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Use of PCR To Detect *Leishmania* (*Viannia*) spp. in Dog Blood and Bone Marrow

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A PCR-based protocol for the detection of *Leishmania* (*Viannia*) parasites in canine blood, buffy coat, and bone marrow was developed and was then tested with field samples taken from a random sample of 545 dogs from villages in Peru where *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *peruviana* are endemic. Comparative tests with cultured parasites mixed with dog blood showed that the PCR assay's sensitivity was significantly dependent on the DNA extraction protocol and the PCR primers used. Mass screening of field samples by the preferred PCR protocol detected American cutaneous leishmaniasis (ACL) in 44 of 545 (8.1%) dogs; 31 of 402 (7.7%), 20 of 223 (9.0%), and 8 of 46 (17.4%) were PCR positive when whole blood, buffy coat, and bone marrow aspirates, respectively, were tested. The high prevalence of *Leishmania* in both asymptomatic (7.6%) and symptomatic (18.0%) dogs provides further circumstantial evidence for their suspected role as reservoir hosts of ACL and indicates that hematogenous dissemination of parasites may be a more common pathological phenomenon than has previously been acknowledged. However, unlike for zoonotic visceral leishmaniasis, the comparatively low prevalence of *Leishmania* (*Viannia*) in the blood of symptomatic dogs indicates that PCR with blood cannot be the "gold standard" for the (mass) screening of samples in epidemiological studies.

Because peri-domestic or domestic transmission of human American cutaneous leishmaniasis (ACL) is increasingly evident and because several studies have reported high rates of canine ACL, there is a growing belief that dogs not only may be the main reservoir host of zoonotic visceral leishmaniasis but may also be the main reservoir host of ACL (13). Sensitive and specific tests for the identification of infected dogs are paramount when considering putative canine leishmaniasis control strategies. Although serological tests should be more specific (i.e., there are many false-positive results by serological tests), they remain the standard tools for the identification of *Leishmania*-infected dogs, because clinical and parasitological diagnoses (e.g., by use of biopsy smears and by parasite culture) are characteristically insensitive and because ACL infections in dogs are frequently asymptomatic (13). Various PCR protocols for the detection of ACL-causing *Leishmania* in humans with either purified DNA (from cultured parasites) or clinical specimens (including lesion and scar biopsy specimens or blood) have been reported (4, 8, 15), but only two have used PCR to identify dogs with ACL. In the first study, PCR detected *Leishmania* DNA in the blood of three asymptomatic dogs (the number tested was not reported) (8), and in the second study, PCR detected *Leishmania* DNA in skin aspirates or biopsy specimens taken from 15 of 276 (5.4%) dogs tested (9).

The present study compared the sensitivity of PCR-based assays for the identification of *Leishmania* (*Viannia*) spp. in dog blood by using four acknowledged DNA extraction methods and four different PCR primer pairs. The preferred protocol was then used for mass screening of dog samples (blood and bone marrow) collected in villages in Peru where *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *peruviana* are endemic.

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MATERIALS AND METHODS

Field samples. Dogs from 16 villages in the Department of Huánuco, Peru, were examined for clinical signs of leishmaniasis, i.e., cutaneous lesions or scars. Impression smears were made of dermal scrapings and/or lesion biopsy specimens from dogs with active cutaneous lesions, stained with Giemsa, and examined microscopically (light microscope, oil immersion, ×100 objective) for amastigotes. Blood (2 to 10 ml) was taken from 545 dogs by venipuncture and was aliquoted into sterile, EDTA-coated, 10-ml polypropylene tubes. The samples were stored at 0 to 4°C and were processed in the laboratory 4 to 10 h after collection. One of the aliquots was centrifuged at 800 × *g* for 20 min, and the buffy coat layer (i.e., buffy coat sample [BCS]) was removed and stored at −20°C; the second blood aliquot (2 to 3 ml) was mixed with an equal volume of 6 M guanidine HCl–0.2 M EDTA (pH 8.0) (i.e., guanidine-blood lysates [GBLs]) and was stored at 4°C (3). Bone marrow (i.e., bone marrow samples [BMSs]) was aspirated from the iliac crest from a random sample of dogs ($n = 46$) by using a mixture of medetomidine (Domitor; SmithKline Beecham, Welwy, United Kingdom) and ketamine hydrochloride (Vetalar; Parke-Davis Veterinary, Ann Arbor, Mich.) as anesthetics, and the BMSs were stored at −20°C.

