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Comparison of *Leishmania* OligoC-TesT PCR with Conventional and Real-Time PCR for Diagnosis of Canine *Leishmania* Infection▽

Connor Carson,[1]* Rupert J. Quinnell,[2] Jennifer Holden,[1]¶ Lourdes M. Garcez,[3] Stijn Deborggraeve,[4] and Orin Courtenay[1]

Populations and Disease Research Group, Department of Biological Sciences, University of Warwick, Coventry, United Kingdom[1]; Institute of Integrative and Comparative Biology, University of Leeds, Leeds, United Kingdom[2]; Departamento de Parasitologia, Instituto Evandro Chagas, Fundação Nacional de Saude, Belém, Brazil[3]; and Department of Parasitology, Institute of Tropical Medicine, Antwerp, Belgium[4]

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There is a need for standardization and simplification of the existing methods for molecular detection of *Leishmania infantum* in the canine reservoir host. The commercially available OligoC-TesT kit incorporates standardized PCR reagents with rapid oligochromatographic dipstick detection of PCR products and is highly sensitive for use in humans but not yet independently validated for use in dogs. Here we compare the sensitivity of OligoC-TesT with those of nested kinetoplast DNA (kDNA) PCR, nested internal transcribed spacer 1 (ITS-1) PCR, and a PCR-hybridization protocol, using longitudinal naturally infected canine bone marrow samples whose parasite burdens were measured by real-time quantitative PCR (qPCR). The sensitivity of OligoC-TesT for infected dogs was 70% (95% confidence interval [CI], 63 to 78%), similar to that of kDNA PCR (72%; 95% CI, 65 to 80%; $P = 0.69$) but significantly greater than those of PCR-hybridization (61%; 95% CI, 53 to 69%; $P = 0.007$) and ITS-1 nested PCR (54%; 95% CI, 45 to 62%; $P < 0.001$); real-time qPCR had the highest sensitivity (91%; 95% CI, 85 to 95%; $P < 0.001$). OligoC-TesT sensitivity was greater for polysymptomatic and oligosymptomatic dogs than for asymptomatic dogs (93%, 74%, and 61%, respectively; $P = 0.005$), a trend also observed for the other qualitative PCR methods tested ($P \leq 0.05$). Test positivity increased with increasing parasite burdens, as measured by real-time qPCR: OligoC-TesT and kDNA PCR detected 100% and 99% of positive samples when parasite burdens exceeded 74 and 49 parasites/ml, respectively. OligoC-TesT has high sensitivity for detection of canine *Leishmania* infections; its ease of operation and ease of interpretation are further advantages for veterinary diagnostic laboratories and for large-scale survey work in developing countries.

Zoonotic visceral leishmaniasis (ZVL), a vector-borne disease caused by the protozoan parasite *Leishmania infantum* [*Leishmania* (*Leishmania*) *infantum chagasi* (11)], results in significant mortality and morbidity in the reservoir host (the domestic dog) and represents a serious public health problem in many regions where it is endemic (the Mediterranean basin, Latin America, and parts of central and eastern Asia). Serological methods are the most technically straightforward of the available tests for diagnosis of canine infection, but these methods lack sensitivity for asymptomatic and early-stage infections (5, 17, 24). Detection of *Leishmania* parasites in canine clinical samples has traditionally been performed by means of microscopic examination of stained tissue specimens and by parasitological culture, which are known to be insensitive. PCR for amplification of defined parasite DNA sequences is highly sensitive for animals with clinical disease and has higher sensitivity than serology for asymptomatic animals and early-stage infections (8, 9, 19, 23, 29). However, the technical complexity of PCR may reduce its practicality for use in developing countries most affected by ZVL. Furthermore, there is a lack of standardization in the selection of target *Leishmania* DNA sequences and experimental PCR protocols used in laboratories worldwide, which complicates objective comparisons of test sensitivity and specificity. In order to address some of these issues, a commercially available PCR test kit (*Leishmania* OligoC-TesT) has been developed and validated for detection of *Leishmania* parasite DNA in human specimens (3, 6). Sensitivities of the test ranged from 77.8% to 100% for clinical samples from patients with visceral leishmaniasis in Sudan and Kenya, with a limit of detection of 1 parasite in 180 μl blood (3). OligoC-TesT has not yet been validated independently for use in dogs. The aims of this study were therefore (i) to measure the sensitivity of OligoC-TesT compared with those of three conventional PCR procedures (nested PCR for amplification of kinetoplast DNA [kDNA], nested PCR of internal transcribed spacer region 1 [ITS-1] of the rRNA gene, and kDNA/rRNA PCR followed by hybridization with specific oligonucleotide probes) and with that of real-time quantitative PCR (qPCR), (ii) to compare the sensitivities of OligoC-TesT and the PCR methods listed above for samples from infected dogs that were positive or negative for clinical signs of leishmaniasis, and (iii) to determine the analytical sensitivity of OligoC-TesT relative to canine bone marrow parasite burdens measured by real-time qPCR. For this study, we used samples collected in a longitudinal study of a cohort of naturally *L. infantum*-infected domestic dogs in Brazil.

