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PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab samples

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ABSTRACT

The efficacy of conjunctival swab (CS) as a sampling method for visceral leishmaniasis (VL) diagnosis by PCR of asymptomatic dogs was evaluated. The CS was compared to blood samples (B) and skin biopsies (SB), two less invasive samples potentially useful for massive screening of dogs. Thirty asymptomatic dogs, with serological and parasitological positive tests, were used. The samples were analyzed by two PCR methods: kDNA PCR-hybridization and ITS-1 nPCR. The DNA sample volume used was of 1.0 μL and 10.0 μL respectively. Using CS samples the kDNA PCR-hybridization was able to detected parasite DNA in 24/30 dogs (80%) using the right conjunctiva (RC) and 23/30 dogs (76.6%) with the left conjunctiva (LC), 17/30 dogs (56.7%) by means of SB and 4/30 dogs (13.3%) with B. The CS positivity obtained combining RC and LC results was of 90% (27/30 dogs). The assay of CS samples by ITS-1 nPCR revealed that 25/30 dogs (83.3%) were positive when using RC and 20/30 dogs (66.6%) were positive when using LC. Via the same method 15/30 dogs (50.0%) were positive by SB and 17/30 dogs (56.7%) with B. The CS positivity obtained by ITS-1 nPCR combining RC and LC was of 83.3%. The CS positivities for RC and LC were significantly higher ($p < 0.05$) than SB and B for kDNA PCR-hybridization method. Statistical difference in relation to SB and B was verified by ITS-1 nPCR only for RC. The kDNA PCR-hybridization and ITS-1 nPCR methods showed similar sensitivities for CS and SB samples. On the other hand, for blood samples, the positivity of ITS-1 nPCR was significantly higher than the one obtained by the kDNA PCR-hybridization, indicating that sensitivity of PCR methods can vary according to the biological sample examined. Our results showed that CS was suitable to detect *Leishmania* DNA in asymptomatic animals when comparing to other low-invasive samples. The CS sensitivities obtained in this study were similar to the ones observed in other studies for VL diagnosis in symptomatic dogs. We concluded that the use of CS for regular screenings of dogs by PCR should be considered.

1. Introduction

Leishmania (*leishmania*) *chagasi* (syn. *Leishmania* (*Leishmania*) *infantum*) is the etiologic agent of visceral leishmaniasis (VL) in Brazil and dogs are the major domestic reservoir of the parasite (Laison and Shaw, 1987). The VL epidemiological control in Brazil involves the elimination of infected dogs, insecticide treatment and systematic

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treatment of human cases (Tesh, 1995). Reliable diagnostic tests for detection of *Leishmania* infection are essential to avoid the disease transmission or the unnecessary culling of dogs. The Brazilian VL control program emphasizes serologic surveys, mainly using the enzyme-linked immunosorbent assay (ELISA) and the immunofluorescence antibody test (IFAT), followed by the elimination of the seropositive dogs. However, these techniques present limitations in terms of reproducibility and specificity (Silva et al., 2006). Sometimes they fail due to the low level of specific antibodies in some dogs and cross-reactivities with Chagas's disease, rickettsiosis, ehrlichiosis and toxoplasmosis (Barbosa de Deus et al., 2002). In addition, symptomatic dogs usually produce high levels of specific antibodies which can be easily detected (Deplases et al., 1995), but the sensitivity of antibody detection is generally lower in early or in asymptomatic canine infections (Leontides et al., 2002). Asymptomatic animals may represent a high percentage (up to 85%) of infected dogs in areas of endemicity (Dantas-Torres et al., 2006) and they serve as reservoir for vector transmission to susceptible animals and humans (Michalsky et al., 2007).

PCR assays have greatly improved the sensitivity of VL diagnosis in dogs (Maia and Campino, 2008). Various canine tissues (including blood, skin biopsies, lymph node, bone marrow and spleen) have been used for PCR detection of the parasite. However, the non-invasive samples assume great importance in this context because they are simpler, painless and more easily allowed by the dog-owners. Non-invasive samplings would represent an essential tool in mass-screening survey for interventional programs. An interesting approach in this context is the conjunctival swab (CS), a method for sample collection that uses a sterile swab for sampling the dog conjunctivas. This method was shown to be highly sensitive when used for diagnosis of symptomatic dogs (Strauss-Ayali et al., 2004; Ferreira et al., 2008; Pilatti et al., 2009). The aim of the present study was to evaluate the canine VL diagnosis by PCR using conjunctival swab from asymptomatic dogs.

