ORIGINAL ARTICLE

PERFORMANCE OF CONVENTIONAL PCRs BASED ON PRIMERS DIRECTED TO NUCLEAR AND MITOCHONDRIAL GENES FOR THE DETECTION AND IDENTIFICATION OF *Leishmania* spp.

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SUMMARY

In visceral leishmaniasis, the detection of the agent is of paramount importance to identify reservoirs of infection. Here, we evaluated the diagnostic attributes of PCRs based on primers directed to cytochrome-B (*cytB*), cytochrome-oxidase-subunit II (*coxII*), cytochrome-C (*cytC*), and the minicircle-kDNA. Although PCRs directed to *cytB, coxII, cytC* were able to detect different species of *Leishmania*, and the nucleotide sequence of their amplicons allowed the unequivocal differentiation of species, the analytical and diagnostic sensitivity of these PCRs were much lower than the analytical and diagnostic sensitivity of the kDNA-PCR. Among the 73 seropositive animals, the asymptomatic dogs had spleen and bone marrow samples collected and tested; only two animals were positive by PCRs based on *cytB*, *coxII*, and *cytC*, whereas 18 were positive by the kDNA-PCR. Considering the kDNA-PCR results, six dogs had positive spleen and bone marrow samples, eight dogs had positive bone marrow results but negative results in spleen samples and, in four dogs, the reverse situation occurred. We concluded that PCRs based on *cytB*, *coxII*, and *cytC* can be useful tools to identify *Leishmania* species when used in combination with automated sequencing. The discordance between the results of the kDNA-PCR in bone marrow and spleen samples may indicate that conventional PCR lacks sensitivity for the detection of infected dogs. Thus, primers based on the kDNA should be preferred for the screening of infected dogs.

KEYWORDS: *Leishmania* spp.; kDNA; Mitochondrial genes; PCR; Phylogeny; Dogs.

INTRODUCTION

The protozoan parasite that causes leishmaniasis belongs to the order Kinetoplastida, family Trypanosomatida and genus *Leishmania*[1]. The protozoan infects the mononuclear phagocyte system mainly in spleen, liver, bone marrow, and lymph nodes[2].

There are reports of at least 30 different *Leishmania* species distributed throughout the Old and New World[1,3]. The species that cause leishmaniasis in Latin America are divided into two taxonomic groups. One group is the subgenus *Viannia*, which comprises the species *Leishmania (Viannia) braziliensis*, *Leishmania (Viannia) panamensis* and *Leishmania (Viannia) guyanensis*, and are responsible for cutaneous or mucocutaneous infections. The other group is the subgenus *Leishmania*, which includes the species *Leishmania (Leishmania) mexicana* and *Leishmania (Leishmania) amazonensis*, responsible for localized or diffuse skin lesions, and *Leishmania (Leishmania) infantum*, which causes visceral leishmaniasis[1,4,5].

In Brazil, the most frequently encountered species is *Leishmania*

(Leishmania) infantum chagasi and the most commonly found vectors are *Lutzomyia longipalpis* and *Lutzomyia cruzi*[6,7]. Based on molecular criteria, Mauricio *et al*. (1999)[8] considered the species *Leishmania (Leishmania) infantum chagasi* and *Leishmania (Leishmania) infantum* synonymous. In the Americas, eleven dermotropic species of *Leishmania* causing disease in humans, and eight species described only in animals are currently recognized. In Brazil, seven dermotropic species have been identified, six of the subgenus *Viannia* and one of the subgenus *Leishmania*. The three main dermotropic species are: *Leishmania (Viannia) braziliensis*, *Leishmania (Viannia) guyanensis* and *Leishmania (Leishmania) amazonensis*. Recently, the species *Leishmania (Viannia) lainsoni*, *Leishmania (Viannia) naiffi*, *Leishmania (Viannia) lindenberg*, and *Leishmania (Viannia) shawi* were identified in North and Northeast States in Brazil[9].

