leishmania (Viannia)* endemicity. The positive-standard control serum as well as 15 other positive-control sera came from lesion and biopsy smear-positive dogs surveyed in the study.

**Data analysis.** Sensitivity, specificity, and positive (PPV) and negative predictive values for each diagnostic test were calculated as described previously (32). Analysis of ELISA data and all statistical analyses were carried out in Microsoft Excel 2000.

**RESULTS**

Samples from 1,104 dogs were tested as follows: (i) 63 samples were tested by PCR on GBL and BCS and by ELISA; (ii) 8 samples were tested by PCR on GBL and BCS; (iii) 282 samples were tested by PCR on GBL and by ELISA; (iv) 677 samples were tested by PCR on BCS and by ELISA; and (v) 15, 21, and 37 samples were tested either by PCR on GBL or BCS or by ELISA alone, respectively. One sample was only tested by PCR on BMS.

**Field samples: clinical diagnosis.** Of surveyed dogs, 21 of 1,104 (1.9%) had active cutaneous lesions and a further 21 of 1,104 (1.9%) had scars. Seventeen of 20 (85%) dogs with active lesions were biopsy smear positive.

**Field samples: reproducibility of PCR assay.** As described in Materials and Methods, all samples were tested by using a PCR-HYB protocol, with PCR amplification products being visualized by gel electrophoresis (PCR) prior to hybridization to a *Leishmania (Viannia)*-specific probe (HYB).

**GBL.** A total of 368 GBL samples were tested at least twice (Table 1). Three hundred ten (84%) of the samples had the same diagnostic outcomes in both replicates, while the remaining 58 samples had to be tested a third time to determine the final diagnosis (Table 1). Hence, a total of 794 (368 plus 368 plus 58) PCR-HYB assays were carried out. For all 22 samples positive by PCR, HYB with the B3 probe detected the B1 and B2 amplification products visible by gel electrophoresis. HYB was positive for a further 90 of 772 (12%) GBL sample replicates where B1 and B2 amplification products were not visible by gel electrophoresis (Table 1), i.e., HYB increased the frequency of positive assays by fivefold.

None of the 58 third PCR-HYB assays were PCR* −*, but 10 were HYB* +* and are therefore believed to have been true positives. The remaining 48 GBL samples that were assayed three times are assumed to have been false positives on the one occasion that the HYB was positive. Thus, although HYB massively increases the frequency of positive diagnoses, a significant proportion of PCR samples with positive HYBs are...
believed to be false positives. In contrast, the PCR assays (i.e., without HYB) appear to be much more specific. Not only were all PCR + assays also HYB +, but 7 of the 8 GBL that were PCR + in one replicate and PCR – in the other were HYB – in both. Hence, there is good reason to believe that samples with inconsistent PCR results are generally true positives.

These results demonstrate the essential requirement for assaying all samples at least twice to minimize the possibility of false-positive HYB results or false-negative PCR results.

**BCS.** A total of 769 BCS were tested at least twice by PCR-HYB. Of the BCS, 701 of 769 (91%) had the same results in the first two PCR replicates, with 39 of 701 (6%) testing PCR-HYB + and 662 of 701 (94%) BCS testing PCR-HYB –. Of the 68 samples that had to be tested a third time, 26 (38%) tested PCR-HYB + and 42 (62%) tested PCR-HYB –.

**BMS.** A total of 42 BMS were tested at least twice by PCR-HYB. Of 42 BMS, 37 (88%) had the same results in both replicates, with 5 of 38 (16%) and 32 of 38 (84%) testing PCR-HYB + and PCR-HYB –, respectively. Of the remainder, 2 (40%) tested PCR-HYB + and 3 (60%) tested PCR-HYB – in the third replicate.

