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Use of PCR–RFLP to identify *Leishmania* species in naturally-infected dogs

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Abstract

Tissue imprints on Giemsa stained slides from dogs were used to investigate the presence of *Leishmania* amastigotes by either optical microscopy (OM) or Polymerase chain reaction (PCR) detection of DNA. Samples from skin, spleen, lymph node, liver and bone marrow from a Leishmaniasis endemic area dogs where *Leishmania (Leishmania) chagasi* and *Leishmania (Viannia) braziliensis* are sympatric were studied. Dogs were initially diagnosed by Indirect Immunofluorescence (IIF), as which 39 were IIF positive ($\geq 1:40$) and 16 negative. The IIF positive dogs were clinically grouped as symptomatic ($n = 15$), oligosymptomatic ($n = 12$) and asymptomatic ($n = 12$). Although PCR positivity was higher in symptomatic dogs, specially their skin samples, there was no significant difference among clinical groups or organs examined. Ten (62.5%) out of 16 IIF and OM negative animals were positive for PCR in at least one organ. Forty-eight positive PCR amplicons were further submitted to RFLP for *Leishmania* identification. All dogs were infected with *L. (L.) chagasi* except one, infected with *L. (V.) braziliensis*. PCR was more efficient than IIF and OM to diagnose canine visceral Leishmaniasis (CVL), regardless of the organ examined and the clinical form present. The use of PCR together with serology helps determining the extension of sub clinical infection in CVL endemic areas and provides a better estimate of the number of dogs to be targeted for control measures. In conclusion, our data reinforce the need for a specific diagnosis of canine infection in areas where diverse *Leishmania* species are sympatric and demonstrate that PCR–RFLP can be used to identify *Leishmania* species in dog tissue imprint stained slides.

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1. Introduction

Leishmaniasis are a group of zoonotic diseases transmitted to humans and animals through infected sand fly bites (Diptera: Psychodidae). Human Visceral

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Leishmaniasis (VL) and Canine Visceral Leishmaniasis (CVL) are mainly caused by *Leishmania* (*L.*) *chagasi* (= *L. infantum*) in South America. However, in a few cases, *Leishmania* (*Viannia*) *braziliensis* the causal agents of human American Tegumentary Leishmaniasis (ATL), has also described to cause the visceral form of the disease in humans. Domestic dogs (*Canis familiaris*) are the main VL peridomicile reservoirs (Reithinger and Davies, 1999). The World Health Organization recommends the treatment of human cases, insecticide vector control and *Leishmania*-seropositive dog sacrifice for the control of VL (Tesh, 1995).

In Brazil, the impact of the control campaign for VL has been both supported (Ashford et al., 1998; Jeronimo et al., 2000) and contested (Dietze et al., 1997; Furtado Vieira and Coelho, 1998), for being too difficult with unknown effectiveness, probably due to the low sensitivity of diagnostic methods (Palatnik-de-Sousa et al., 2001) and the delay in infected dog removal (Machado Braga et al., 1998). The control campaign official data (Furtado Vieira and Coelho, 1998) demonstrated however, that the increase in seropositive dog removal efficiency led to maintaining human annual cases of VL at basal levels (Palatnik-de-Sousa et al., 2001).

Due to a considerable increase in ATL transmission in the domestic environment and to studies reporting ATL in dogs (Reithinger and Davies, 1999; Madeira et al., 2003), canines are also believed to serve as ATL reservoirs. VL and ATL may become a public health problem in urban areas as they are opportunistic infections in HIV-infected people (Reithinger and Davies, 1999). In South America, HIV/ATL co-infections have already been described in Brazil (Nogueira-Castanon et al., 1996), Peru (Echevarria et al., 1993) and Venezuela (Hernandez et al., 1995).

In the last few years the standard reference diagnosis for VL has either been parasite visualization through optical microscopy (OM) or a culture of spleen, lymph node or bone marrow aspirates. Unfortunately, sensitivity in humans and dogs is variable and relatively low (Schnur and Jacobson, 1987; Osman et al., 1997; Reale et al., 1999). In the last years, polymerase chain reaction (PCR) has been proven to be sensitive and specific to detect *Leishmania* DNA (Pirmez et al., 1999; Passos et al.,

1999; Marques et al., 2001; Volpini et al., 2004). Canine tissues, such as spleen, lymph nodes, skin and even conjunctival biopsy are prime candidates for PCR diagnosis and blood and bone marrow are usually the most common canine tissues used for *Leishmania* PCR diagnosis (Ashford et al., 1995; Andrade et al., 2002; Lachaud et al., 2002; Manna et al., 2004). Skin however, is considered an important parasite reservoir tissue, regardless of the presence of lesions and/or other disease indications (Abranches et al., 1991; Solano-Gallego et al., 2001).