The Polymerase Chain Reaction (PCR) is largely used for the detection and identification of pathogens and is a valuable tool for molecular and epidemiological studies. In leishmaniasis, as in many other systemic infections, the detection of the agent is of paramount importance for the confirmation of infection in seropositive animals, and in studies carried

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out with vectors and free-living animals to identify reservoirs of infection. In addition, PCR allows the molecular identification of the agent when phylogenetically-informative markers are amplified and sequenced, such as DNA barcode within mitochondrial and nuclear genomes[10,11]. Mitochondrial genes have shown a high degree of resolution in molecular/phylogenetic studies because they are a result of maternal transmission in metazoans[12]. Besides, mitochondrial genes phylogenies are not affected by multiple nucleotide substitutions driven by adaptive selection, since the majority of the substitutions found in these loci are synonymous[13].

The aim of the present study was to determine the analytical and diagnostic performance of PCRs based on primers directed to conserved markers located in the mitochondrial (-maxicircle-kDNA and minicircle-kDNA) and the nuclear genome for the direct diagnosis of *Leishmaniasis* using animal tissues.

In addition, we aimed to compare the performance of a PCR based on the minicircle-kDNA detection in bone marrow and spleen samples for the *post-mortem* diagnosis of canine leishmaniasis (CVL) in seropositive, asymptomatic dogs. In this kind of animals (seropositive but asymptomatic), *Leishmania* testing is still controversial.

MATERIAL AND METHODS

Parasites

Promastigotes of *Leishmania* spp. grown in Schneider medium with 20% fetal bovine serum were used. The samples were provided by the Leishmaniasis Research Laboratory, *Oswaldo Cruz* Foundation and belonged to the Collection of *Leishmania* of the *Instituto Oswaldo Cruz (CLIOC)*.

The following reference strains were used: *Leishmania* (*leishmania*) *mexicana* (MHOM/BZ/1982/BEL21), *Leishmania* (*Viannia*) *guyanensis* (MHOM/BR/1975/M4147), *Leishmania* (*Viannia*) *braziliensis* (MHOM/BR/1975/M2903), *Leishmania* (*Leishmania*) *tropica* (MHOM/SU/1958/STRAIN OD), *Leishmania* (*Leishmania*) *amazonensis* (IFLA/BR/1967/PH8), *Leishmania* (*Viannia*) *lainsoni* (MHOM/BR/1981/M6426), *Leishmania* (*Viannia*) *naiffi* (MDAS/BR/1979/M5533), *Leishmania* (*Viannia*) *shawi* (MCEB/BR/1984/M8408), *Leishmania* (*Leishmania*) *donovani* (MHOM/ET/1967/L82;HV3;LV9), *Leishmania* (*Viannia*) *major* (MHOM/IL/1980/FRIEDLIN), *Leishmania* (*Leishmania*) *infantum chagasi* (MHOM/BR/2002/LPC-RPV), *Leishmania* (*Leishmania*) *infantum* (MHOM/TN/1993/LV10), *Leishmania* (*Leishmania*) *hertigi* (MCOE/PA/1965/C8), Leishmania (*Leishmania*) *infantum chagasi* (MHOMBR/1974/PP75), and *Leishmania* (*Viannia*) *colombiensis* (IGOM/PA/1985/E582.34).

Dog samples

Samples of 73 CVL seropositive and asymptomatic dogs from the city of *Espírito Santo do Pinhal*, *São Paulo* State, were collected in 2010. The dogs were neither evaluated by radiological and ultrasound methods, nor by pathological ones. The asymptomatic condition was defined after visual inspection and the confirmation of the absence of emaciation, skin lesions, onychogryphosis, alopecia and hair loss.

The serological status of the animals was determined at the time of sampling using ELISA (EIE-*leishmaniose-visceral-canina-Bio-Manguinhos, Rio de Janeiro*, *RJ*, Brazil) and IFAT (IFI-*leishmaniose-visceral-canina-Bio-Manguinhos, Rio de Janeiro*, *RJ*, Brazil) consecutively, as recommended by the Brazilian sanitary authorities. After euthanasia, the animals were necropsied and fragments of the spleen and aspirates of bone marrow were collected. The samples were kept in 1.5 mL plastic microtubes and stored at -20 °C. Tissues of a CVL-seronegative dog from a non-endemic area were used as negative controls, and in the analytical sensitivity experiments.

Preparation of *Leishmania* and canine tissue suspensions

Promastigotes of *Leishmania* spp. reference strains were concentrated and re-suspended in 100 μL of Schneider medium and used for DNA extraction.