**Comparison of the reproducibility and sensitivity of PCR for GBL, BCS, and BMS.** No difference between the reproducibility (i.e., in the proportion of samples that were consistent) of the PCR-HYB protocol on GBL, BCS, or BMS was observed (chi-square test, Yates-corrected, \( \chi^2 = 0.72, df = 2, P = 0.7 \)). Samples were considered PCR-HYB + if out of a maximum of three replicates, two replicates were PCR + and HYB – or PCR – and HYB + (for GBL samples only), or HYB – (for BCS and BMS). Additionally, GBL samples were considered positive if one replicate was PCR + and HYB + and one replicate was PCR – and HYB +. Samples were considered PCR-HYB – if out of a maximum of three replicates, two were negative after HYB. Using these definitions, 32 of 368 (8.7%), 65 of 769 (8.5%), and 7 of 42 (16.7%) were PCR-HYB + on GBL, BCS, and BMS, respectively (Table 2); this observed difference in the proportion positive for GBL, BCS, and BMS was not significant (chi-square test, Yates-corrected, \( \chi^2 = 3.35, df = 2, P = 0.19 \)). When more than one sample was assayed from dogs, there were highly significant associations between the results: for example, among those 42 dogs with bone marrow samples, all four positive by GBL (and 4 of 4 positive by BCS) were also positive by BMS.

**TABLE 2. Detection of Leishmania (Viannia) spp. in field dogs by PCR-HYB and ELISA a**

<table>
<thead>
<tr>
<th>Yr and sample source</th>
<th>No. of positive samples/total no. of samples (% positive) by:</th>
<th>PCR</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GBL</td>
<td>BCS</td>
<td>BMS</td>
</tr>
<tr>
<td>1997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs with active lesions</td>
<td>4/11 (36.4)</td>
<td>3/7 (42.9)</td>
<td>0/4 (0.0)</td>
</tr>
<tr>
<td>Dogs with scars</td>
<td>2/5 (40.0)</td>
<td>2/5 (40.0)</td>
<td>0/1 (0.0)</td>
</tr>
<tr>
<td>Dogs without lesions or scars</td>
<td>26/352 (7.4)</td>
<td>16/209 (7.7)</td>
<td>7/37 (18.9)</td>
</tr>
<tr>
<td>Dogs, BMS taken</td>
<td>4/33 (12.1)</td>
<td>4/41 (9.8)</td>
<td>7/42 (16.7)</td>
</tr>
<tr>
<td>Dogs, BMS not taken</td>
<td>5/30 (16.7)</td>
<td>6/30 (20.0)</td>
<td>8/30 (26.7)</td>
</tr>
<tr>
<td>Dogs, BMS not taken, BCS ND</td>
<td>23/297 (7.7)</td>
<td>11/142 (7.7)</td>
<td>18/130 (13.8)</td>
</tr>
<tr>
<td>Dogs, BMS not taken, GBL ND</td>
<td>0/8 (0.0)</td>
<td>0/8 (0.0)</td>
<td></td>
</tr>
<tr>
<td>1997 total</td>
<td>32/368 (8.7)</td>
<td>21/221 (9.5)</td>
<td>7/42 (16.7)</td>
</tr>
<tr>
<td>1998</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs with active lesions</td>
<td>1/8 (12.5)</td>
<td>6/8 (75.0)</td>
<td></td>
</tr>
<tr>
<td>Dogs with scars</td>
<td>3/9 (33.3)</td>
<td>3/8 (37.5)</td>
<td></td>
</tr>
<tr>
<td>Dogs without lesions or scars</td>
<td>15/270 (5.6)</td>
<td>39/270 (14.4)</td>
<td></td>
</tr>
<tr>
<td>1998 total</td>
<td>19/287 (6.6)</td>
<td>48/286 (16.8)</td>
<td></td>
</tr>
<tr>
<td>1999</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs with active lesions</td>
<td>0/2</td>
<td>0/2 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Dogs with scars</td>
<td>3/4 (75.0)</td>
<td>1/4 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Dogs without lesions or scars</td>
<td>22/255 (8.6)</td>
<td>57/252 (22.6)</td>
<td></td>
</tr>
<tr>
<td>1999 total</td>
<td>26/291 (9.0)</td>
<td>58/286 (20.5)</td>
<td></td>
</tr>
<tr>
<td>1997–1999</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs with active lesions</td>
<td>4/11 (36.4)</td>
<td>4/17 (23.5)</td>
<td>0/4 (0.0)</td>
</tr>
<tr>
<td>Dogs with scars</td>
<td>2/5 (40)</td>
<td>8/18 (44.4)</td>
<td>0/1 (0.0)</td>
</tr>
<tr>
<td>Dogs without lesions or scars</td>
<td>26/352 (7.4)</td>
<td>53/734 (7.2)</td>
<td>7/37 (18.9)</td>
</tr>
<tr>
<td>1997–1999 total</td>
<td>32/368 (8.7)</td>
<td>65/769 (8.5)</td>
<td>7/42 (16.7)</td>
</tr>
</tbody>
</table>