2. Material and methods

2.1. Dogs

Thirty mongrel dogs, designated to compulsory euthanasia, were donated by the Municipal Zoonotic Diseases Control Department of Belo Horizonte, Minas Gerais State, Brazil. The dogs were simultaneously positive for ELISA and IFAT. All dogs were asymptomatic and positive in the parasitological diagnosis. Six healthy serum negative dogs (from a non-endemic area) were used as negative controls. This study was approved by the Animal Experimentation Ethics Committee of the Federal University of Minas Gerais (CETEA/UFMG) protocol number183/2008.

2.2. Samples

Exfoliative epithelial cells were collected from the right (RC) and left (LC) conjunctiva of each animal using sterile cotton swabs manufactured for bacteriological isolation. Each sample was analyzed separately to provide a replicate diagnosis for each animal. The cotton tips were broken and only the cotton parts were transferred to sterile tubes and stored at −20 °C until use. Peripheral blood (2.7 mL) (B) was collected from each dog in tubes containing EDTA, kept in ice and processed as soon as possible. Skin biopsies (SB) were collected from the internal side of the ear using a 5 mm diameter punch. The biopsies, weighing approximately 15.0 mg were stored at −20 °C until processed. Bone marrow aspirates were obtained in a sterile manner using a thin biopsy needle, kept at 4 °C and used in the following 24 h for parasitological diagnosis.

2.3. DNA extraction

The DNA purification from CS was carried out as described by Strauss-Ayali (2004), with minor modifications. Each cotton received 300 μL of lysis buffer (50 mMol/L Tris, 50 mMol/L NaCl, and 10 mMol/L EDTA, pH 8.0) containing proteinase K (250 (g/mL) and Triton X-100 (1%). After the incubation (2 h at 56 °C) the solution was eluted from the cotton, transferred into phase-lock gel tube PLG-H (Eppendorf) and was mixed with 500 μL of 75% Tris-saturated phenol (Sigma), 25% chloroform-isoamyl alcohol. The organic phase was separated from the aqueous phase by centrifugation at 12,000 × *g* for 5 min which was transferred to a new phase-lock gel tube PLG-H. The extraction was repeated with 500 μL of 50% phenol, 50% chloroform-isoamyl alcohol and once with 100% chloroform-isoamyl alcohol. The DNA precipitation was done with one volume of isopropanol-sodium acetate, followed by wash with 75% ethanol. The DNA pellet was suspended in 30 μL of Tris-EDTA buffer (10 mMol/L Tris and 1mMol/L EDTA, pH 8.0) and was kept at −20 °C until being used.

The DNA extraction from 1.0 mL of whole blood (B) and skin biopsies (SB) were performed using the commercial kits illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare) and Wizard® SV Genomic DNA Purification System (Promega), respectively, according to the manufacturer's protocol. The purified DNA, 80 μL and 160 μL respectively, was stored at −20 °C.

2.4. PCR

The samples were analyzed by two PCR protocols: kDNA PCR-hybridization and internal transcribed spacer 1 nested PCR (ITS-1 nPCR). Positive control with *L.* (*L.*) *chagasi* (strain MHOM/1973/BH46) genomic DNA was used at 1.0 ng/μL. A negative control without DNA was included in all tests. The DNA sample volume used for kDNA PCR-hybridization was of 1.0 μL and for the ITS-1 nPCR was of 10.0 μL.

2.4.1. kDNA PCR-hybridization

The PCR reaction mixture contained 0.2 mM of each dNTP, 0.2 nMol of each primer [5′-(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACAACCCC-3′ and 5′-GGGGAGGGGCGTTCTGCGAA-3′], 2.5 U of AmpliTaq Gold® (Applied Biosystems), 2.5 μL of 10X buffer (Tris–HCl 50 mM, [pH 8.3], KCl 50 mM), 2.0 mM $MgCl_2$ in a final volume of 50 μL. The amplifying conditions were: initial denaturation at 95 °C for 15 min, 30 cycles including 94 °C at 30 s, 50 °C at 30 s, 72 °C at 30 s, and a final extension at

72 °C for 10 min. All amplification products were analyzed on 2% agarose gel and were stained with ethidium bromide. The target for amplification was a 120 base pairs (bp) sequence of *Leishmania* kDNA minicircle conserved region (Degrave et al., 1994).