Recently, Volpini et al. (2004) have demonstrated that PCR and restriction fragment length polymorphism (RFLP) of *Leishmania* conserved region of minicircle kinetoplast DNA (mkDNA) is able to differentiate *L. (L.) amazonensis* from *L. (V.) braziliensis* in infected humans. The same technique may also differentiate *L. (L.) amazonensis* and *L. (V.) braziliensis* from *L. (L.) chagasi* (Volpini, 2003 unpublished data). In VL and ATL endemic areas where *L. (L.) chagasi* and *L. (V.) braziliensis* are sympatric, it is important to have diagnostic tests which not only confirm the presence of parasite in dogs but also identify and distinguish the *Leishmania* species. In this work, we have employed PCR–RFLP mkDNA for this purpose.

2. Material and methods

2.1. Animals and samples

Tissue samples from 55 mongrel dogs, with unknown age, were used in this study. Of these, 39 animals were identified as naturally infected with *Leishmania* during the seroepidemiological survey for canine visceral Leishmaniasis (CVL), carried out by “Departamento de Zoonoses da Prefeitura de Belo Horizonte”, in the city of Belo Horizonte, Minas Gerais state, Brazil. Indirect Immunofluorescence (IIF) was used as the diagnostic test. IIF-positive (cut off 1:40) dogs were clinically classified according to Mancianti et al. (1998) as: asymptomatic ($n = 12$), oligosymptomatic ($n = 12$) and symptomatic ($n = 15$). The reference group ($n = 16$) from the same endemic area, presented negative IIF, negative parasitological tests and no clinical manifestations. Biopsy tissue imprints on glass slides from skin, spleen, lymph node,

liver and bone marrow smears were obtained in triplicate from all dogs. The slides were prepared accordingly with Marques et al. (2001) and further stained with Giemsa for routine optical microscopy (OM) examination.

2.2. *Leishmania* DNA extraction and amastigote finding

The presence of *Leishmania* amastigotes was initially investigated with OM on three stained slides of each organ. *Leishmania* DNA detection was carried out by PCR. DNA was extracted from the slides by pouring Milli Q[®] water (Millipore, Billerica, MA, US) over an area with visible and well-stained imprints, scraping the material with a sterile toothpick and then transferring the suspension (50 µl) to a 0.5 ml Eppendorf tube. Samples were heated at 70 °C for 10 min, centrifuged at 10,000 × g for 5 min and the supernatant (DNA preparation) was maintained at –20 °C until use (Volpini et al., 2006).

Polymerase chain reaction (PCR) was performed out using the primers 150 forward: [5'-GGG(G/T)AGGGGCGTTCT(G/C)CGAA-3'] and 152reverse: [5'-(G/C)(G/C)(G/C)(A/T)CTAT(A/T)TTACACCA-ACCC-3'] that amplifies a DNA fragment of 120 base pairs (bp) from the conserved region of *Leishmania* minicircle kDNA (Degraeve et al., 1994). Reactions were carried out in a final volume of 10 µl containing 1.0 µl of DNA preparation, 0.2 mM dNTPs, 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 10 pmol of each primer and 1U Taq polymerase (Invitrogen). PCR amplifying conditions were: initial denaturation at 94 °C for 5 min, 30 cycles: denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Positive controls with genomic DNA of *L. (V.) braziliensis* (MHOM/BR/1975/M2903), *L. (L.) amazonensis* (IFLA/BR/1967/PH8) and *L. (L.) chagasi* (MCAN/BR/1986/CCC17580) were used. These *Leishmania* strains are deposited at CLIOC – Coleção de *Leishmania* do Instituto Oswaldo Cruz (WDCM 731) at Rio de Janeiro. A negative control without DNA were included in all tests. After amplification, samples were submitted to electrophoresis in 6% polyacrylamide gel and silver-stained for the PCR product identification.

2.3. PCR–RFLP mkDNA

PCR–RFLP mkDNA was carried out according to Volpini et al. (2004). Briefly, 5 µl of PCR products were digested by 1 U HaeIII (Invitrogen, Carlsbad, CA, USA) and ApaLI (Amersham Biosciences, Piscataway, NJ, USA) enzymes separately and incubated for 3 h at 37 °C in the manufacturer's buffer. Restriction fragments were separated in 10% polyacrylamide gel and silver stained. The fragments generated were compared with those from the DNA of *Leishmania* reference strains.