* Field samples were amplified with primer pair B1-B2, and products were then hybridized with [γ-32P]-labeled B3 probe. The ELISA was carried out as described in Materials and Methods by using the 4.16-LAU/ml cutoff. ND, not done. BCS, GBL, and BMS were all prepared in 1997, as a PCR protocol for dogs.
Field samples: definition of PCR-HYB positivity. A dog was considered PCR-HYB⁺ when one of the PCR-HYB assays for either GBL, BCS, or BMS was positive (Table 2). Thus, using the B1 and B2 primers and the B3 probe, the PCR-HYB assay detected Leishmania (Vianna) parasites in 8.4% (90 of 1,066) of tested dogs, with 32% (13 of 41) of the clinically symptomatic and 7.5% (77 of 1,025) of the clinically asymptomatic dogs testing PCR-HYB⁺ (chi-square test, Yates-corrected, \( \chi^2 = 26.8, P < 0.001 \)) (Table 2). However, this CO is conservative and it is likely that it will underestimate the true proportion of infected dogs.

ELISA. The mean antibody levels for the three groups of negative controls were 5,584 (standard deviation [SD] = 2,315) (Lima), 2,602 (SD = 1,707) (Belém), and 4,803 (SD = 4,728) (Cambridge) U/ml (Fig. 1). Whereas the mean log anti-LAU/milliliter of Lima and Belém (\( t \) test, \( t = 4.12, df = 29, P < 0.001 \)) and Cambridge and Belém (\( t \) test, \( t = 2.21, df = 24, P = 0.04 \)) dogs were significantly different, the mean LAU/milliliter of Lima and Cambridge dogs were not (\( t \) test, \( t = 1.26, df = 29, P = 0.22 \)) (Fig. 1, inset).

The mean antibody levels for positive control (i.e., lesion biopsy smear positive) and scar positive dog sera were 1,243,388 (SD = 3,635,762) U/ml and 154,825 (SD = 419,317) U/ml, respectively, with the mean LAU/milliliter significantly different between the two groups (\( t \) test, \( t = 2.81, df = 33, P = 0.01 \)) (Fig. 1, inset).

A total of 1,059 field dogs were tested by ELISA, the frequency distribution of which is represented in Fig. 1. Two different methods were used to analyze the data. First, the standard definition of positive cutoff (CO), i.e., the mean level of antibody units/milliliter of negative controls plus 3 SD was used (11, 28). The mean level of antibody units/milliliter of all negative controls was 4,742 (SD = 3,299) U/ml; hence, the positivity CO was 14,369 U/ml (i.e., 4.16 LAU/ml). Using this CO, 230 of 1,059 (21.7%) dogs were positive, 25 of 40 (62.5%) dogs were symptomatic, and 205 of 1,019 (20.1%) dogs were asymptomatic. This proportion of infected dogs is close to the best estimate of 249 (23.5%) dogs predicted by the fit of the log-lognormal distributions (i.e., the area under the log-lognormal distribution fitted to the right-hand tail of the ELISA LAU/milliliter distribution).