Aspirates of bone marrow and spleen fragments of the seronegative dogs were homogenized in TE 20% w/v (10 mM Tris–HCl pH 8.0; 1 mM EDTA pH 8.0) and the suspensions were mixed with decreasing amounts of *Leishmania* (*Leishmania*) *infantum chagasi* promastigotes (MHOM/BR/2002/LPC-RPV) (see below).

A suspension containing 1.0×10^4 parasites/μL of promastigotes of *Leishmania* (*Leishmania*) *infantum chagasi* (MHOM/BR/2002/LPC-RPV) in Schneider medium was prepared (counted in a Neubauer chamber), then tenfold dilutions using TE buffer to a final volume of 20 μL were prepared. The dilution procedure was performed in duplicate.

Each serially diluted *Leishmania* suspension was mixed with either 100 μL of bone marrow suspension or spleen suspension obtained from the seronegative dog, resulting in eight suspensions of bone marrow and eight suspensions of spleen containing from 200,000 to 0.02 promastigotes in a final volume of 120 μL.

DNA extraction

The bone marrow and spleen suspensions containing decreasing amounts of *Leishmania* promastigotes were washed twice in TE by centrifugation at 12,000 X g for 5 min, the supernatant was discarded and the pellet was re-suspended in 500 μL of lysis buffer (10 mM Tris-HCl pH 8.0; 25 mM EDTA pH 8.0; 100 mM NaCl, 1% SDS, 10 μg/mL proteinase K). The suspension was incubated at 37 °C overnight. The DNA extraction was performed according to a phenol, chlorophorm, isoamyl-alcohol (25:24:1) protocol followed by a precipitation step with ethanol as described elsewhere[14]. The precipitated DNA was re-suspended in 30 μL of TE and stored at -20 °C until used in PCR.

Aspirates of bone marrow and spleen fragments of the 73 seropositive dogs were homogenized in TE 20% w/v (10 mM Tris–HCl pH 8.0; 1 mM EDTA pH 8.0) and 100 μL of the resulting suspensions were used in the DNA extraction, as previously described.

Primers

Five sets of primers were used: one set directed to the minicircles-kDNA, two sets directed to the mitochondrial maxicircle-kDNA and two sets directed to the nuclear DNA. The primers for the minicircles-kDNA were described by Rodgers *et al*. (1990)[15]. The primers targeting the

mitochondrial genes were based on cytochrome B (*cytB*) and cytochrome oxidase subunit II (*coxII*) gene sequences (maxicircle-kDNA). The primers directed to the nuclear DNA were based on the 18S rRNA sequence and cytochrome C (*cytC*) coding sequences. The primers targeting *cytB*, *coxII*, and *cyt*C genes were designed in this study, and were based on sequences of several species of the genus *Leishmania* available in GenBank database. The sequences obtained from GenBank were aligned and consensus primers were designed to amplify gene fragments of all the sequences, indiscriminately. The primers directed to 18S rRNA (for PCR and sequencing) were described elsewhere[16]. All the primers used in this study are described in Table 1.

PCR

The PCR cycling conditions were 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, T°C for 30 s and 72 °C for 30 s (T value for each set of primers is found in Table 1). The PCR was ended with a final extension of 72 °C for 5 min. Primers, dNTP and MgCl$_2$ were used at a final concentration of 0.5 µM, 200 µM and 1.5 mM, respectively. Taq DNA polymerase platinum (Invitrogen™, Carlsbad, CA, USA) was used at a final concentration of 1.25 Units/50 µL. Five microliters of the buffer supplied with the enzyme and 5 µL of template DNA were added to the PCR mixture. To visualize the amplified products, 10 µL of samples were mixed with 3 µL of loading buffer and subject to electrophoresis in 2.0% agarose gels stained with ethidium bromide (0.5 µg/mL).