For the hybridization step 10.0 μL of each amplification product was mixed to 110.0 μL of NaOH 0.4 M, EDTA 25 mM [pH 8.0] solution and spotted on nylon membrane (Hybond–XL Amersham) using a Bio Dot apparatus (Hybridot manifold-BRL®). After that, the membrane was rinsed with 2× SSC (0.3 M NaCl, 0.3 mM sodium citrate) and dried. The DNA was fixed to the filter by UV light (0.12 J/cm^2). Cloned kDNA minicircles from *L.* (*L.*) *chagasi* were used as probes. The probe was labeled with ^{32}P[α]dCTP using the Random Primer DNA Labeling System® (Invitrogen). Hybridization conditions were as previously described (Andrade et al., 2001). Briefly, the filters were pre-soaked at 58 °C for 30 min in 0.5% non-fat milk, 1% sodium dodecyl sulphate (SDS) and 2× SSC solution. The kDNA probe was added to the solution after being heated for 3 min in a boiling water bath. The filters were incubated for 14 h at 58 °C, with shaking, then placed in 2× SSC at room temperature for 20 min and washed in 0.5× SSC, 0.5× SDS, at 65 °C for 30 min. Finally, it was dried and exposed in the cassette (BAS 2325 Fujifilm) for 2 h. The image was obtained using a Bio-Imaging Analyzer® (Fujifilm).

2.4.2. ITS-1 nPCR

This method was adapted from Schõnian et al. (2003). Primers addressed to internal transcribed spacer 1 (ITS-1) between the genes coding for SSU rRNA and 5.8S rRNA were used. For the first amplification 10.0 μL of DNA solution was added to 40.0 μL of PCR mix containing 15 pMol of the primers 5′-CTGGATCATTTTCCGATG-3′ and 5′-TGATACCACTTATCGCACTT-3′ and 0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, 5 mM KCl, 75 mM Tris–HCl pH 9.0, 2.0 mM $(NH_4)SO_4$, and 1.4 U of Taq DNA polymerase (Ludwig Biotec). The cycling conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. Amplification products were visualized on 2% agarose gel stained with ethidium bromide. The PCR product size stays between 300 and 350 bp. For the second amplification 10.0 μL of a 1:40 dilution of the first PCR product was added to 15 μL of PCR mix under the same conditions as the first amplification but with the following primers (15 pMol each): 5′-CATTTTCCGATGATTACACC-3′ and 5′-CGTTCTTCAACGAAATAGG-3′. Positive samples yielded a PCR product between 280 and 330 bp.

For the restriction fragment length polymorphism (RFLP) analysis the non-purified PCR products (17 μL) were digested with 1 U of Hae III enzyme, for 4 h, at 37 °C. The restriction fragments were visualized on 2% agarose gel stained with ethidium bromide.

2.5. Statistical analysis

The results were analyzed using the Pearson chi square test. The difference between the results was considered significant when $p < 0.05$.