DNA extraction. (i) STA. The choice of DNA extraction protocol and primers to be used for mass screening of field samples was based on a series of sensitivity titration assays (STAs). One hundred-fold dilutions of 10^8 water-lysed *L. braziliensis* MHOM/BR/75/M2903 were added to 200-μl aliquots of guanidine blood lysate, yielding a concentration range from 0.01 to 10^6 parasites per spiked sample. Water was added to a separate aliquot as a negative control. DNA was extracted by standard protocols with either phenol-chloroform (PC), Chelex 100 resin (Bio-Rad, Hemel Hempstead, United Kingdom), or the DNeasy DNA extraction kit (Qiagen, Crawley, United Kingdom). GBLs were heated for 10 min in boiling water to denature the concatenated minicircle DNA molecules which constitute most of the *Leishmania* kinetoplast DNA (kDNA) network and were allowed to cool to room temperature. After one extraction with PC, the DNA was back-extracted with TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and was then extracted with chloroform and precipitated with ethanol, resuspended in 50 μl of TE, and stored at 4°C. Chelex and DNeasy DNA extractions were carried out as described by Walsh et al. (17) and according to the manufacturer's protocol, respectively. To increase the DNA yield from the samples extracted with Chelex, 300 μl of the extract's supernatant was precipitated with ethanol and was resuspended in 30 μl of TE.

(ii) Field samples. BCSs and BMSs were mixed with an equal volume of DNA extraction buffer (10 mM Tris-HCl [pH 8.0], 0.1 M EDTA [pH 8.0], 0.5% sodium dodecyl sulfate [SDS]), proteinase K was added to a final concentration of 50 μg/ml, and the samples were incubated for 5 h at 50°C. Aliquots (200 μl of GBLs, BCSs, and BMSs) were taken and DNA was extracted with PC as described above.

PCR. (i) Sensitivity titration assay. Spiked samples and the original culture water-lysate dilutions were amplified by four different PCR assays (three replicates), each one with a different set of primer pairs: primers B1 (5′-GGGGTT GGTGTAATATAGTGG-3′) and B2 (5′-CTAATTGTGCACGGGGAGG-3′)

(6), primers MP1L (5′-TACTCCCCGACATGCCTCTG-3′) and MP3H (5′-GAACGGGGTTTCTGTATGC) (11), primers Min11B (5′-GGATCGCTGGGAACAATC-3′) and Min22 (5′-CATGAATGGCTTTCGTTTCAG-3′) (7), and primers R221 (5′-GGTTCCTTTCCTGATTTACG-3′) and R332 (5′-GGCCGGTAAAGGCCGAATAG-3′) (16). Briefly, 1 μl (2 to 5 ng) of DNA was amplified on a Biometra Thermocycler (Biometra, Göttingen, Germany) in a total reaction volume of 25 μl overlaid with 30 μl of mineral oil (Sigma, Poole, United Kingdom). Table 1 summarizes the reaction conditions. Amplification products were analyzed by electrophoresis on 1.5% agarose gels in 1× Tris-acetate EDTA buffer (14). To evaluate sample degradation or PCR inhibition, sample DNA was also amplified for a canine housekeeping gene, acidic ribosomal phosphoprotein fragment, by using primers PO3 (5′-GGAGAAGGGGGAGATGTT-3′) and PO5 (5′-TCATTGTGGGAGCAGACA-3′) (2). When samples did not yield amplification products, they were extracted again until amplification products were obtained. Each amplification cycle included negative controls (no DNA, DNA from an uninfected dog) and positive controls (water-lysates of cultures obtained from Huánuco dog isolates). PCR-grade H_2O was used throughout the study. To avoid cross-contamination, separate areas were used for DNA extraction, PCR sample preparation, and amplification.

(ii) Hybridization. Agarose gels were processed by standard procedures, i.e., in denaturation buffer and in neutralization buffer for 20 min each, and were Southern blotted onto a nylon membrane (Boehringer Mannheim, Basel, Switzerland). The DNA was fixed to the membrane by UV cross-linking (14). The membranes were prehybridized at 42°C and hybridized with either an [α-^{32}P]dATP- or [γ-^{32}P]ATP-labelled probe for 8 to 12 h (Table 1) and were then washed at 42 or 65°C twice for 15 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and in 0.1× SSC–0.1% SDS, before being exposed for autoradiography for 36 and 72 h at −70°C (14).