Sequencing

The PCR products were sequenced using the same primers and the Big Dye® reagent (Applied Biosystems, Foster City, CA, USA). Sequencing products were analyzed on an ABI377 automated sequencer. Both strands of each PCR product were sequenced at least four times in both directions to increase the reliability of sequencing. The sequences were assembled and the contig formed with the phred-base calling were analyzed using the phrap-assembly tool available in the suite Codoncode aligner v.1.5.2. (Codoncode Corp. Dedham, Massachusetts). The PCR derived sequences were submitted to the BLAST search (blastn, www.ncbi.nlm.nig.gov/BLAST) and similar sequences were downloaded to be used in the sequence analysis. Genetic sequences were deposited in GenBank under the accession numbers: KF302704 to KF302753; KU674349, KU674350.

Sequence analysis

The evolutionary history was inferred using the Neighbor-Joining method[17]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) were calculated as described elsewhere[18]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[19]. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analyses were conducted by using MEGA5 (maximum likelihood, evolutionary distance, and maximum parsimony methods)[20].

Tests of samples

Promastigotes of *Leishmania* spp. were tested with all the sets of primers, as well as the promastigote-spiked samples. The PCR products were sequenced, and these sequences were analyzed as already described. Aspirated bone marrow and spleen suspension from the 73 seropositive dogs were also tested by PCR using the minicircle-kDNA primers and the ones directed to cytochrome coding sequences.

Ethics

This work is in agreement with the Ethical Principles in Animal Research adopted by the Bioethics Commission of the School of Veterinary Medicine and Animal Science, University of Sao Paulo, Brazil (protocol number 1501/2008).

Table 1
Nucleotide sequences of the primers used in the molecular analysis, the fragment length they amplify, and annealing temperature for each pair used in PCR

Name	Gene	Sequence	Fragment lenght / T value
13A	kDNA	GTG GGG GAG GGG CGT TCT	116 / 56°C
13B	kDNA	ATT TTA CAC CAA CCC CCA GTT	
609F	18S	CAC CCG CGG TAA TTC CAG C	900 / 55°C
706R	18S	TTG AGG TTA CAG TCT CAG	
1156F	18S	CGT ACT GGT GCG TCA GAG G	Sequencing primers
1156R	18S	CCT CTG ACG CAC CAG TAC G	Sequencing primers
CytB/R2	Cytochrome B	GAA CTT CKA CAA TAH ACA AAT CAT AAT A	377 / 52°C
CytB/F1	Cytochrome B	ATG CAT TTR TTT TGT TTA CAT TAT TTT A	
CytOxII/R2	Cytochrome Oxidase II	GCA TAA ATC CAT GTA AAA CAC CAC A	602 / 52°C
CytOxII/F1	Cytochrome Oxidase II	TGG CTT TTA TWT TAT CAT TTT GAA TG	
CytC/F3	Cytochrome C	GYG GYG AGA AGC TGT TCA AG	230 / 60°C
CytC/R2	Cytochrome C	CGA CAT CTT CGT GCC AGG CAT AA	

Statistical analysis

The agreement between the diagnostic techniques was evaluated using the kappa test, with the following definitions: no agreement (k<0), slight agreement (0<k<0.2), fair agreement (0.2<k<0.4), moderate agreement (0.4<k<0.6), substantial agreement (0.6<k<0.8) and almost perfect agreement (k>0.8)[21].

RESULTS

All the reference strains were successfully detected using primers targeting the minicircle-kDNA, maxicircle-kDNA (*cytB* and *coxII*) and the nuclear genes (*cytC* and *18SrRNA*). The evolutionary histories of standard isolates used in this study were reconstructed by the analysis of the nucleotide sequences obtained by each PCR. The topology of the trees revealed that all the markers could unequivocally differentiate the species (Fig. 1).

However, the PCRs based on primers directed to *cytB*, *cytC* and *coxII* were about 10 times less sensitive than the primers directed to the minicircle-kDNA. The PCR targeting *cytB*, *cytC* and *coxII* were able to detect, theoretically, 20 to 200 *Leishmania* parasites diluted in 120 μl of suspension, while the PCR directed to the minicircle-kDNA detected two to 20 parasites. The procedures to determine the analytical sensitivity were performed twice. The analytical sensitivity of the primers directed do 18S rRNA was not determined.

The number of positive samples detected by the primers targeting *cytB*, *cytC*, and *coxII* genes was significantly lower than the number of positive samples revealed by the PCR targeting the minicircle-kDNA. Eighteen dogs tested positive by PCR targeting the minicircle-kDNA while only two dogs were positive by PCR based on *cytB*, *cytC*, and *coxII* primers. The two dogs that had positive PCR results in both bone marrow and spleen samples using all of the primers, and the corresponding sequencing results revealed that *Leishmania (Leishmania) infantum chagasi* was the cause of infections.