2.4. Statistical analysis

SPSS 11.0 program was used to apply χ^2 -test and Kappa index (κ). Significance level of 5% was adopted for χ^2 . For κ , values <0.4 were considered as low concordance, values ≥ 0.4 and ≤ 0.7 as good concordance and values >0.7 as strong concordance. Sensitivity (*S*) and specificity (*SP*) for PCR were calculated using IIF and OM as a gold standard.

3. Results

PCR and OM performances for CVL diagnosis were initially determined considering IIF as a gold standard and the five organs together (Table 1). PCR presented *S* = 92% and *SP* = 40%. OM exhibited *S* = 85% and *SP* = 100%. Interestingly, 10 (62.5%) out of the 16 negative IIF and OM animals, yielded PCR positive. Table 2 displays the performance of PCR for

Table 1

Performance of optical microscopy (OM) and polymerase chain reaction (PCR) considering indirect immunofluorescence (IIF) as gold standard to diagnose canine leishmaniasis

	IIF		<i>S</i> (%)	<i>SP</i> (%)
	Pos.	Neg.		
PCR				
Pos.	36	10	92	40
Neg.	3	6	92	40
OM				
Pos.	33	0	85	100
Neg.	6	16	85	100

S, sensitivity; *SP*, specificity; Pos., positive results; Neg., negative results.

Table 2

Performance of polymerase chain reaction (PCR) per organ considering indirect immunofluorescence (IIF) as gold standard to diagnose canine leishmaniasis

PCR/Organ	IIF		S (%)	SP (%)
	Pos.	Neg.		
Skin				
Pos.	34	7	87.2	56.2
Neg.	5	9		
Spleen				
Pos.	33	4	84.6	75.0
Neg.	6	12		
Liver				
Pos.	28	5	80.0	68.7
Neg.	7	11		
Lymph node				
Pos.	30	5	76.9	68.7
Neg.	9	11		
Bone marrow				
Pos.	26	5	66.7	68.7
Neg.	13	11		

S, sensitivity; SP, specificity; Pos., positive results, Neg., negative results. Concordance between PCR and IFA $\kappa < 0.4$ ($p = 0.001$).

each organ considering IIF as a gold standard to diagnose CVL. There is a low concordance between PCR and IIF ($\kappa < 0.4$). The highest PCR sensitivity was for skin samples (87.2%), followed by spleen (84.6%), liver (80.0%), lymph node (76.9%) and finally bone marrow (66.7%). The highest PCR specificity was for spleen (75.0%), followed by bone marrow/liver/lymph node (68.7%) and skin (56.2%).

When the PCR performance was compared to OM as a gold standard, the concordance was good for all organs ($0.4 \geq \kappa \leq 0.7$). S varied from 75.9 to 96.4% and SP from 50.0 to 70.4% according to the organ. PCR detected more *Leishmania* DNA than OM revealed amastigotes in all organs ($p < 0.005$) (Table 3).

Results from the PCR comparison to confirm presence of *Leishmania* in different organs and clinical groups of animals are presented in Table 4. PCR positivity varied from 31.2 to 43.7% in animals of the reference group; from 41.7 to 75.0% in the asymptomatic group; from 66.7 to 83.3% in the oligosymptomatic group and from 86.7 to 100% in the symptomatic group. The highest PCR positivity for each clinical group was observed in samples from skin and spleen. However the sample size was not large enough to qualify for statistical significance.

Table 3

Comparison of *Leishmania* detection with polymerase chain reaction (PCR) considering optical microscopy (OM) as gold standard

PCR/Organ	OM		S (%)	SP (%)	χ^2	κ
	Pos.	Neg.				
Spleen						
Pos.	27	10	96.4	63.0	$p < 0.001$	0.4
Neg.	1	17				
Lymph node						
Pos.	27	8	96.4	70.4	$p < 0.001$	0.7
Neg.	1	19				
Liver						
Pos.	21	12	95.4	57.1	$p < 0.001$	0.5
Neg.	1	16				
Skin						
Pos.	27	13	93.1	50.0	$p = 0.001$	0.6
Neg.	2	13				
Bone marrow						
Pos.	22	9	75.9	65.4	$p = 0.003$	0.4
Neg.	7	17				

PCR detected a greater number of positives than OM. χ^2 , chi-square test; κ , Kappa index; S, sensitivity; SP, specificity; Pos., positive results; Neg., negative results.