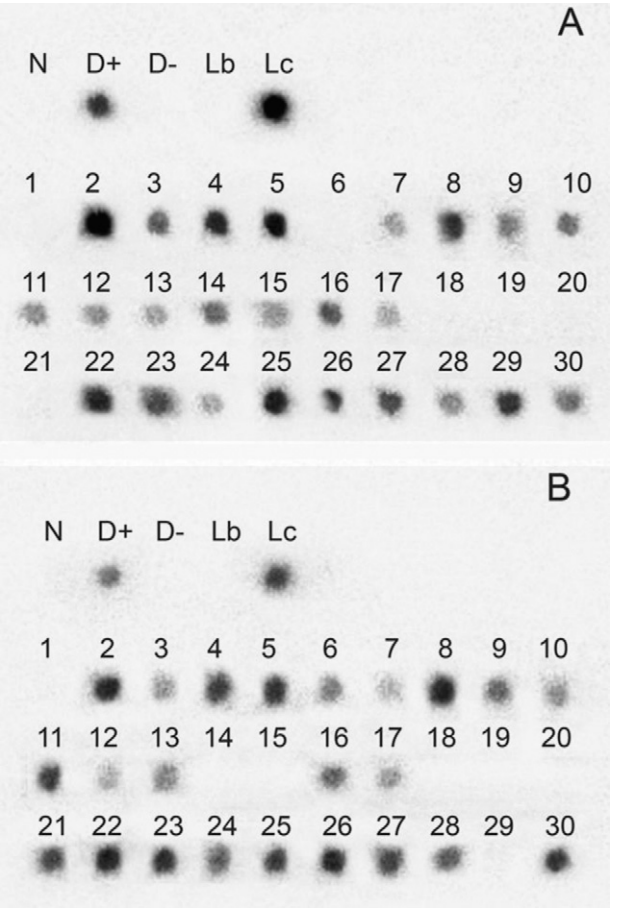

Fig. 1. CS samples Autoradiogram. The PCR products were hybridized with *L.* (*L.*) *chagasi* cloned kDNA minicircle probes labeled with ^{32}P for the right conjunctiva (A) and the left conjunctiva samples (B). Negative control (N); positive control dog (D+); negative control dog (D−); *L.* (*V*). *braziliensis* control (Lb) (strain MHOM/BR/1975/M2903); *L.* (*L*) *chagasi* control (Lc) (strain MHOM/1973/BH46); dogs samples (1–30).

3. Results

The non-invasive conjunctival swab (CS) was compared with blood (B) and skin biopsies (SB), two other less invasive samples potentially useful for massive screening of dogs by PCR. The samples were analyzed by two PCR methods: kDNA PCR-hybridization and ITS-1 nPCR.

Using CS samples the kDNA PCR- hybridization was able to detected parasite DNA in 24/30 dogs (80%) using the right conjunctiva (RC) and 23/30 dogs (76.6%) with the left conjunctiva (LC), 17/30 dogs (56.7%) by means of SB and 4/30 dogs (13.3%) with B. The CS positivities for RC and LC were significantly higher ($p < 0.05$) than the positivities obtained for SB and B. The SB showed a significantly higher positivity ($p < 0.05$) than found by the B samples. The CS positivity obtained combining the results of RC and LC was of 90% (27/30 dogs). The CS samples autoradiogram was showed in Fig. 1.

The assay of CS samples by ITS-1 nPCR revealed that 25/30 dogs (83.3%) were positive when using RC and 20/30 dogs (66.6%) were positive when using LC. Via the same