(iii) Field samples. On the basis of the results of the STA, all field samples were amplified by using the PO3-PO5 and B1-B2 primer pairs. Hybridization was carried out as described above by using the [γ-^{32}P]ATP-labelled oligonucleotide primer probe B3 (5′-TTGAACGGGGTTTCTGTATG-3′).

RESULTS

STA. Table 1 summarizes the sensitivities of the PCR assays according to the DNA extraction protocol and primer pairs used. Briefly, the Min11B and Min22 primer pair was 10^6- to 10^8-fold more sensitive than the MP1L-MP3H primer pair and 10^2- to 10^4-fold more sensitive than both the B1-B2 and R221-R332 primer pairs in amplifying DNA from culture dilutions on PC- or DNeasy-extracted samples (Table 1). None of the samples extracted with Chelex only could be amplified. PCR with PC-extracted samples was 2- and $>10^4$-fold more sensitive than reactions with DNeasy and Chelex-ethanol-extracted samples, respectively. Hybridization generally increased the assay's sensitivity by 10^2-fold but increased the sensitivity by up to 10^4-fold for DNeasy-extracted samples amplified with the B1-B2 primer pair. All but the Chelex-only-extracted samples were successfully amplified with PO3-PO5. PC was used in the DNA extraction protocol for field samples, as it was almost as good as the DNeasy kit in extracting parasite DNA from blood (Table 1), but at a significantly lower cost. The B1-B2 primer pair was chosen for mass screening because (i) with hybridization it yielded the greatest sensitivity, along with the Min11B-Min22 primer pair (Table 1); (ii) it did not yield any PCR product artifacts (unlike Min11B-Min22 and MP1L-MP3H); and (iii) it has previously been tested with clinical field samples (although not blood) (6). In our hands, PC extraction combined with the use of the B1-B2 primer pair and the B3 probe could detect parasitemias at a level of one *Leishmania* parasite/40 ml of canine blood.

Field samples. Of the surveyed dogs, 11 of 545 (2.0%) had active cutaneous lesions and a further 11 of 545 (2.0%) had scars and/or ulcers. All dogs with active lesions were biopsy smear positive. By using B1-B2, the PCR-based assay detected *Leishmania* parasites in 4 of 22 (18.0%) of the clinically symptomatic dogs and in 40 of 523 (7.6%) of the asymptomatic dogs. When more than one sample from dogs was assayed, there were highly significant associations between the results: for example, among those 46 dogs with BMSs taken, all five with a positive GBL (and five of six dogs with a positive BCS) also had a positive BMS. B1-B2 amplification products were detected by agarose gel electrophoresis in 14 of 402 (3.5%) GBLs, 8 of 223 (3.6%) BCSs, and 5 of 46 (10.9%) BMSs. Hybridization with the B3 probe detected all amplification products visible by gel electrophoresis and in a further 17 GBLs, 12 BCSs, and 3 BMSs not visible by gel electrophoresis (Table 2); i.e., hybridization doubled the sensitivity.