Field samples: PCR-HYB and ELISA. Table 3 summarizes the ELISA and PCR-HYB results of field samples and negative and positive controls, depending on which ELISA CO was used. The frequency distribution of LAU/milliliter relative PCR-HYB positivity is represented in Fig. 3. None of the 44 negative control samples were positive by PCR-HYB or ELISA (CO₄₁₆), but 3 dogs (one from Lima and two from Cambridge) were positive with the CO₄₁₆ (Table 3). Thus, the specificity of PCR and ELISA₄₁₆ was 100%, whereas the specificity of ELISA₄₁₄₆ was 95%. Five of 16 and 13 of 16 positive-control samples were positive by PCR and ELISA (no matter which CO was used), i.e., sensitivities for PCR and ELISA were 31 and 81%, respectively (Table 3). The proportion of samples PCR positive was shown to be associated with LAU/milliliter (linear regression analysis after arc-sine transformation of the data, \( r = 0.54, df = 50, P < 0.001 \)) (Fig. 3), the frequency distributions of PCR⁺ and PCR⁻ samples is shown in Fig. 4.

Based on the results of both PCR and ELISA, dogs were diagnosed as positive when they were positive by either PCR or ELISA₄₁₄₆ or both. The reason we decided to choose CO₄₁₆ as the positive CO for the final diagnosis is because of the higher specificity when using CO₄₁₄₆. Hence, out of 1,022 dogs that were tested by both PCR and ELISA, 262 of 1,022

![FIG. 1. Distribution of ELISA anti-LAU/milliliter of field samples (main frame) and controls (inset). Ab, antibody.](http://jcm.asm.org/Downloaded/from/picture.png)
(25.6%) were diagnosed as positive (Table 3); 30 of 39 (76.9%) were clinically symptomatic and 232 of 983 (23.6%) were clinically asymptomatic dogs.

DISCUSSION

We assessed the utility of PCR as a diagnostic mass-screening tool for epidemiological surveys and compared its sensitivity and specificity to ELISA, a commonly used test to detect *Leishmania* (*Viannia*) in dogs. We chose ELISA rather than the immunofluorescence antibody test as a comparative test because previous studies had shown ELISA to be more sensitive and specific than the immunofluorescence antibody test for the detection of ACL in dogs (30).

**PCR as a diagnostic mass-screening tool.** The classic diagnostic techniques for ACL diagnosis have a number of limitations. Microscopic examination of skin scrapings or lesion biopsy samples (Fig. 3), though rapid and low cost, has limited sensitivity, particularly in chronic lesions (e.g., mucocutaneous leishmaniasis) (39, 40). While in vitro culture techniques are slightly more sensitive than microscopic examination of lesion biopsy smears or histological samples, they are labor intensive and costly and are susceptible to microbiological contamination (16). They also are hampered by the particular growth requirements of different *Leishmania* strains, and as some strains grow better than others in vitro, the dominant strains can be inadvertently selected when culturing mixed infections (2). MST detects specific cutaneous delayed-type hypersensitivity but cannot distinguish between active and past infections (39). Also, logistical problems would be associated with its application in epidemiological dog surveys (e.g., dogs would have to be kenneled or monitored for 48 h prior reading the MST induration size). PCR has been shown to be particularly useful for the diagnosis of *Leishmania* (*Viannia*) infection, as parasite numbers in clinical samples are typically sparse (4, 6, 9, 17, 21, 27, 29, 33, 34, 40, 41).

Although a positive PCR was associated with higher LAU/milliliter (Fig. 3), the ELISA was much more sensitive than PCR in detecting *Leishmania* infection in symptomatic (chi-square test, *Y* = 6.5, *P* = 0.1) and asymptomatic (chi-square test, *Y* = 58.9, *P* < 0.001) field dogs. Considering the 100% specificity of the ELISA result and the absence of any potential cross-reacting parasites in the study area, it is very likely that the 163 ELISA-positive but PCR-negative dogs were true positives (see below).