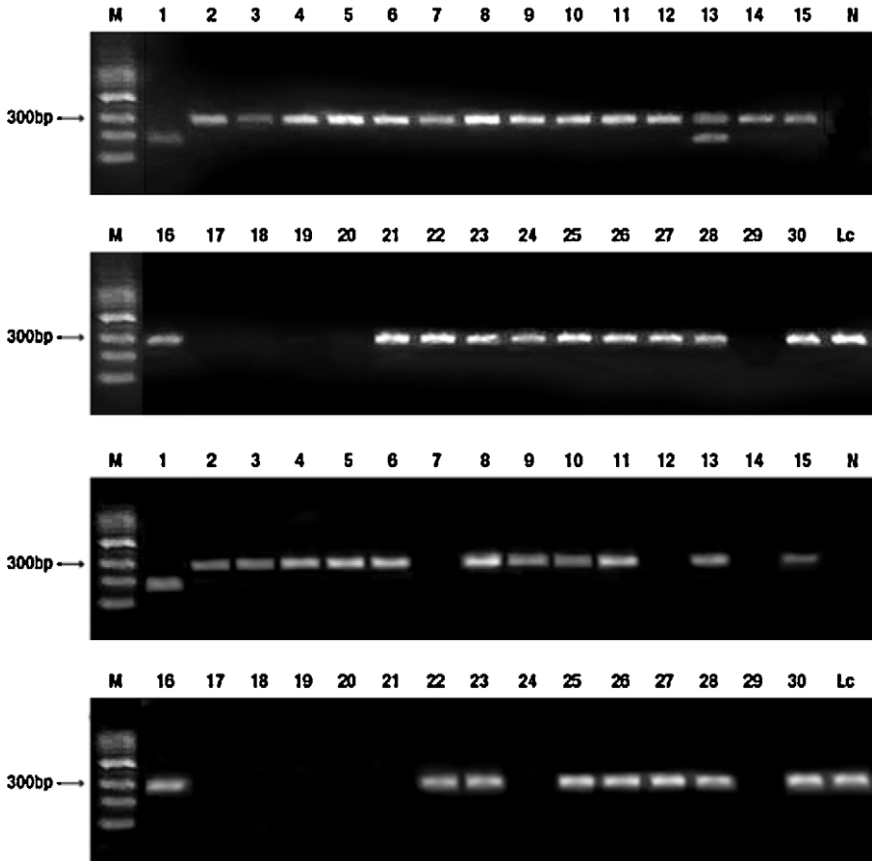

Fig. 2. ITS-1 nPCR analysis of CS samples. Electrophoresis of the ITS-1 nPCR products for the right conjunctiva (A) and the left conjunctiva samples (B). Molecular weight standard (M); negative control (N); *L.* (*L.*) *chagasi* control (Lc) (strain MHOM/1973/BH46); dogs samples (1–30).

method 15/30 dogs (50.0%) were positive by SB and 17/30 dogs (56.7%) with B. The CS positivity combining RC and LC was of 83.3%. The CS positivity for RC was significantly higher ($p < 0.05$) than obtained for SB and B samples. However, the CS positivity for LC did not show statistical difference in relation to SB and B ($p > 0.05$). For the ITS-1 nPCR no statistical difference was verified between the positivity of SB and B samples ($p > 0.05$). The ITS-1 nPCR uses two successive rounds of amplifications (nested) using two different groups of primers addressed to internal transcribed spacer 1 region of the ribosomal rRNA genes. The CS samples electrophoresis by this method were showed in Fig. 2.

The kDNA PCR- hybridization and ITS-1 nPCR methods showed similar sensitivities for CS (RC and LC) and SB samples. Moreover, for blood samples the positivity of ITS-1 nPCR (56.7%) was significantly higher than the positivity obtained by the kDNA PCR-hybridization (13.3%).

The RFLP analysis of the ITS 1 nPCR positive samples (Fig. 3) showed that the dog 01 was infected with *Leishmania* (*Viannia*) *braziliensis.* In agreement with this result the sample 01 was positive in the PCR step of the kDNA PCR-hybridization method, but negative in the hybridization step with *L.* (*L.*) *chagasi* kDNA minicircle probe (Fig. 1). The RFLP profile of sample 13 suggests a case of co infection. All other animals were infected with *L.* (*L.*) *chagasi* (= *L.* (*L*) *infantum*). The six serum negative control dogs were proven negative in the PCR analysis for all samples tested. They were also negative for the parasitological test.

4. Discussion

In the present study only non-invasive (CS) or low-invasive (SB and B) samples were compared since our main objective was to choose a screening method useful for large-scale epidemiological surveys. Invasive procedures are not adequate for this purpose due the difficulty to obtain these samples outside of a veterinary centre and the dog owners' opposition.

Some works have obtained satisfactory results for canine VL diagnosis using B samples (Reale et al., 1999; Hu et al., 2000; Ikonomopoulos et al., 2003; Maia et al., 2009), while others point that blood samples frequently presenting problems related to DNA preparation and PCR inhibitors (Reale et al., 1999; Silva et al., 2001; Lachaud et al., 2002b; Nunes et al., 2007). Another disadvantage of using blood samples is that the parasite load in blood tends to lower in the course of infection. Good sensitivities using skin biopsies have also been reported in some studies (Manna et al., 2004; Quaresma et al., 2009). This tissue may be a good reference for PCR diagnosis since sandflies are infected by