* Corresponding author. Mailing address: Populations and Disease Research Group, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom. Phone: 44 24 7657 5874. Fax: 44 24 7652 4619. E-mail: c.carson@warwick.ac.uk.

¶ Present address: Department of Pathology, Cambridge University, Tennis Court Road, Cambridge, United Kingdom.

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MATERIALS AND METHODS

Sampling and selection of dogs. Bone marrow samples from naturally exposed outbred dogs were tested. These samples came from a previous longitudinal field study in Marajó Island, Pará State, Brazil (24), in which serum (for serological testing by crude parasite enzyme-linked immunosorbent assay [ELISA]) and bone marrow (for PCR and *in vitro*/*in vivo* parasitological culture) were obtained at approximately 2-month intervals for up to 24 months after initial natural exposure. Dogs were also examined clinically at each time point and were assigned a semiquantitative clinical score by scoring each of 6 typical signs of leishmaniasis (alopecia, dermatitis, cutaneous ulceration, conjunctivitis, onychogryposis, and lymphadenopathy) on a scale of 0 to 3: dogs with total scores of 0 to 2 were considered asymptomatic, those with scores of 3 to 6 were defined as oligosymptomatic, and those with scores of 7 to 18 were defined as polysymptomatic (23). In the current study, we used archived longitudinal bone marrow samples from dogs which showed evidence of infection anytime after initial exposure, where infection was defined as positivity by serology (crude parasite ELISA) and/or *in vitro*/*in vivo* culture. Identification of cultured parasites in 34 canine bone marrow samples from the study population by use of monoclonal antibodies yielded only *L. infantum*, as previously described (24). Samples were aligned by time point of first detection of infection (t_0). A total of 163 samples (from 67 infected dogs) were tested, with a mean of 2.4 time points per dog (range, 1 to 5 time points), from 2 months before first detection of infection (t_{-2}) to 12 months postinfection (t_{+12}). One hundred forty-seven samples were from dogs with confirmed infection (i.e., samples taken on or after the time of confirmed infection), and 15 samples were taken 2 months before confirmed infection, when dogs are likely to be in the prepatent period (24). One sample was not included in the analysis due to PCR inhibition (see below). Clinical scores were unavailable for 2.5% (4/163 samples) of the samples.

DNA extraction. Previously, bone marrow samples were aspirated from the iliac crests of anesthetized dogs, collected in 200 μl buffer (15 mM Tris, pH 8.3, 1 mM EDTA, 150 mM NaCl) containing 1% SDS, incubated at 65°C for 2 h, and stored at −20°C, as described previously (23). In the present study, DNA extraction was performed on 100-μl aliquots of bone marrow, which were digested in 100 μl PK buffer (50 mM KCl, 10 mM Tris, pH 8.0, 0.05% Tween 20, 100 μg/ml proteinase K). After overnight incubation at 56°C, a phenol-chloroform protocol was used to extract DNA as follows. In a phase-lock gel tube (VWR International, United Kingdom), 100 μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added and mixed, followed by centrifugation at 13,000 rpm for 5 min to separate the aqueous phase. In a new phase-lock tube, 100 μl of chloroform-isoamyl alcohol (24:1) was added and mixed, and the sample was centrifuged as before. Five hundred microliters of 30% polyethylene glycol 6000 (PEG 6000)–1.6 M NaCl solution was then added to the aqueous phase, and the sample was allowed to stand for 1 h at room temperature. Samples were centrifuged as before, and the DNA pellet was washed with 70% ethanol and air dried before elution in 100 μl molecular biology-grade H_2O. Total DNA quantity was measured by determining the optical absorbance at 260 nm, using a Nanodrop 1000 spectrophotometer (Thermo Scientific, United Kingdom), and purity was estimated from the ratio of optical absorbances at 260 and 280 nm ($A_{260/280}$). The median $A_{260/280}$ was 1.81 (interquartile range = 1.74 to 1.85), indicating high purity of the extracted DNA. Each bone marrow sample was subjected to DNA extraction once only, since our objective was to compare PCR procedures post-extraction rather than to validate the reproducibility of the phenol-chloroform method.