Amplicons from the 48 positive canine tissue samples and DNA from reference *Leishmania* strains were submitted to RFLP mkDNA with HaeIII and ApaLI endonucleases. HaeIII digested the 120 bp fragment product of PCR of *L. (V.) braziliensis* in two fragments, one of 80 bp and the other of 40 bp but did not digest the amplified product of *L. (L.) amazonensis*. Digestion of *L. (L.) chagasi* strain amplicons produced a distinct profile with 120, 80, 60 and 40 bp bands using the same enzyme. On the other hand, ApaLI only digested the PCR product of *L. (V.) braziliensis* in two fragments, one of 88 bp and the other of 32 bp, with *L. (L.) amazonensis* and *L. (L.) chagasi* strains remaining uncut. Comparing DNA fragments generated by digestion of amplicons from dog tissue samples with those of the reference strains of the *Leishmania* species, we observed that all samples were identified as *L. (L.) chagasi* except one (dog 28), which was classified as *L. (V.) braziliensis* (Fig. 1).

4. Discussion

Domestic dogs have not only been reported as the main reservoir from *L. (L.) chagasi* but also host for *L.*

Table 4

Positivity of polymerase chain reaction (PCR) to detect *Leishmania* in different organs and clinical groups of animals

Organ	Clinical group			
	Reference (n = 16) Pos. (%)	Asymptomatic (n = 12) Pos. (%)	Oligosymptomatic (n = 12) Pos. (%)	Symptomatic (n = 15) Pos. (%)
Skin	7 (43.7)	9 (75.0)	10 (83.3)	15 (100)
Spleen	7 (43.7)	9 (75.0)	9 (75.0)	15 (100)
Lymph node	5 (31.2)	7 (58.3)	8 (66.7)	15 (100)
Liver	5 (31.2)	6 (50.0)	9 (75.0)	15 (100)
Bone marrow	5 (31.2)	5 (41.7)	8 (66.7)	13 (86.7)

The sample were not large enough to have statistical significance.

(V) *braziliensis* (Reithinger and Davies, 1999; Madeira et al., 2003). However the role of the dog in ATL transmission is not completely understood. Thus identifying *Leishmania* species causing canine Leishmaniasis has become essential to Leishmaniasis diagnosis, epidemiological understanding and guide the control measures to be taken. Herein we have demonstrated that PCR–RFLP mkDNA may be utilized for this purpose.

In our study, PCR and OM of skin, spleen, lymph node, liver and bone marrow tissue samples on Giemsa stained slides have been used to detect the presence of *Leishmania* in dogs from Belo Horizonte, MG, Brazil, an area of species sympatry, and PCR–RFLP mkDNA was employed with the aim toward species identification. For PCR amplification we used a pair of primers which amplifies a 120 bp DNA fragment from the

Leishmania mkDNA. These pair of primers presented the best positivity out of the five tested to detect *Leishmania* DNA by Lachaud et al. (2002). In addition, the amplified fragment enabled a further enzymatic digestion (RFLP) for identification of the specific *Leishmania* species present in this Brazilian area (Volpini et al., 2004). DNA was extracted efficiently and economically using tissue imprint on Giemsa stained slides (Volpini et al., 2006).

Sensitivity, specificity, simplicity and cost make serological tests standard tools for *Leishmania* identification in endemic areas (Reithinger and Davies, 1999). The application of PCR together with serology not only helps in determining the extension of subclinical infections in CVL endemic areas but also allows estimation of the number of dogs to be targeted for control measures, as PCR was able to