DISCUSSION

Investigators carrying out PCR assays rarely justify choice of DNA extraction protocol and PCR primers (10), but both were shown here to have a significant effect on assay sensitivity. Furthermore, most reported STAs were based either on pure *Leishmania* parasite culture lysates or on standard amounts of background host DNA added to known quantities of parasite DNA (6, 7, 11, 16). Both fail to mimic the situation encountered in the field: the concentration of background host and parasite DNA will vary considerably by biopsy sample, thereby influencing the outcome of the PCR assay, as will other factors related to the host's medical condition (e.g., hematocrit) (5). The present STA demonstrates that DNA from less than one *Leishmania* parasite can be amplified by PCR in the presence of host canine background DNA but generally less readily than from pure parasite culture lysates (Table 1). Hybridization with a ^{32}P-labelled probe usually increased the sensitivity of the assay by 10^2- to 10^4-fold (Table 1). Contrary to previous reports (4, 11), the M1L-M3HL primer pair performed rather poorly. Although the target DNA to be amplified was the smallest, M1L-M3HL was 10^4- to 10^6-fold less sensitive than the other primer pairs used. Also, a particular problem associated with the use of M1L-M3HL was the difficult visual separation of the amplification product and primer dimers on standard agarose gels (and subsequently on the probed filters). Although organic solvents are known to persist in DNA extracts and can inhibit the PCR, extraction with PC was comparable to extraction with the DNeasy kit in preparing samples for PCR. Commercial DNA extraction kits (e.g., DNeasy) may have the advantage of speed and a reduced safety hazard (10), but they are expensive compared to PC extraction and (at least in our hands) are no more efficient. Quicker and easier DNA extraction techniques with Chelex were not as successful (10^3- to 10^4-fold less sensitive) as the DNeasy kit or PC extraction procedures when preparing samples for PCR. The reason why none of the Chelex-only-extracted samples amplified the target DNA may be due to the presence of a PCR inhibitor not removed by the extraction method or remaining Chelex particles. Although ideal for screening large numbers of samples because of the minimal manipulations required and the reduced risk of specimen-to-specimen contamination (17), this extraction protocol appears to be unsuitable for DNA extraction when one is using clinical specimens containing very small numbers of parasites or large numbers of potential PCR inhibitors, e.g., heme. In contrast, *Leishmania* (*Viannia*) sp. DNA has been successfully extracted from lesion scrapings with Chelex resin (4). The advantage of using guanidine HCl is that blood samples can be stored at 4°C (and possibly at room temperature) (3), which is useful in the field, where there is often no access to freezers. As for *Trypanosoma cruzi* (3), the *Leishmania* DNA in guanidine HCl remained undegraded for months, and we successfully amplified *Leishmania* DNA originating from samples stored at 4°C for 1.5 years. However, it should be noted that guanidine HCl is a salt which could inhibit PCR amplification, so dilutions of extracted DNA may be required for successful amplification.

PCR is particularly useful for the diagnosis of *Leishmania*

TABLE 1. PCR conditions[a]

Characteristic	B1-B2	M1L-M3HL	Min11-Min22	R221-R332	PO3-PO5
PCR product	Whole kDNA minicircle (750 bp)	kDNA minicircle fragment (75 bp)	Subtelomeric DNA repeat (491 bp)	Ribosomal DNA repeat (603 bp)	Genomic DNA repeat (469 bp)
Reaction mixture					
PCR buffer composition	10 mM Tris-HCl (pH 8.3) 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin	10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.01% gelatin	10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin	10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin	10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin
dNTP[b] concn (mM each)	0.2	0.2	0.2	0.2	0.2
Amt of primer (pmol)	50	50	50	50	45
Amt of *Taq* polymerase (U)	1	1	0.5	0.5	1
Cycles					
Initial denaturation	95°C for 6 min	94°C for 3 min	95°C for 30 s	95°C for 5 min	95°C for 5 min
Initial annealing	64.5°C for 2 min	54°C for 2 min	57°C for 45 s	60°C for 30 s	55°C for 30 s
Extension	72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 30 s	72°C for 30 s
Denaturation	95°C for 30 s	95°C for 1 min	95°C for 30 s	95°C for 30 s	93°C for 30 s
Annealing	60.5°C for 1 min	54°C for 2 min	57°C for 45 s	60°C for 30 s	55°C for 30 s
No. of cycles	35	35	35	35	34
Final extension	72°C for 10 min	72°C for 10 min	72°C for 10 min	72°C for 10 min	72°C for 10 min
Hybridization probe	B3 oligonucleotide, end labelling (γ-^{32}P labelled)	Positive control, random primer (α-^{32}P labelled)	Positive control, random primer (α-^{32}P labelled)	Positive control, random primer (α-^{32}P labelled)	
Sensitivity of PCR[c]					
Gel electrophoresis					
1 Pure culture	0.1	10^5	0.001[d]	10	
2 PC extraction	0.8	8000	0.008	0.8	
3 DNeasy	1.9	1.9×10^4	0.019	1.9	
4 Chelex 100 resin					
5 Chelex-ethanol	6.7×10^4			6.7×10^4	
Hybridization					
1 Pure culture	0.001[d]	10^3	0.001[d]	0.1	
2 PC extraction	0.008	80	0.008	0.8	
3 DNeasy	0.00019[d]	190	0.00019[d]	0.019	
4 Chelex 100 resin					
5 Chelex-ethanol	6.7×10^4		6.7×10^4	6.7×10^4	

[a] PCR and hybridization were carried out as described in Materials and Methods.
[b] dNTP, deoxynucleoside triphosphate.
[c] The sensitivity of the PCR STA is given as the minimum number of parasites in the PCR sample required for successful amplification.
[d] Most dilute sample tested by the assay.