PCR-hybridization. In the previous study, DNAs extracted from bone marrow samples (using a similar phenol-chloroform extraction protocol to that described here) were tested for the presence of *L. infantum* by conventional PCR as described previously (23). Briefly, PCRs were carried out using primers DBY and AJS31 (specific for *L. donovani* group parasites) first, followed by gel electrophoresis and hybridization with the digoxigenin-labeled oligonucleotide probe B4Rsa to amplify and detect an 805-bp fragment of minicircle kDNA (27, 28), and then primers R221 and R332, to amplify a 604-bp fragment of the small ribosomal subunit RNA genes of all *Leishmania* species, using probe R331 for detection (32). Positive samples were defined as samples that were positive by both primer sets.

Nested ITS-1 PCR. DNA extracts were subjected to nested PCR targeting the ITS-1 region of the rRNA genes of all *Leishmania* species. First-round reactions were carried out to amplify the complete ITS-1 region, including the 5.8S rRNA gene, with primers IR1 and IR2 (2). Two microliters of DNA extract was added to a 20-μl reaction mix with 2.5 mM $MgCl_2$, and all other reaction and cycling conditions were as described previously (20, 21). Second-round PCR was performed in a separate tube, using 1 μl of a 1:10 dilution of the first-round PCR product and primers ITS1F and ITS2R4, as described previously (21). A thermal PCR cycler (iCycler, Bio-Rad, United Kingdom) was used for this procedure and for all conventional PCR amplification methods reported below. PCR products were electrophoresed in a 1.5% agarose gel with ethidium bromide and examined under UV light to confirm a second-round reaction product of approximately 480 bp. A positive control (bone marrow DNA from a polysymptomatic Brazilian dog with *L. infantum* infection, confirmed by *in vitro* parasitological culture) was included in each PCR run. Negative DNA controls (blood samples) from dogs from an area where leishmaniasis is not endemic (United Kingdom), and with no history of foreign travel, were also included in each batch of samples, in addition to contamination controls for PCR reagents and sham DNA extracts.

Nested kDNA PCR. A nested PCR was used to amplify a variable region of *Leishmania* kDNA, as previously described (18). Primers were not species specific for *L. infantum*, but species can be discriminated based on PCR product size. First-round reactions were carried out with the external primers CSB2XF and CSB1XR, with 2 μl DNA extract in a 20-μl reaction mix. Second-round PCR was performed in a separate tube, using 1 μl of a 1:10 dilution of the first-round PCR product and primers 13Z and LiR. Reaction and cycling conditions were as described previously (18). Electrophoresis on an agarose gel, as described previously, enabled identification of a 680-bp PCR product, consistent with the amplification of *L. infantum* kDNA. Positive and negative PCR controls were as described above.

Real-time quantitative PCR. Primers and a fluorophore-labeled probe were used in a previously validated protocol to amplify a conserved region of *L. infantum* kDNA, which produces a 120-bp amplicon (7). Primers LEISH-1 and LEISH-2 and a 6-carboxyfluorescein (FAM)-labeled TaqMan MGB probe (Applied Biosystems, United Kingdom) were added at 900 nM, 900 nM, and 250 nM, respectively, to a 25-μl total reaction volume containing 2× TaqMan Universal PCR master mix containing UNG Amperase (Applied Biosystems, United Kingdom) to avoid carryover contamination and 5 μl sample DNA at a 1:10 dilution. All bone marrow DNA samples were amplified in duplicate. The thermal cycling profile was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, carried out on an Applied Biosystems 7500 Fast real-time PCR system. Quantification of *Leishmania* DNA was performed using an absolute method based on comparison of threshold cycle (C_T) values with those from a standard curve constructed from 10-fold dilutions of *L. infantum* DNA extracted from cultured parasites, from 1×10^6 to 0.1 parasite equivalent/ml (strain MHOM/MA/67/ITMAP-263), which were run in triplicate on every plate. Predeveloped TaqMan assay reagents (Applied Biosystems, United Kingdom) were used to amplify the 18S rRNA gene as an internal reference of canine genomic DNA to detect PCR inhibition. Negative controls were DNAs extracted from blood samples of 10 dogs from the United Kingdom with no history of foreign travel. The interassay coefficient of variability (CV) of the *Leishmania* real-time qPCR, estimated by running a reference sample in duplicate on every plate, was 1.90%. The mean intra-assay CV of reference sample duplicates was 0.29% (range, 0.12% to 0.52%).

