Short Communication

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Detection of canine visceral leishmaniasis by conjunctival swab PCR

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ABSTRACT

Introduction: Conjunctival swab PCR was evaluated as a tool to diagnose visceral leishmaniasis in dogs. **Methods:** Conjunctival swab PCR was compared to indirect immunofluorescence antibody test and blood PCR. **Results:** Indirect immunofluorescence was significantly correlated with conjunctival swab PCR ($p < 0.05$), but not with blood PCR ($p > 0.05$). In addition, conjunctival swab PCR was significantly associated with presence of clinical symptoms ($p < 0.05$), whereas blood PCR was associated with absence of clinical symptoms ($p < 0.05$). **Conclusions:** Results indicate that conjunctival swab PCR is useful in epidemiological surveys of canine visceral leishmaniasis.

Keywords: Canine visceral leishmaniasis. Conjunctival swab. Epidemiological survey.

Visceral leishmaniasis, a vector-borne zoonosis of global importance, is caused by the protozoan *Leishmania infantum* (syn. *Leishmania chagasi*) in Brazil[1]. The disease is transmitted to dogs, humans, and other hosts primarily through the bite of infected sand flies[1]. It is considered a serious and chronic illness in dogs, with clinical symptoms dramatically different from one animal to another in most cases[1]. For instance, dogs may present subclinical infection that is sometimes self-limiting, or may present severe symptoms that can lead to death.

Leishmania infection may be diagnosed by several methods. For PCR-based diagnosis, DNA can be extracted from various clinical specimens, including blood, skin biopsies, lymph nodes, bone marrow, and spleen. However, collection of such specimens is invasive, and requires skilled labor or appropriate facilities. In contrast, conjunctival swabs are fast and easy to collect[2] [3]. Thus, we assessed the suitability of conjunctival swabs as a biological sample for PCR-based diagnosis of canine visceral leishmaniasis. Its performance was compared to blood PCR and indirect immunofluorescence antibody test.

Blood samples and conjunctival swabs were collected between July and August 2011 from 213 dogs in Ilha Solteira (20° 25′ 58″ S and 51° 20′ 33″ W), a city in the northwest region of the Brazilian State of São Paulo. During sample collection, each dog was evaluated for clinical symptoms consistent with visceral leishmaniasis. Animals were considered to be symptomatic if at least one of the following was observed: skin disorders, apathy, lymphadenomegaly, dry fur, alopecia, onychogryphosis, erosions, ulcers, prostration, and/or cachexia[1]. The study (Protocol No. 2203/2011) was approved by the Ethics Committee in Animal Experimentation and Animal Welfare of the Faculty of Veterinary Medicine and Animal Science from the University of Sao Paulo, and was compliant with national guidelines (Law No. 11.794, 8/10/2008).

Indirect immunofluorescence antibody test was performed according to published methods[4] based on canine anti-IgG conjugated to fluorescein isothiocyanate (Sigma-Aldrich, Bellefonte, PA, USA, Catalog No. F7884) and diluted 1:600. Sera were considered positive when samples were fluorescently stained, with a 1:40 dilution as cutoff point[4].

For PCR-based diagnosis, DNA was purified from conjunctival swabs and blood by phenol-chloroform[5] and salting-out[6], respectively. DNA was stored at -20°C until analysis. A conserved 120 bp fragment in *Leishmania* spp. kinetoplast DNA minicircle was amplified according to a published protocol[7], using two pairs of primers, including primers 13A (5′-dGTG GGG GAG GGG CGT TCT-3′) and 13B (5′-dATT TTA CAC CAA CCC CCA GTT-3′). Reactions consisted of 1 U Platinum® Taq DNA polymerase (Invitrogen, Camarillo, CA, USA), 15.25µL ultrapure water, 1× PCR buffer, 1.5mM $MgCl_2$, 0.31mM each of dATP, dCTP, dGTP,

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and dTTP, 0.26μM of each primer, and 2.5μL of extracted DNA in a final volume of 25μL. Positive control reactions contained DNA extracted from an *in vitro* culture of *L. infantum*. Negative control reactions contained DNA extracted from blood and conjunctival swabs of dogs previously confirmed to be uninfected. Amplified products were resolved on 2% agarose, stained with ethidium bromide, and photographed with a Cybershot 7.2 megapixel digital camera (DSC-W70, Sony). All samples were tested in triplicate.

To eliminate false negatives due to issues with DNA loading, sample degradation, or PCR failure, a real-time PCR reaction was performed to amplify β-actin[8]. The gene was amplified with forward primer 5′-dCTG GCA CCA CAC CTT CTA CAA-3′, reverse primer 5′-dGCC TCG GTC AGC AGC A-3′, and fluorogenic probe 5′-CCAC GCG CAG CTC G-3′[8]. To construct an absolute standard curve for this reaction, canine DNA was serially diluted nine times so that each point of the curve corresponded to one log. Template DNA concentration was estimated by absorbance at 260 and 280nm.

Agreement between diagnostic techniques was evaluated using the kappa statistic, with values considered to indicate no agreement ($k < 0$), slight ($0 < k < 0.2$), fair ($0.2 < k < 0.4$), moderate ($0.4 < k < 0.6$), substantial ($0.6 < k < 0.8$), and almost perfect ($k > 0.8$) agreement [9]. Sensitivity, specificity, and confidence intervals were also calculated for all diagnostic tests, using indirect immunofluorescence as gold standard. Data were analyzed in SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA) by Pearson chi-square test at 95% confidence level.