Based on negative- and positive-control samples, the PPV was 100% for PCR and ELISA, and 81% for ELISA. The negative predictive value was 80% for PCR and 94% for ELISA. If one estimates the PPV from the PCR GBL replicate data, then the PPV for PCR and HYB' is similar, i.e., 95% (of the 22 PCR assays that were PCR' and HYB' at least once, 21 were diagnosed as positive) (Table 1). However, the PPV for PCR and HYB' is only 31% (of 80 samples that were PCR' and HYB' at least once, 25 were diagnosed as positive). This discrepancy is most probably due to samples having parasite numbers equal to or lower than the PCR positivity CO.

**TABLE 3. Comparative diagnosis of *Leishmania* (*Viannia*) spp. in dog blood**

<table>
<thead>
<tr>
<th>ELISA result</th>
<th>No. of negative controls (n = 44)</th>
<th>No. of positive controls (n = 16)</th>
<th>No. of other isolates (n = 1,006)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with PCR result:</td>
<td>with PCR result:</td>
<td>with PCR result:</td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>(3)</td>
<td>4 (4)</td>
<td>9 (9)</td>
</tr>
<tr>
<td>Negative</td>
<td>44 (41)</td>
<td>1 (1)</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

* Results are based on those found by ELISA, and ELISA (shown in parentheses). Positive controls and other isolates were field samples.

* PCs on negative controls from Belém and Cambridge were carried out on serum samples, not BCS.

* Dogs with active lesions that were biopsy smear positive. Of 20 dogs with active lesions (Table 2), 17 were biopsy smear positive and 16 were tested by both ELISA and PCR.
(i.e., 1 parasite per 400 μL of blood) (31). Whether future PCR protocols and new PCR primers will be able to reduce that false-negative rate remains to be established. Replicate results of established PCR protocols are rarely reported (18, 19, 28) but are of importance when interpreting epidemiological data because they show that the diagnostic test used is either not reliable (i.e., there is contamination or lack of reproducibility of the protocol) or not performing at its highest level of sensitivity. Considering that, in our case, a positive PCR result means that a dog is potentially infectious, the lack of replicates may have a significant impact on the outcome on dog intervention (e.g., culling) campaigns.

A PCR-based diagnostic test on blood is advantageous because samples can be obtained less invasively from the patient (human or dog) and are relatively easy to process. We also show that the choice of biopsy material does not appear to be as critical as for studies with PCR for diagnosing human ACL patients (e.g., skin snips and lesion aspirates), as the sensitivities of the PCR on GBL, BCS, or BMS were not significantly different. Studies which used PCR to detect L. infantum in dogs reported similar observations to the ones made here, showing that sensitivity was higher in clinically symptomatic dogs than in asymptomatic dogs (3, 5, 22, 32, 36). Only in two studies did the sensitivity of PCR vary according to biopsy origin (22, 36). The reason we could not demonstrate such an association is probably due to the pathogenesis of canine Leishmania (Viannia) infections, where parasites remain localized at the site of infection (38).

Only one large (>50 tested samples) study has used PCR as a mass-screening tool in epidemiological studies of ACL and reported lower ACL infection rates in dogs than the ones reported here (20). Similarly, other small studies (<50 tested samples) which used PCR to detect ACL in putative animal reservoirs failed to observe high ACL infection rates. Incidentally, in all studies, a second, comparative diagnostic test such as serology was not carried out.