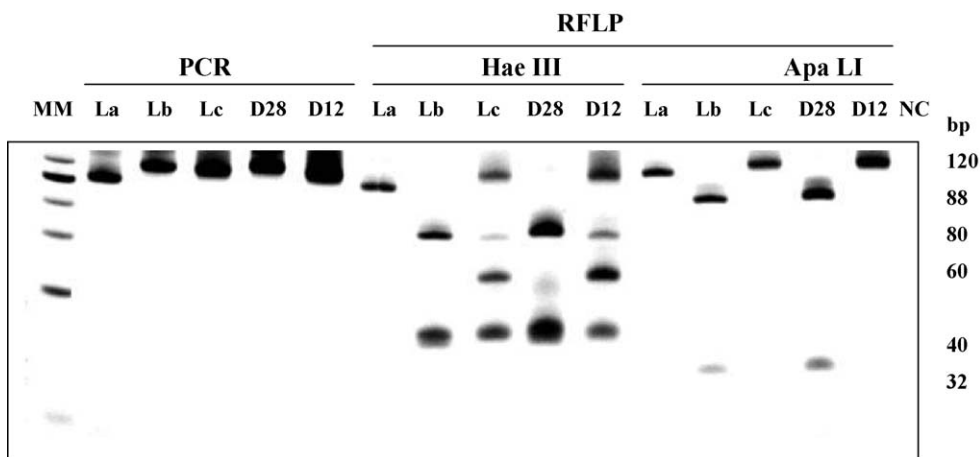


Fig. 1. PCR amplified products from the conserved region of *Leishmania* minicircle kDNA before and after Hae II and Apa LI digestion. MM, 25 bp molecular weight ladder (Invitrogen); reference strain of *Leishmania*: La, *L. (L.) amazonensis* (IFLA/BR/1967/PH8); Lb, *L. (L.) braziliensis* (MHOM/BR/1975/M2903); Lc, *L. (L.) chagasi* (MCAN/BR/1986/CCC17580); D-28, dog no. 28; D-12, dog no. 12.

detect sub clinical canine infection by *L. infantum* (Solano-Gallego et al., 2001). Here, PCR detected 62.5% of infected animals in the reference group, which was initially classified as non-infected, by routine CVL diagnostic tests (IIF and OM). The absence of bands in PCR negative controls (without DNA) allows us to assure that PCR products observed were not related to contamination but to the presence of *Leishmania* DNA in the biological material tested. This result reinforces the hypothesis that the diagnostic methods used in endemic areas underestimate the number of infected animals. Thus, a considerable number of positive animals may remain as reservoirs interfering in the evaluation of dog elimination impact on VL control.

Considering IIF as a gold standard and regardless of the organ, PCR presented 92.3% sensitivity and 37.5% specificity. We believe that this low level of PCR specificity is attributed to two factors: (1) a serological and non a parasitological test was used as a gold standard and (2) 10 out of 16 negative animals for IIF were PCR positive. The first hypothesis was strengthened when the parasitological test, optical microscopy (OM) was used as a gold standard. The PCR sensitivity remained at nearly at the same level (94%) and the specificity increased considerably (78%). While analyzing organs and maintaining IIF as a gold standard, PCR sensitivity values varied from 66.7 to 87.2%, and they were in accordance with most of the previous reports on PCR sensitivity of 60% (Mathis and Deplazes, 1995), 71.4% (Andrade et al., 2002), 87% (Lopez et al., 1993) and even 100% (Ashford et al., 1995). PCR detected more *Leishmania* DNA than OM the amastigotes in all organs ($p < 0.001$). PCR has already been proven to be more sensitive than OM (Osman et al., 1997; Reale et al., 1999). However, not many comparative analysis of the sensitivity of PCR and OM in detect *Leishmania* DNA or amastigotes in different organs has been reported.

There have been few studies comparing CVL diagnosis in different organs. Through PCR, Solano-Gallego et al. (2001) achieved 51.0% positivity in canine skin, 32.0% in conjunctiva and 17.8% in bone marrow. Barrouin-Melo et al. (2004) reported that spleen tissue is better than lymph node for parasite isolation in culture. Manna et al. (2004) observed that skin, regardless of the presence of cutaneous lesions

was better than blood and lymph node to detect *Leishmania* DNA by PCR. We searched for *Leishmania* DNA and amastigotes in five organs previously described in the literature as a source of parasites.

The animals in our study were recruited from an area where *L. (L.) chagasi* and *L. (V.) braziliensis* are sympatric (Passos et al., 1996; Silva et al., 2001). Autochthonous ATL has been reported in the rural area of Minas Gerais state since 1950 (Mayrink et al., 1979). In the last 20 years, the incidence of new ATL cases in peri-urban and urban areas has been increasing (Passos et al., 1999; Marques et al., 2001). In Brazil, the presence of *L. (V.) braziliensis* or *Leishmania* from the *Viannia* subgenus in dogs has already been described in the States of Bahia (Cuba Cuba et al., 1985), Ceará (Vasconcelos et al., 1988), Rio de Janeiro (Pirmez et al., 1999), São Paulo (Yoshida et al., 1990), Espírito Santo (Falqueto et al., 1991) and Minas Gerais (Passos et al., 1996).