The performance of PCR using minicircle-kDNA primers for *Leishmania* detection in bone marrow and spleen samples of the 73 seropositive dogs resulted in a poor agreement according to the kappa coefficient test (k = 0.4). Six animals had both samples positive (spleen and bone marrow), eight dogs had positive only the bone marrow samples positive, and four animals had only the spleen samples positive (Table 2).

DISCUSSION

This work aimed to evaluate the analytical and diagnostic performance of PCR assays based on primers targeting conserved genes for DNA barcoding within the mitochondrial and the nuclear genome of *Leishmania* spp.

Genes encoding proteins of the cytochrome family are valuable markers for molecular/phylogenetic studies, as they allow the reconstruction of the evolutionary histories in various classes of organisms, including the genus *Leishmania*[21,22,23]. For this reason, we designed new primers targeting the mitochondrial DNA, in particular *cytB*, and *coxII*. The *cytC* gene encodes a mitochondrial product, but it
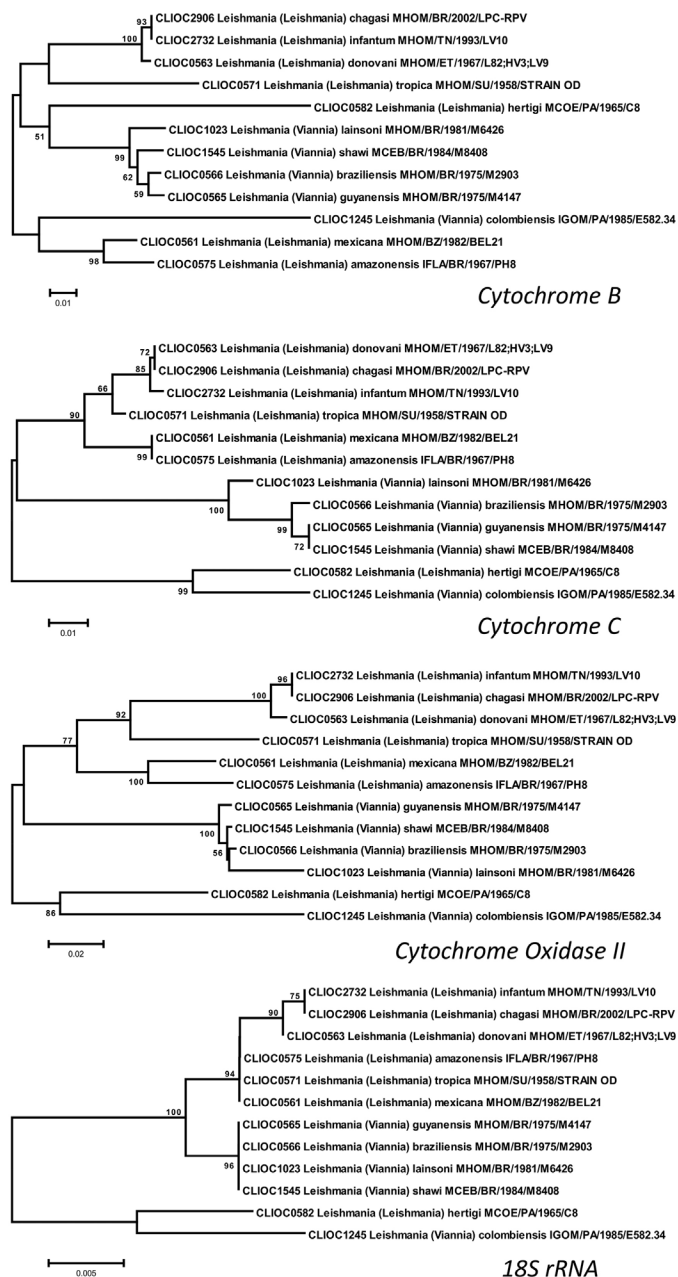

Fig. 1 - Evolutionary relationships within *Leishmania* spp. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved nucleotide sequences of locus coding for 18S rRNA, Cytochrome B, Citochrome oxidase II and Citochrome C. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.

is located in the nuclear genome of the parasite[25]. *CytC* was selected for primer design because this gene also codes for a universally conserved protein and should have the same applicability in terms of phylogenetic studies as is the case of the genes located in the mitochondrial genome.