**Epidemiological considerations.** The high prevalence shown in both asymptomatic and symptomatic dogs provides further evidence for their suspected role as (peridomestic) reservoir hosts of ACL (30). The detection of Leishmania (Viannia) DNA in canine blood implies that infected dogs should be infectious to blood-feeding sandfly vectors, but xenodiagnosis studies will be required to prove this. Although Leishmania (Viannia) DNA was detected in the blood and bone marrow of 8.4% (90 of 1,066) of the dogs tested, indicating that metastasis by hematogenous dissemination may be a more common phenomenon than previously acknowledged (38), blood samples from the majority of dogs (68% [28 of 41]) with active (and biopsy smears positive) lesions were PCR−. This is probably because, following inoculation by sandfly bite, Leishmania (Viannia) parasites are first localized in the dermis, with hematogenous dissemination occurring after an undefined interval (38). An interesting observation is the 52 PCR− but ELISA<sub>CO4.16</sub>-negative asymptomatic field dogs. Because the PCR was 100% specific, it is unlikely that they were false positives. They may have been false ELISA negatives, but considering that their mean level of LAU/milliliter (i.e., 3.61) was much lower than the 4.16 LAU/ml CO, this seems improbable. One explanation is that, although infected, the dogs had yet to develop an immune response to infection (i.e., prepatent period); 3 of 14 (21%) PCR− but ELISA-negative dogs that were followed up became ELISA positive. Another explanation is that dogs have developed cellular immunity after self-cure (e.g., studies of L. infantum in dogs have shown that up to 50% of asymptomatic dogs have an anti-Leishmania cellular immune response) (1, 8, 25, 36), as it now becomes increasingly evident that persistence of parasites is required to maintain cell-mediated immunity in the long term (38). Of note is that despite the high prevalence of infection only 4% of surveyed dogs had clinical symptoms of ACL, which is considerably lower than the figures observed in cross-sectional studies of dogs in areas of ACL endemicity (30). This observation could be explained if the circulating Leishmania (Viannia) parasites are of low virulence (i.e., a small proportion of infection causes clinical disease) but high pathogenicity (i.e., a high proportion of parasite inoculations cause infections), which is characteristic of L. peruviana (38). Unfortunately, currently no rapid, easy-to-use diagnostic test exists to differentiate the closely related Leishmania (Viannia) spp., L. braziliensis, and L. peruviana. Surprisingly little known is known about the clinical and
parasitological course of *Leishmania* (*Viannia*) in dogs (as in humans and other mammalian hosts) and the role of cellular immunity during infection. It also remains to be established whether *Leishmania* (*Viannia*)-resistant dogs could have an important role in ACL disease transmission.

This is the first large-scale study to test the feasibility of the use of PCR to detect *Leishmania* (*Viannia*) spp. parasites in host blood and to test whether PCR could be used as a diagnostic mass-screening tool in epidemiological studies. As is the case for zoonotic visceral leishmaniasis (3, 5, 22, 32, 36), PCR on blood alone does not appear to be the elusive gold standard for diagnosing ACL infections in dogs (or humans). Unless a more sensitive PCR protocol (e.g., PCR-ELISA or different PCR primers) is developed to detect asymptomatic ACL infections, the use of PCR should be restricted to the diagnosis of active cases (e.g., hospitals in Europe, the United States, and elsewhere increasingly rely on PCR for *Leishmania* diagnosis in human patients) (6, 41) and, in particular, MCL cases where common diagnostic tests (e.g., parasite culture, biopsy smears, and histopathology) are less sensitive. Nonetheless, the use of PCR on blood will have an important epidemiological application in studies monitoring the clinical and chemotherapeutic follow-up of ACL patients (10, 15, 34). Detection of disseminating *Leishmania* parasites in patient blood would indicate that they are at risk of developing mucocutaneous lesions, the treatment of which is more complicated than the treatment of the single lesions characteristic of ACL (38). Also, PCR combined with specific DNA probing and sequencing should help to identify and characterize those strains that are drug resistant and that cause the different clinical pathologies associated with ACL.

Thus, although there are concerns about the specificity of serological tests, they will remain the main diagnostic tool for epidemiological *Leishmania* mass-screening surveys. The main advantages of serological tests are that large numbers of samples can be processed readily and inexpensively, with comparatively low technical expertise required. Significantly, the recent identification of specific recombinant *Leishmania* antigens (e.g., the *Leishmania donovani*- and *L. infantum*-specific rK39) (7) suggests that serological tests may become more specific in the future. Several studies have shown that serological tests based on recombinant antigens (e.g., rK39-ELISA) are able to distinguish not only between present and past infections but between symptomatic and asymptomatic infections as well. Surprisingly, serological tests based on known *Leishmania* (*Viannia*) spp.-specific recombinant antigens (23) have yet to be tested as a diagnostic tool either for active cases or in epidemiological surveys.

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