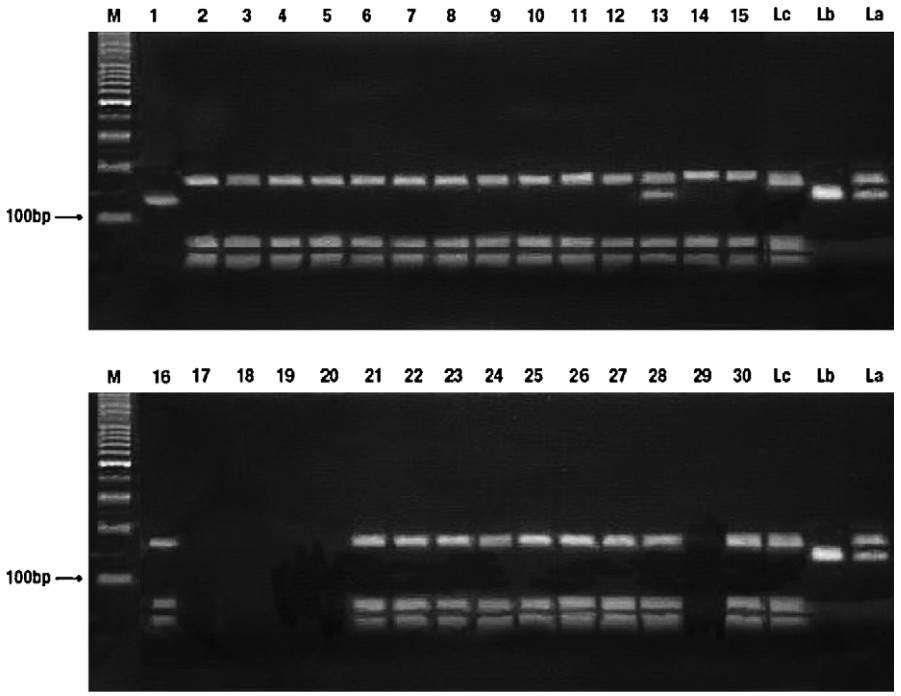

Fig. 3. Restriction fragment length polymorphism analysis of ITS-1 nPCR amplicons. Molecular weight standard (M); *L.* (*L.*) *chagasi* control (Lc) (strain MHOM/1973/BH46); *L.* (*V.*) *braziliensis* (Lb) (strain MHOM/BR/1975/M2903); *L.* (*L.*) *amazonensis* control (La) (strain IFLA/BR/67/PH8); dogs samples (1–30). The RFLP fragments were of 136 and 123 bp for *L.* (*V.*) *braziliensis*, 166 and 122 bp for *L.* (*L.*) *amazonensis* and 164, 72 and 35 bp for *L.* (*L.*) *chagasi*. The sample 1 was identified as *L.* (*V.*) *braziliensis*. All the other dogs were infected with *L.* (*L.*) *chagasi*. The RFLP profile of sample 13 suggested co infection. Samples 17–20 and 29 were negative for ITS-1 nPCR.

biting the dog skin. However, the drawback of skin biopsies is that it is relatively painful and bloody.

The PCR diagnosis with DNA from CS by the kDNA PCR-hybridization method exhibited a significantly higher sensitivity when compared to the one obtained with SB and B samples. Using ITS-1 nPCR statistical difference in relation to SB and B was verified only for RC. The use of combined results of both conjunctivas is recommended for the best performance of the CS procedure. The sensitivities of 90% (for PCR-hybridization) and 83.3% (for ITS-1 nPCR) obtained in this study using CS samples of both conjunctiva, were similar to the ones observed in other studies, also combining the conjunctivas, for VL diagnosis in symptomatic dogs. Strauss-Ayali et al. (2004) observed 92% of positivity using CS. Ferreira et al. (2008) detected parasite DNA in 91.7% of the dogs using CS. Pilatti et al. (2009) obtained between 73.9% and 95.6% of positivity by CS, depending of the PCR method used. Our results point out that the CS is equally effective for asymptomatic dogs.

The high CS sensitivity in asymptomatic dogs highlights this method as promising tool in mass-screening surveys by PCR, mainly because of the high prevalence of asymptomatic dogs in endemic areas and the lower sensitivity of tests based on antibody detection in asymptomatic infections (Leonides et al., 2002), In addition, CS is non-invasive, painless, fast, easily repeatable and acceptable by the dog owners.

Two PCR methods were used in this work: PCR-hybridization and ITS-1 nPCR. In a previous study Pilatti et al. (2009) demonstrated that the PCR-hybridization was significantly more sensitive than ITS-1 nPCR. To compensate the lower sensitivity of ITS-1 nPCR, the DNA sample volume used for this method was increased in the present study. For ITS-1 nPCR were used 10.0 μL of DNA sample and for PCR-hybridization 1.0 μL was used. These conditions did not show statistical difference in the sensitivity of both methods for CS and SB samples. Surprising, for B samples the sensitivity of ITS-1 nPCR was significantly higher (56.7%) than verified for PCR-hybridization (13.3%), demonstrating that sensitivities of PCR methods can vary according to the biological sample.

This was the first report of CS for VL diagnosis by PCR in asymptomatic dogs. CS is very sensitive method and superior, in sensitivity and practicability, to other low-invasive samples. We concluded that the use of CS for the regular screenings of dogs by PCR must be considered.

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