OligoC-TesT kit. OligoC-TesT kits were provided by the manufacturer (Coris Bioconcept, Gembloux, Belgium) and were used according to product recommendations for genus-specific detection of *Leishmania* DNA (3). The test targets the *Leishmania* 18S rRNA gene to carry out PCR amplification of a 115-bp product, alongside which an internal PCR control template is coamplified with the same primers, allowing detection of PCR inhibition. Five microliters of DNA extract was amplified in a 50-μl total reaction volume with 1 U *Taq* polymerase (Truestart *Taq*; Fermentas, United Kingdom), using a conventional thermal cycler (iCycler, Bio-Rad, United Kingdom). Thermal cycling conditions were as follows: 1 cycle of 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s and then 1 cycle of 72°C for 5 min and 94°C for 30 s.

Detection of the specific PCR product was carried out within 30 min of completion of the PCR, using double-sided dipsticks to replace detection by gel electrophoresis. Briefly, 40 μl PCR product was mixed with 40 μl of migration buffer in individual assay tubes. *Leishmania* and internal control PCR amplicons hybridize with gold-labeled detection probes and specific biotinylated capture probes in the lower part of the strip and migrate upwards to capture areas, where they accumulate on Neutralite avidin lines and become visible to the naked eye as a red line. Following the manufacturer's recommendations, a positive result was recorded after 10 min of incubation at 55°C when both the *Leishmania* test line and the internal control line were visible, together with migration control lines, which indicate correct running of the test strip buffer. A positive result was also recorded when only the *Leishmania* test line and migration control lines were visible. Negative results were recorded when only the internal control line and migration control lines were visible. PCR inhibition was recorded when migration control lines were visible but neither *Leishmania* test nor internal control lines were visible. Positive and negative controls and sham DNA extracts were also tested. Positive results were obtained with control DNAs extracted

TABLE 1. Percentages of canine bone marrow samples testing positive by conventional and nested PCRs, OligoC-TesT, and real-time qPCR[a]

Test[b]	% Positive samples (95% CI)				Result of comparison to OligoC-TesT[c]
	Polysymptomatic group (n = 29)	Oligosymptomatic group (n = 34)	Asymptomatic group (n = 82)	Overall (n = 145)	
Real-time qPCR	100 (88.1–100)	91.2 (76.3–98.1)	87.8 (78.7–94.0)	91.0 (85.2–95.1)	+
kDNA PCR**	96.6 (82.2–99.9)	76.5 (58.8–89.3)	62.2 (50.1–72.7)	72.4 (64.4–79.5)	=
OligoC-TesT**	93.1 (77.2–99.2)	73.5 (55.6–87.1)	61.0 (49.6–71.6)	70.3 (62.2–77.6)	
PCR-hybridization*	82.8 (64.2–94.2)	58.8 (40.7–75.4)	53.7 (42.3–64.7)	60.7 (52.2–68.7)	−
ITS-1 PCR**	89.7 (72.6–97.8)	52.9 (35.1–70.2)	41.5 (30.7–52.9)	53.8 (45.3–62.1)	−

[a] Samples were collected from dogs with *L. infantum* infection, as confirmed by ELISA and/or *in vitro*/*in vivo* culture. The clinical status of dogs was defined as asymptomatic (total clinical score of ≤2), oligosymptomatic (total clinical score of 3 to 6), or polysymptomatic (total clinical score of 7 to 18). Samples taken from dogs with no clinical score data were excluded.

[b] PCR tests which showed higher sensitivities for samples from dogs with clinical signs of ZVL (oligo- and polysymptomatic groups combined) than for samples from asymptomatic dogs are indicated by asterisks (χ^2 test). *, $P \leq 0.05$; **, $P \leq 0.01$.

[c] Result of pairwise comparison of the overall sensitivity of each test with that of OligoC-TesT (McNemar's χ^2 test). +, more sensitive than OligoC-TesT; −, less sensitive than OligoC-TesT; =, no significant difference between tests.

from cultured parasites of *L. infantum* MHOM/FR/78/LEM75, *L. tropica* MHOM/SU/74/K27, *L. major* MHOM/SU/73/5-ASKH, and *L. donovani* MHOM/IN/80/DD8. Negative control DNAs from dogs from the United Kingdom and sham extracts were OligoC-TesT negative. A single inconclusive OligoC-TesT result (1/163 samples) due to PCR inhibition was excluded from further analysis.

Statistics. Pairwise agreement between tests with dichotomous results was measured using Cohen's kappa coefficient (κ), with agreement characterized as excellent (κ = 1.00 to 0.81), good (κ = 0.80 to 0.61), moderate (κ = 0.60 to 0.41), weak (κ = 0.40 to 0.21), or negligible (κ = 0.20 to 0.00), as described by others (12). Differences in proportions of positive samples by each test were compared by McNemar's chi-square (χ^2) test for paired data. Between-group differences in positive proportions were compared using Pearson's chi-square test. Generalized estimating equations (XT-GEE) were used to measure the association between the positive proportion for each test and the time from first detection of infection or the total clinical score and to measure associations between test results and log-transformed (n + 1) parasite burdens or clinical scores, allowing for autocorrelation (i.e., nonindependent repeat samples from the same animal) by clustering on individual dogs. P values of <0.05 were considered statistically significant. All analyses were carried out using Stata, version 9.0 (30).