Table 2
Results of the PCR based on primers to minicircle-kDNA (PCR-kDNA) performed with samples of bone marrow and spleen from seropositive dogs for Canine Visceral Leishmaniasis

		PCR-kDNA spleen		Total
		+	−	
PCR-kDNA	+	6	8	14
bone marrow	−	4	55	59
Total		10	63	73

The experiment to determine the analytical specificity of PCRs targeting *cytB*, *cytC* and *coxII* showed that a broad range of species of the genus *Leishmania* could be successfully detected and identified. The topology of the trees reconstructed using each marker (*cytB*, *cytC*, and *coxII* and *18SrRNA*) were fully consistent when examined independently or compared two by two revealing that all the markers can be used together or separately for the molecular identification of *Leishmania* species.

The phylogeny of *Leishmania* isolates from China was reconstructed using *coxII* and *cytB* in two evolutionary studies[26,27]. *CoxII* phylogenies allowed the former authors to assign some of the autochthone isolates to define species within the genus *Leishmania*. In addition, a number of undescribed *Leishmania* species causing visceral leishmaniasis were identified by using *cytB*[27]. Phylogenies inferred with ITS-1, *coxII* and *cytB* had already been demonstrated as congruent[26,27,28] and here we could extend these findings after adding more species to the analysis, and another marker, the *cytC*. As pointed elsewhere[26], the complementation of the molecular identification by using other mitochondrial and nuclear markers is advantageous for the microorganisms typing, and the four markers tested here have shown equal capability to discriminate each one of the well-defined species within the genus *Leishmania*. *CytC* seems to have the same evolutionary rate as *cytB*, as judged from their equivalent overall similarity (not shown). Unlikely, *coxII* has shown a higher evolutionary rate, which has been reported elsewhere[26].

Regarding the experiments on the analytical sensitivity of PCRs, a remarkable superiority of primers designed by Rodgers *et al.* (1990)[15] was observed. The locus targeted by the primers 13A and 13B of the minicircle-kDNA is present in thousands of copies in the parasite genome, as well as the dozens of copies of the maxicircle-DNA hosting the *cytB* and *coxII* genes[29]. The higher number of copies could explain the higher analytical sensitivity of the PCR primers of the minicircle-kDNA. Another important factor that could explain the differences in among the PCR assays is related to the molecular weight of the amplified products. It is well known that the PCR efficiency is greater when the amplified product is smaller[30,31,32]. The molecular weight of the amplified products with primers 13A and 13B are substantially smaller than the ones of the other PCRs.

Such a high difference in the PCR performances was also noted by Lachaud *et al.* (2002)[33], when conventional PCR directed to kDNA had an absolute sensitivity 500-fold higher than the PCRs directed to the genes located in the nuclear genome (18S rRNA).

Considering the diagnostic sensitivity, the primers directed to the

minicircle-kDNA were more sensitive than the other primers. Based on these results, only two dogs were considered positive by the primers targeting the genes encoding cytochromes (*cytB, cytC* and *coxII*).

The spleen and bone marrow kDNA-PCR results revealed that they were concordant in six of the 18 positive dogs, indicating that the use of only one type of sample for the detection of *Leishmania* spp. has a considerable chance of yielding a false negative result. Therefore, the association of two samples might be a necessary measure to increase the sensitivity of the molecular diagnosis of leishmaniasis.

Although molecular methods for CVL diagnosis based on nucleic acid amplification assays are considered highly sensitive[34], in the present study the conventional PCR did not seem to be more sensitive than the serological techniques. In the present study, all the dogs tested positive by ELISA and IFAT (used consecutively), and this combination of tests was the official system used for the detection of infected dogs in Brazil until 2011. Nevertheless, seropositive results by ELISA and IFAT do not necessarily mean a true *Leishmania* spp. infection because of the controversial specificity of both tests. It is noteworthy that since 2011, the official criteria for the diagnosis of CVL in Brazil has been based on the use of a Dual Path Platform (DPP®CVL, *Biomanguinhos, Rio de Janeiro, RJ*, Brazil) comprising specific recombinant proteins (rK26 and rK39) as the screening test, followed by the indirect ELISA as the confirmatory test[35].