Leishmania species identification is still mainly performed with isoenzyme electrophoresis and/or with monoclonal antibodies that require parasite isolation and cultivation. PCR-RFLP mkDNA (Volpini et al., 2004), such as other molecular methods, was developed as a method to be used in biopsies of ATL patients for identification of the most common *Leishmania* species in Brazil. We carried out PCR-RFLP mkDNA in *Leishmania* amplicons from 48 dogs using HaeIII and ApaLI endonucleases. Comparing DNA fragments generated in amplicons from dog samples with those from *Leishmania* reference strains, 47 (97.6%) samples were identified as *L. (L.) chagasi* and 1 (2.1%) as *L. (V.) braziliensis*. Interestingly the *L. (V.) braziliensis* infected dog was IIF and OM negative in all tissues whereas PCR was positive in all tissues except lymph node. The identification of parasites from both dogs and humans does not determine whether dogs are accidental or reservoir hosts, but merely shows that they are susceptible to infection (Reithinger and Davies, 1999).

In this study, PCR proved to be superior to IIF and OM for CVL diagnosis, regardless of the canine organ or the clinical manifestation in the dog. The need for a specific CVL diagnosis is reinforced in areas where several of *Leishmania* species are sympatric, and PCR-RFLP mkDNA may be applied for this purpose using Giemsa stained slides of diverse canine tissue samples.