RESULTS

Overall test sensitivity for infected dogs. For bone marrow samples taken from dogs on or after the time of first detection of infection by serology and/or *in vitro*/*in vivo* culture, the overall sensitivity of OligoC-TesT was 70.3% (102/145 samples) (Table 1). The proportion of positive samples by OligoC-TesT was significantly greater than the proportion detected by the PCR-hybridization method (60.7% [88/145 samples]; McNemar's $\chi^2 = 8.17$; $P = 0.007$) or ITS-1 nested PCR (53.8% [78/145 samples]; McNemar's $\chi^2 = 22.2$; $P < 0.001$) but was not significantly different from the proportion detected by kDNA nested PCR (72.4% [105/145 samples]; McNemar's $\chi^2 = 0.36$; $P = 0.69$). The most sensitive method was real-time qPCR, which detected *L. infantum* DNA in 91.0% (132/145 samples) of the samples tested, which is significantly higher than the sensitivity of any of the other procedures (McNemar's $\chi^2 \geq 25.14$ and $P < 0.001$ in all pairwise comparisons).

Sensitivity for symptomatic and asymptomatic dogs. The proportions of positive test results for bone marrow samples from dogs stratified into 3 clinical groups (oligo-, poly-, and asymptomatic, as defined above) are shown in Table 1. Comparing symptomatic (oligo- and polysymptomatic groups combined) with asymptomatic dogs, the test sensitivity was significantly higher for bone marrow samples from the former group for OligoC-TesT, kDNA PCR, ITS-1 PCR, and PCR-hybridization ($\chi^2 \geq 3.91$; $P \leq 0.05$) but was not significantly different between clinical groups for real-time qPCR ($\chi^2 = 2.41$; $P = 0.12$) (Table 1). There was a significant positive association between the severity of clinical signs of leishmaniasis in infected dogs (total clinical score range, 0 to 18) and the probability of testing positive by any of the diagnostic PCR methods, including real-time qPCR ($z \geq 2.66$; $P \leq 0.008$). Similarly, total clinical scores in infected dogs were positively correlated with log-transformed *Leishmania* parasite burdens measured in bone marrow samples by real-time qPCR ($z = 4.33$; $P < 0.001$).

Test agreement. Pairwise agreement between different tests for individual samples was only moderate between OligoC-TesT and both ITS-1 PCR ($k = 0.57$; standard error [SE] = 0.07; $P < 0.01$) and PCR-hybridization ($k = 0.59$; SE = 0.08; $P < 0.01$), reflecting the superior sensitivity of OligoC-TesT. Agreement was weak between OligoC-TesT and real-time qPCR ($k = 0.38$; SE = 0.06; $P < 0.01$) due to the superior sensitivity of real-time qPCR. Despite the similar sensitivities of the OligoC-TesT and kDNA PCR, their pairwise agreement was only moderate: 16 samples were kDNA positive and OligoC-TesT negative, whereas for 14 samples the reverse was true ($k = 0.56$; SE = 0.08; $P < 0.01$). In 29 of 30 of these apparently discordant samples, parasite burdens estimated by real-time qPCR were low (<100 parasites/ml) (mean = 19.5; 95% confidence interval [CI] = 13.5 to 25.5; one outlier was excluded).

A single bone marrow sample that tested negative by real-time qPCR was positive by OligoC-TesT, PCR-hybridization, and kDNA PCR. The specificities of the tests were not measured for dogs from the area of endemicity, since all Brazilian dogs in the present study were selected based on criteria of infection at some time point. However, using a small number of healthy United Kingdom control dogs from areas where leishmaniasis is not endemic ($n = 10$), the specificity was 100% for all tests.