The determination of the sensitivity and specificity of diagnostic tests for CVL has been a moot point due to the difficulty in establishing a gold standard. The parasitological examination remains the gold standard for the diagnosis of CVL, and a positive result in any of the other diagnostic tests might be misinterpreted as a false positive, because of the low sensitivity of the parasitological exam.

The finding of a considerable amount of dogs that were positive by PCR performed on spleen samples, but negative in bone marrow samples and vice versa is a strong indication that PCR can fail quite often in detecting infected individuals. The characteristics of a diagnostic test for CVL may vary depending on the clinical condition of the dog[36]. In fact, the conventional PCR showed a low sensitivity for the detection of *Leishmania* spp. in samples of asymptomatic dogs[34], and a strong association between the frequency of animals with symptoms and the frequency of positive animals by PCR has been shown[37]. Thus, although improving strategies for CVL diagnosis is important, samples should be carefully selected to avoid inappropriate interpretation of results.

In the present study, the detection thresholds of primers based on kDNA differed greatly from the results obtained by other authors. Nunes *et al.* (2007)[38] reported the ability to detect as few as 0.1 parasite in 500 μL of spiked blood. In the present study, we were able to detect as few as two parasites in 120 μL of bone marrow or spleen suspensions, corresponding to eight parasites in 500 μL. The differences in the two analytical sensitivities may be due to variances in the method of counting cells performed in both studies, as well as differences in the efficiency of DNA extraction, purification and nucleic acids concentration. Nevertheless, in both cases, it is evident that PCR does not seem to be a sensitive technique to detect infection in asymptomatic dogs. Whereas Nunes *et al.* (2007)[38] found 55 positive samples by PCR in a total of 80 blood samples from seropositive dogs (revealed by IFAT); we recorded

18 positive samples (in at least one of the tested samples) among the 73 seropositive dogs.

Lachaud *et al.* (2002)[33] found a remarkable diagnostic sensitivity of a kDNA-PCR that was able to detect 25 seropositive, asymptomatic dogs among 29 tested animals. However, it is noteworthy that serology in this case was performed using two methods, IFAT and counter immune electrophoresis, which has certainly conferred a high positive predictive value to the serologic investigation.

There is no consensus among authors, particularly in Brazil, on which primers are to be used for the diagnosis of CVL as there are a multitude of oligonucleotide sequences available for the molecular diagnosis of this disease. Procedures for nucleic acids extraction have different performances, as well as methods for the handling of samples used in molecular diagnosis. Different performances of molecular techniques in several laboratories, make it difficult to compare results.

Carry-over contamination by amplicons is an issue that is rarely discussed by authors worldwide[33] when they present results of surveys based on PCR and certainly has to be considered in the interpretation of techniques of exquisite sensitivity as is the case of amplification assays. The use of negative controls at various stages, disposable gloves frequently exchanged between the stages of the molecular procedures, automatic pipettes coupled to filter tips, and physical separation between working areas (DNA extraction, DNA amplification and manipulation of amplified products) are measures which are not always observed by technicians and, can seriously compromise the reliability of the results obtained by molecular techniques. In the present study, in addition to all the above measures, we used, in all stages of samples manipulation, at least one sample of ultrapure water for each three tested samples. The ultrapure water samples were used throughout the procedure.

Molecular diagnosis of CVL is considered successful if real time PCR is used[34,39]. In this case, the safety measures mentioned above should be even more rigorous, since the real-time PCR is usually performed in microtiter plates and many test samples are manipulated simultaneously, making the whole process more susceptible to cross contamination.

The results presented here allow us to conclude that the primers directed to *cytB, cytC,* and *coxII* can be used to identify isolates of parasites of the genus *Leishmania*, when used in combination with an automated sequencing technique. The conventional PCR is a technique that may have a low sensitivity for detection of infected dogs due to the possibility of generating a non-negligible number of false negative results when asymptomatic dogs infected by *Leishmania* are tested. Finally, primers based on the kDNA should be preferred for the screening of infected dogs.

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