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References

- Abranches, P., Silva-Ferreira, M.C.D., Conceição-Silva, F., Santos-Gomes, G.M., Janz, J.G., 1991. Canine Leishmaniasis: pathological and ecological factors influencing transmission of infection. *J. Parasitol.* 77, 557–561.
- Andrade, H.M., Toledo, V.P.C., Marques, M.J., Franca Silva, J.C., Tafuri, Wg.L., Mayrink, W., Genaro, O., 2002. *Leishmania (Leishmania) chagasi* is not vertically transmitted in dogs. *Vet. Parasitol.* 103, 71–81.
- Ashford, D.A., David Jr., Freire, M., David, R., Sherlock, I., Eulálio, Mc., Pedral Sampaio, D., Badaró, R., 1998. Studies on control of visceral Leishmaniasis: impact of dog control on canine and human visceral Leishmaniasis in Jacobina, Bahia, Brazil. *Am. J. Trop. Med. Hyg.* 59, 53–57.
- Ashford, D.A., Bozza, M., Freire, M., Miranda, J.C., Sherlock, I., Eulálio, C., Lopes, U., Fernandes, O., Degrave, W., Barker Jr., R.H., Badaró, R., David, J.R., 1995. Comparison of the polymerase chain reaction and serology for the detection of canine visceral Leishmaniasis. *Am. J. Trop. Med. Hyg.* 53, 251–255.
- Barrouin-Melo, S.M., Larangeira, D.F., Trigo, J., Aguiar, P.H., dos-Santos, W.L., Pontes-de-Carvalho, L., 2004. Comparison between splenic and lymph node aspirations as sampling methods for the parasitological detection of *Leishmania chagasi* infection in dogs. *Mem. Inst. Oswaldo Cruz.* 99, 195–197.
- Cuba Cuba, C.A., Miles, M.A., Vexenat, A., Barker, D.C., McMahon Pratt, D., Butcher, J., Barreto, A.C., Marsden, P.D., 1985. A focus of mucocutaneous Leishmaniasis in Tres Bracos, Bahia, Brazil: characterization and identification of *Leishmania* stocks isolated from man and dogs. *Trans. R. Soc. Trop. Med. Hyg.* 79, 500–507.
- Degrave, W., Fernandes, O., Campbell, D., Bozza, M., Lopes, U., 1994. Utilization of molecular probes and PCR for detection and typing of *Leishmania*—a mini-review. *Mem. Inst. Oswaldo Cruz.* 89, 463–469.
- Dietze, R., Baptista Barros, G., Teixeira, L., Harris, J., Michelson, K., Falqueto, A., Corey, R., 1997. Effect of eliminating seropositive canines on the transmission of visceral Leishmaniasis in Brazil. *Clin. Inf. Dis.* 25, 1240–1242.
- Echevarria, J., Campos, P., Chang, J., Cuellar, L., Gotuzzo, E., Paz, L., Llanos-Cuentas, A., 1993. Mucocutaneous Leishmaniasis and AIDS: case report. *Trans. R. Soc. Trop. Med. Hyg.* 87, 186.
- Falqueto, A., Sessa, P.A., Varejão, J.B., Barros, G.C., Momen, H., Grimaldi Junior, G., 1991. Leishmaniasis due to *Leishmania braziliensis* in Espírito Santo State, Brazil. Further evidence on the role of dogs as a reservoir of infection for humans. *Mem. Inst. Oswaldo Cruz.* 86, 499–500.
- Furtado Vieira, J.B., Coelho, E.G., 1998. Leishmaniose visceral ou calazar: aspectos epidemiológicos e de controle. *Rev. Soc. Bras. Med. Trop.* 31, 85–92.
- Hernandez, D.E., Rodriguez, N., Wessolossky, M., Convit, J., 1995. Visceral Leishmaniasis due to a *Leishmania* variant that shares kinetoplast DNA sequences with *Leishmania braziliensis* and *Leishmania mexicana* in a patient infected with human immunodeficiency virus: identification of the *Leishmania* species with use of the polymerase chain reaction. *Clin. Infect. Dis.* 21, 701–702.
- Jeronimo, S.M., Teixeira, M.J., Sousa, A.D., Thielking, P., Pearson, R.D., Evans, T.G., 2000. Natural history of *Leishmania (Leishmania) chagasi* infection in Northeastern Brazil: long-term follow-up. *Clin. Inf. Dis.* 30, 608–609.
- Lachaud, L., Marchegui-Hammami, S., Chabbert, E., Dereure, J., Dedet, J.P., Bastien, P., 2002. Comparison of six PCR methods using peripheral blood for detection of canine visceral Leishmaniasis. *J. Clin. Microbiol.* 40, 210–215.
- Lopez, M., Inga, R., Cangalaya, M., Echevarria, J., Llanos-Cuentas, A., Orrego, C., Arevalo, J., 1993. Diagnosis of *Leishmania* using the polymerase chain reaction: a simplified procedure for field work. *Am. J. Trop. Med. Hyg.* 49, 348–356.
- Machado Braga, M.D., Coelho, I.C.B., Lima Pompeu, M., Evans, T.G., Tavares, M.I., Teixeira, M.J., Oliveira Lima, J., 1998. Controle do calazar canino: comparação dos resultados de um programa de eliminação rápida de cães sororreagentes por ensaio imuno-enzimático com outro de eliminação tardia de cães sororreagentes por teste de imunofluorescência indireta de eluato de papel filtro. *Rev. Soc. Bras. Med. Trop.* 31, 419–424.
- Madeira, M.F., Uchuôa, C.M.A., Leal, C.A., Silva, R.M.M., Duarte, R., Magalhães, C.M., Serra, C.M.B., 2003. *Leishmania (Vianina) braziliensis* em cães naturalmente infectados. *Rev. Soc. Bras. Med. Trop.* 36, 351–355.
- Mancianti, F., Gramiccia, M., Gradoni, L., Pieri, S., 1998. Studies on canine Leishmaniasis control. I. Evolution of infection of different clinical forms of canine Leishmaniasis following antimonial treatment. *Trans. R. Soc. Trop. Med. Hyg.* 82, 566–567.
- Manna, L., Vitale, F., Reale, S., Caracappa, S., Pavone, L.M., Morte, R.D., Cringoli, G., Staiano, N., Gravino, A.E., 2004. Comparison of different tissue sampling for PCR-based diagnosis and follow-up of canine visceral leishmaniosis. *Vet. Parasitol.* 125, 251–262.
- Marques, M.J., Volpini, A.C., Genaro, O., Mayrink, W., Romanha, A.J., 2001. Simple form of clinical sample preservation and *Leishmania* DNA extraction from human lesions for diagnosis of American cutaneous Leishmaniasis via polymerase chain reaction. *Am. J. Trop. Med. Hyg.* 65, 902–906.
- Mathis, A., Deplazes, P., 1995. PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. *J. Clin. Microbiol.* 33, 1145–1149.
- Mayrink, W., Williams, P., Coelho, M.V., Dias, M., Martins, A.V., Magalhaes, P.A., Da Costa, C.A., Falcao, A.R., Melo, M.N., Falcao, A.L., 1979. Epidemiology of dermal Leishmaniasis in the Rio Doce Valley, State of Minas Gerais, Brazil. *Ann. Trop. Med. Parasitol.* 73, 123–137.
- Nogueira-Castanon, M.C., Pereira, C.A., Furtado, T., 1996. Unusual association of American cutaneous Leishmaniasis and acquired immunodeficiency syndrome. *Int. J. Dermatol.* 35, 295–297.
- Osman, O.F., Oskam, L., Zijlstra, E.E., Kroon, N.C., Schoone, G.J., Khalil, E.T., El-Hassan, A.M., Kager, P.A., 1997. Evaluation of PCR for diagnosis of visceral Leishmaniasis. *J. Clin. Microbiol.* 5, 2454–2457.