Analytical sensitivity. The detection limit of OligoC-TesT, as stated by the manufacturer, is 0.05 *Leishmania* parasite per PCR (3). The analytical sensitivity of both kDNA nested PCR and ITS-1 PCR, estimated from 10-fold serial dilutions of DNA from cultured *L. infantum* promastigotes (MHOM/MA/67/ITMAP-263), was 0.2 parasite per PCR, though subjective signal strength (band intensity) in endpoint PCR was stronger

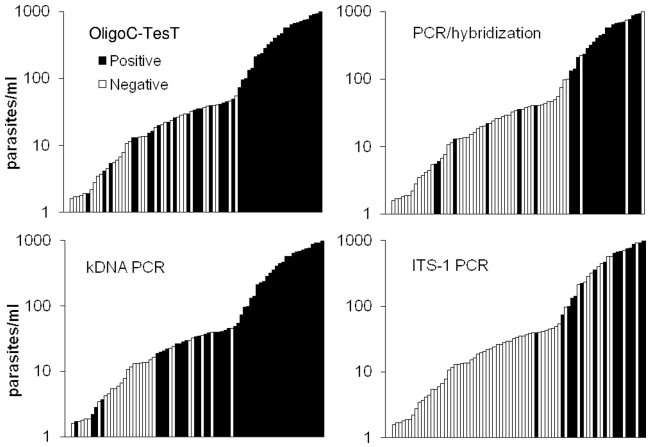

FIG. 1. Distribution of Oligo-C TesT and conventional PCR positive and negative results for individual bone marrow samples, sorted according to parasite burdens measured by real-time qPCR. Samples are sorted in ascending order with respect to parasite burden, truncated at 1,000 parasites/ml for clarity.

for kDNA PCR. The analytical sensitivity of the previously performed PCR-hybridization reaction was not determined. The detection limit for real-time qPCR was 0.005 parasite per PCR, similar to the previously published estimate of 0.001 parasite per PCR (7). When tested in clinical samples (Fig. 1), OligoC-TesT gave positive results for 100% of canine bone marrow DNA samples with estimated *Leishmania* burdens of ≥74 parasites/ml (i.e., ≥0.4 parasite/PCR); its sensitivities to detect 10 to 100 parasites/ml (0.05 to 0.5 parasite/PCR) and <10 parasites/ml (<0.05 parasite/PCR) were 50.0% and 21.1%, respectively (Fig. 2). kDNA PCR showed a slightly lower cutoff, of 49 parasites/ml (0.1 parasite/PCR), for bone marrow samples, missing only one positive sample above this

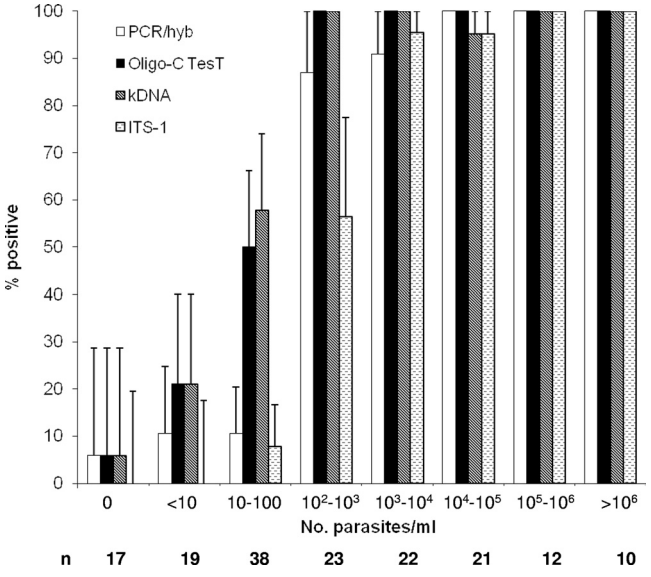

FIG. 2. Sensitivities of OligoC-TesT and conventional PCR methods (with 95% upper binomial CI) with bone marrow samples, grouped by parasite burdens measured by real-time qPCR. *n*, number of samples.