TABLE 2. Detection of *Leishmania* (*Viannia*) spp. in dog field samples by PCR and hybridization[a]

Group	No. of dogs positive/total no. of dogs (%)		
	GBL	BCS	BMS
Dogs with active lesions	2/11 (18.18)	2/11 (18.18)	
Dogs with scars	2/11 (18.18)	2/11 (18.18)	
Dogs without lesions or scars	27/380 (7.11)	16/201 (7.96)	8/46 (17.39)
Dogs from which BMS were taken	5/46 (10.87)	6/46 (13.04)	8/46 (17.39)
Dogs from which BMS were not taken	4/34 (11.76)	5/34 (14.71)	
Dogs from which BMS were not taken and BCSs were not tested	22/322 (6.83)		
Dogs from which BMS were not taken and GBLs were not tested		9/143 (6.29)	

[a] NOTE Field samples were amplified with the B1-B2 primer pair and the products were then hybridized with γ-^{32}P-labelled B3 probe as described in Materials and Methods.

(*Viannia*) infection, as the parasite numbers in clinical samples are typically sparse (4, 6, 8, 15). A PCR-based assay with blood is advantageous, as samples can be obtained less invasively from the patient (human or dog) and are easy to process. This is the first large-scale study to test the feasibility of using PCR to detect *Leishmania* (*Viannia*) DNA in host blood. The high prevalence shown in both asymptomatic and symptomatic dogs provides further evidence of their suspected role as (peridomestic) reservoir hosts of ACL (13), and the detection of *Leishmania* DNA in canine blood implies that infected dogs should be infectious to blood-feeding sandfly vectors. However, xenodiagnostic studies will be required to prove this. Although *Leishmania* DNA was detected in the blood and bone marrow of a relatively large proportion of the dogs tested, indicating that metastasis by hematogenous dissemination may be a more common phenomenon than has previously been acknowledged (1, 18), blood samples from the majority of dogs with active (and biopsy smear-positive) lesions were PCR negative. This is probably because *Leishmania* (*Viannia*) parasites are first localized at the site of infection in the dermis, with hematogenous dissemination occurring after an undefined interval (if at all) (1, 18). Hence, unlike for zoonotic visceral leishmaniasis (2, 12, 13), PCR with blood alone is unlikely to provide the elusive "gold standard" for the diagnosis of ACL in dogs. Mass screening of dogs (or humans) in epidemiological studies should therefore use another diagnostic test, such as enzyme-linked immunosorbent assay or the Montenegro skin test, in conjunction with PCR. The use of PCR in conjunction with, for example, serology or the Montenegro skin test should also help to determine the true extent of subclinical infections in areas where ACL is endemic and give an estimate of the number of dogs to be targeted within a putative control program. Current dog control programs are based on culling of only seropositive dogs and suffer from the poor sensitivity and specificity of the serological tests used (13). Consequently, dog control programs that have been implemented have proven to be ineffective; for example, despite culling of more than 25,000 dogs per year, canine and human visceral leishmaniases have steadily increased in Brazil during the past 20 years. The use of PCR with blood will, however, have an important epidemiological application in studies that monitor the clinical and chemotherapeutic follow-up of patients with ACL (8, 15). Detection of disseminating *Leishmania* parasites in patient blood would indicate that they are at risk of developing mucocutaneous lesions, the treatment of which is much more complicated than the treatment of the single cutaneous lesions characteristic of ACL (18). Also, PCR combined with specific DNA probing and sequencing should help to identify and characterize those *Leishmania* species and/or strains that are drug resistant and that cause the different clinical pathologies associated with ACL.

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ERRATUM

Use of PCR To Detect *Leishmania* (*Viannia*) spp. in Dog Blood and Bone Marrow

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Volume 38, no. 2, p. 748–751. Page 749, column 1, line 11 from bottom: "40 ml" should read "400 μl."