- Palatnik-de-Sousa, C.B., Santos, W.R., França-Silva, J.C., Costa, R.T., Barbosa Reis, A., Palatnik, M., Mayrink, W., Genaro, O., 2001. Impact of canine control on the epidemiology of canine and human visceral Leishmaniasis in Brazil. *Am. J. Trop. Med. Hyg.* 65, 510–517.
- Passos, V.M., Andrade, A.C., Silva, E.S., Figueiredo, E.M., Falcao, A.L., 1996. A canine survey in a recent focus of cutaneous Leishmaniasis in the city of Sabará, the metropolitan area of Belo Horizonte. *Rev. Soc. Bras. Med. Trop.* 29, 323–329.
- Passos, V.M., Fernandes, O., Lacerda, P.A., Volpini, A.C., Gontijo, C.M., Degraive, W., Romanha, A.J., 1999. *Leishmania (Viannia) braziliensis* is the predominant species infecting patients with American cutaneous Leishmaniasis in the State of Minas Gerais, Southeast Brazil. *Acta Trop.* 72, 251–258.
- Pirmez, C., da Silva Trajano, V., Paes-Oliveira Neto, M., da-Cruz, A.M., Goncalves-da-Costa, S.C., Catanho, M., Degraive, W., Fernandes, O., 1999. Use of PCR in diagnosis of human American tegumentary Leishmaniasis in Rio de Janeiro, Brazil. *J. Clin. Microbiol.* 37, 1819–1823.
- Reale, S., Maxia, L., Vitale, F., Glorioso, N.S., Caracappa, S., Vesco, G., 1999. Detection of *Leishmania infantum* in dogs by PCR with lymph node aspirates and blood. *J. Clin. Microbiol.* 37, 2931–2935.
- Reithinger, R., Davies, C.R., 1999. Is the domestic dog (*Canis familiaris*) a reservoir host of American cutaneous Leishmaniasis? A critical review of the current evidence. *Am. J. Trop. Med. Hyg.* 61, 530–541.
- Schnur, L., Jacobson, R.L., 1987. Parasitological techniques. In: Peters, W., Killick-Kendrick, R. (Eds.), *The Leishmaniasis in Biology and Medicine/Clinical Aspects and Control*, vol. 1. Academic Press, New York, pp. 499–542.
- Silva, E.S., Gontijo, C.M., Pacheco, R.S., Fiúza, V.O., Brazil, R.P., 2001. Visceral Leishmaniasis in the metropolitan region of Belo Horizonte, state of Minas Gerais, Brazil. *Mem. Inst. Oswaldo Cruz.* 96, 285–291.
- Solano-Gallego, L., Morell, P., Arboix, M., Alberola, J., Ferrer, L., 2001. Prevalence of *Leishmania infantum* infection in dogs living in an area of canine Leishmaniasis endemicity using PCR on several tissues and serology. *J. Clin. Microbiol.* 39, 560–563.
- Tesh, R., 1995. Control of zoonotic visceral Leishmaniasis. Is it time to change strategies? *Am. J. Trop. Med. Hyg.* 52, 287–292.
- Vasconcelos, I.A., Vasconcelos, A.W., Momen, H., Grimaldi Jr., G., Alencar, J.E., 1988. Epidemiological studies on American Leishmaniasis in Ceara State, Brazil. Molecular characterization of the *Leishmania* isolates. *Ann. Trop. Med. Parasitol.* 82, 547–554.
- Volpini, A.C., Passos, V.M., Oliveira, G.C., Romanha, A.J., 2004. PCR–RFLP to identify *Leishmania (Viannia) braziliensis* and *L. (Leishmania) amazonensis* causing American cutaneous Leishmaniasis. *Acta Trop.* 90, 31–37.
- Volpini, A.C., Marques, M.J., Santos, S.L., Machado-Coelho, G., Mayrink, W., Romanha, A.J., 2006. *Leishmania* identification by PCR on Giemsa stained lesion imprint slides stored for up to 36 years. *Clin. Microbiol. Infect.* 12, in press.
- Yoshida, E.L., Correa, F.M., Marques, S.A., Stolf, H.O., Dillon, N.L., Momen, H., Grimaldi Jr., G., 1990. Human, canine and equine (*Equus caballus*) Leishmaniasis due to *Leishmania braziliensis* (= *L. braziliensis*) in the south-west region of Sao Paulo State, Brazil. *Mem. Inst. Oswaldo Cruz.* 85, 133–134.