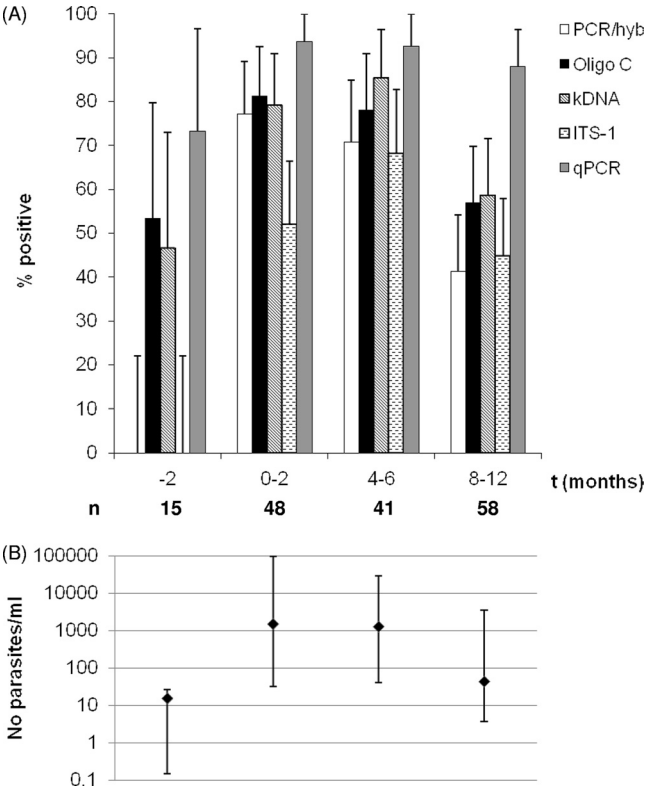

FIG. 3. (A) Percentages of positive canine bone marrow samples (with 95% upper binomial CI) by conventional and nested PCRs, OligoC-TesT, and real-time qPCR at time *t* after first detection of *L. infantum* infection by culture and/or serology (t_0). (B) Median parasite burdens (parasites/ml sample) measured by real-time qPCR at time *t*. Error bars show interquartile ranges (25% to 75%). *n*, number of samples.

threshold (i.e., 98.9% sensitivity) and detecting 57.9% and 21.1% of positive samples at 10 to 100 and <10 parasites/ml, respectively. PCR-hybridization and ITS-1 PCR were less sensitive with low parasite burdens in clinical samples and did not reach 90% sensitivity until the parasite concentration exceeded 1,000 per ml of sample.

Test sensitivity and time course of infection. The results of the three conventional PCR tests, OligoC-TesT, and real-time qPCR for bone marrow samples collected at the time of detection of infection are shown in Fig. 3A. The most striking differences in test sensitivity occurred at t_{-2}, when the proportions positive by ITS-1 PCR and PCR-hybridization were both 0, whereas real-time qPCR, OligoC-TesT, and kDNA PCR were positive in 73.3%, 53.3%, and 46.7% of cases, respectively. The positive proportion was highest in the first 6 months after confirmed infection and decreased thereafter. Excluding samples taken at t_{-2} and controlling for clinical score, there was a significant negative relationship between the proportion of positive samples and increasing time from confirmed infection when samples were tested by OligoC-TesT ($z = -2.71$; $P = 0.007$), kDNA PCR ($z = -2.93$; $P = 0.003$), PCR-hybridization ($z = -4.05$; $P < 0.001$), or real-time qPCR ($z = -2.52$; $P = 0.01$), and this trend was borderline significant in the case of ITS-1 PCR ($z = -1.70$; $P = 0.09$). This trend of lower sensitivity with increasing duration of infection was associated

with a similar negative correlation between bone marrow parasite burdens measured by real-time qPCR and increasing duration of infection, controlling for clinical score ($z = -4.48$; $P < 0.001$) (Fig. 3B).

DISCUSSION

This study shows that the overall sensitivity of OligoC-TesT for detection of *Leishmania* DNA in bone marrow aspirates from infected dogs (70%) is comparable to or greater than the sensitivities of the conventional PCR methods tested (range, 54 to 72%). In polysymptomatic clinical cases of canine ZVL, the sensitivity of OligoC-TesT was 93%, in comparison with 83 to 97% sensitivity for the conventional PCR methods. This is comparable to previously reported OligoC-TesT sensitivities for humans with symptomatic visceral leishmaniasis (78% to 100%) (3). The positive correlation reported here between parasite burdens in canine bone marrow samples and the severity of clinical signs of ZVL was consistent with our finding that OligoC-TesT was significantly more sensitive for symptomatic than asymptomatic dogs. This was similar to the trend shown by the other conventional PCR tests and corroborates previous work demonstrating similar relationships (15, 26). Therefore, for the diagnosis of ZVL by veterinarians in suspected clinical cases, OligoC-TesT is likely to be highly sensitive, whereas for detection of asymptomatic and early-stage infections in epidemiological surveys or trials of vaccines against canine ZVL, additional diagnostic procedures may be required. The ease of use of OligoC-TesT will be an advantage in both veterinary diagnostic and public health contexts. In particular, OligoC-TesT provides high sensitivity without the need for nested PCR protocols, which are prone to contamination problems, or hybridization steps, which increase the processing time and in some cases do not improve sensitivity (10). Conversely, real-time qPCR showed the highest sensitivity of all methods tested, though this procedure has the most requirements for trained personnel and costly equipment.

OligoC-TesT and kDNA PCR, despite having similar overall sensitivities, showed only moderate agreement, indicating a degree of discordance. Importantly, almost all of the instances of discordance (29/30 instances) occurred in samples with low parasite burdens (<100 parasites/ml), which likely reflects lower analytical sensitivity at the detection limit for both these tests. The apparent lower sensitivity of the PCR-hybridization method could have resulted from a lower DNA extraction efficiency during the previous study (23), whereas all other tests in the present study were performed on our DNA extracts from the same archived bone marrow samples. In addition, the PCR-hybridization method relied on agreement between rRNA and kDNA PCRs, so any samples which were positive in one test but not the other would have been classed as negative.

Despite the wide range of PCR protocols that have been used to detect *Leishmania* infection, relatively few studies have directly compared the sensitivities of different protocols. More-sensitive conventional methods than the ones used here have been described (9, 10). A detection limit of 10^{-3} parasite/ml seeded canine blood has been reported for primers targeting kDNA sequences, though as previously discussed, theoretical analytical sensitivity does not always reflect diagnostic performance with clinical samples (10). For example, in other work comparing the sensitivity of conventional kDNA PCR with that of real-time qPCR for use on canine bone marrow samples, only samples with parasite loads in excess of 30 parasites/ml were positive by kDNA PCR (7). With blood samples from human patients suffering from visceral leishmaniasis, nested PCR was 100% sensitive when parasitemia exceeded 22 parasites/ml, though parasite burdens as low as 0.12 parasite/ml were detected sporadically (16). The nested kDNA PCR tested here showed 99% sensitivity at parasite burdens above 49 parasites/ml (Fig. 1), while OligoC-TesT reliably produced positive results when parasite densities exceeded 74 parasites/ml of sample. In contrast, the sensitivities of nested ITS-1 PCR and PCR-hybridization remained low until bone marrow parasite burdens were 10- to 100-fold higher. The diagnostic advantage of OligoC-TesT and kDNA PCR was most apparent for samples with low parasite burdens, such as samples from asymptomatic dogs and dogs in the early stages of infection (2 months before the first detection of infection by serology and/or parasitological culture). The most sensitive test was real-time qPCR, which is likely to become the gold standard for parasite detection in population (prevalence) studies, although it is not necessarily the best method for diagnostic use. As pointed out by others (10), the advantages of a highly sensitive test are offset by a reduced positive predictive value for clinically patent disease, which has been correlated with increased transmission of parasites from dogs to sand flies in previous studies (1).

A negative relationship between the sensitivity of conventional PCR and the duration of infection has been reported in previously published results obtained using the PCR-hybridization test and larger numbers of samples from these Brazilian dogs. That study showed a decline to ca. 50% sensitivity at 300 days postinfection (23). In the present study, we confirmed a similar trend of lower test sensitivity with increasing duration of infection for three of the four qualitative PCR methods tested. The fall in sensitivity with increasing time from confirmed infection was associated with lower bone marrow parasite burdens and a consequent decrease in the number of samples with parasite levels above the detection limit for each test. Lower parasite burdens are likely to reflect both parasite clearance from more-resistant animals and the loss of highly parasite-positive susceptible dogs from the population through an increased mortality rate (23).

Species of *Leishmania* other than *L. infantum*, such as *L. amazonensis* and *L. braziliensis*, have previously been isolated from dogs (4, 13, 22, 25, 31), although the epidemiological significance of the dog as a reservoir host for these parasites is unclear. Mixed infections with *L. braziliensis* and *L. infantum* have also been documented (14). It is necessary to confirm that the comparative high sensitivities of OligoC-TesT and kDNA PCR did not result from detection of species of *Leishmania* other than *L. infantum*, since both OligoC-TesT and kDNA PCR show broad specificity for all *Leishmania* spp. However, product sizes in kDNA PCR were in all cases consistent with *L. infantum* and enabled exclusion of *L. amazonensis* (but not *L. braziliensis*) (18), and previous identification of cultured parasites from the study population yielded only *L. infantum* (24). Moreover, examination of health records from several decades of monitoring the human population at the study site did not provide any evidence to support autochthonous transmission

of cutaneous leishmaniasis in this study area (L. M. Garcez, unpublished observations).

In conclusion, we have demonstrated that OligoC-TesT is an effective method for detection of *Leishmania* DNA in the reservoir host of ZVL, with a comparable or superior sensitivity to those of the conventional PCR methods tested. Due to the speed and ease of operation and interpretation and to standardized methodology, it is highly suitable for use in areas of endemicity for veterinary laboratory diagnostic purposes and for use by public health authorities. As with all molecular methods for detection of *Leishmania*, DNA extraction and amplification using a thermal cycler are required, so OligoC-TesT is not yet suitable for point-of-care veterinary clinical use. In regions where multiple species of *Leishmania* are (potentially) circulating, direct sequencing of PCR products may be necessary to allow species identification (3); however, prototype *Leishmania* species-specific oligochromatographic kits are also in development.

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