Of 213 dogs evaluated, 28.2% (60/213) tested positive for visceral leishmaniasis on at least one of three diagnostic tests. In particular, 13.6% (29/213) tested positive on indirect immunofluorescence only, and 13.1% (28/213) tested positive by conjunctival swab or blood PCR only. Notably, the tests detected visceral leishmaniasis in different subsets of dogs. For example, 7.9% (17/213) tested positive on both indirect immunofluorescence and conjunctival swab PCR, while 81.2% (173/213) tested negative on both. In addition, 5.1% (11/213) of dogs were infected based on conjunctival swab PCR, while 5.6% (12/213) were infected based on indirect immunofluorescence. Thus, conjunctival swab PCR had sensitivity 58.6%, specificity 94%, positive predictive value 61%, and negative predictive value 94% **(Table 1)**.

On the other hand, 3.3% (7/213) tested positive on both indirect immunofluorescence and blood PCR, 10.3% (22/213) tested positive by indirect immunofluorescence only, 9.9% (21/213) tested positive by blood PCR only, and 76.5% (163/213) tested negative on both. Based on these data, blood PCR had sensitivity 24.1%, specificity 88.5%, positive predictive value 25%, and negative predictive value 88% **(Table 1)**.

Notably, the sensitivity of conjunctival swab PCR was lower than that of other diagnostic approaches based on conjunctival swabs. For instance, previous studies demonstrated sensitivity as high as 92%[5], and 91.7%[10]. In addition, 90% sensitivity was achieved by hybridization, and 83.3% by nested PCR[2]. *L. infantum* DNA was detected in conjunctival swabs from 95% of symptomatic dogs and 77.5% of asymptomatic dogs that also tested positive on other serological and parasitological tests[3]. These results, however, were obtained from highly sensitive techniques. In particular, hybridization can enhance the sensitivity of PCR and verify results[10]. However, this technique requires more elaborate infrastructure to handle radioisotopes. Similarly, nested PCR has been used to enhance sensitivity, but is more time consuming and is more susceptible to contamination[11].

In line with previous results[12], the adjusted kappa index between indirect immunofluorescence and conjunctival swab PCR was 0.53, suggesting moderate[9], but statistically significant agreement ($p < 0.05$, **Table 2**). However, the number of seronegative dogs that tested positive on conjunctival swab PCR (n = 11) was similar to the number of seropositive dogs that tested negative on conjunctival swab PCR (n = 12). This result may reflect parasite load, which may go above or below the limit of detection of each assay, depending on the phase of infection. Thus, the use of both tests would enhance diagnosis of visceral leishmaniasis in dogs.

For indirect immunofluorescence and blood PCR, the adjusted kappa index was 0.13, indicating slight agreement[9]. Indeed, these two tests were not significantly correlated ($p > 0.05$). Accordingly, blood and conjunctival swab PCR were also not significantly correlated ($p > 0.05$). These results are summarized in **Table 2**, and are in line with published results[12].

TABLE 1 - Sensitivity, specificity, positive predictive value, and negative predictive value of conjunctival swab and blood PCR for canine visceral leishmaniasis, using indirect immunofluorescence antibody test as gold standard. A total of 213 dogs were tested by all three methods.

Parameter	IFAT × CS-PCR	IFAT × blood PCR
Sensitivity (%)	58.6 (17/29)	24.0 (7/29)
Specificity (%)	94.0 (173/184)	88.5 (163/184)
Positive predictive value (%)	61.0 (17/28)	25.0 (7/28)
Negative predictive value (%)	93.5 (173/185)	88.0 (163/185)

IFAT: immunofluorescence antibody test; **PCR:** polymerase chain reaction; **CS-PCR:** conjunctival swab- polymerase chain reaction.

TABLE 2 - Kappa agreement among indirect immunofluorescence antibody test, conjunctival swab, and blood PCR for canine visceral leishmaniasis.

	CS-PCR			Blood PCR		
IFAT	**+**	**-**	**total**	**+**	**-**	**total**
+	17	12	29	7	22	29
-	11	173	184	21	163	184
Total	**28**	**185**	**213**	**28**	**185**	**213**
kappa		0.53*			0.13	

IFAT: immunofluorescence antibody test; **PCR:** polymerase chain reaction; **CS-PCR:** conjunctival swab-polymerase chain reaction.*$p < 0.05$ by chi-square test.

Clinical symptoms were apparent in 43 dogs. Of the 29 dogs that tested positive by indirect immunofluorescence, 51.7% (15/29) were symptomatic. Similarly, symptoms were apparent in 57.1% (16/28) of dogs that tested positive by conjunctival swab PCR. In contrast, clinical symptoms were observed in only 28.6% (8/28) of dogs that tested positive on blood PCR. Clinical signs were also observed in 70.6% (12/17) of animals that tested positive on both conjunctival swab PCR and indirect immunofluorescence. Three of five animals that tested positive on all three tests were symptomatic.

Notably, statistical analysis **(Table 3)** indicated that a positive result on conjunctival swab PCR is significantly associated with the presence of clinical signs ($p < 0.05$), while a positive result on blood PCR is significantly associated with the absence of clinical signs ($p < 0.05$). Indeed, conjunctival swab PCR has been demonstrated to have high sensitivity for *Leishmania* in clinically diseased[12,13], and asymptomatic dogs[2]. We note, however, that higher sensitivity was achieved in asymptomatic dogs by other groups[2].

TABLE 3 - Association of a positive test on indirect immunofluorescence, conjunctival swab PCR, and blood PCR with presence or absence of clinical symptoms.

	Clinical symptoms		
Positive diagnostic test	+	-	**p value, χ^2**
Conjunctival swab PCR	16	12	< 0.05
Blood PCR	8	20	< 0.05
IFAT	15	14	> 0.05

IFAT: immunofluorescence antibody test; **PCR:** polymerase chain reaction.

Because of high specificity and sensitivity, molecular techniques provide precise and accurate diagnosis of canine visceral leishmaniasis, and may identify the infecting *Leishmania* species. For example, blood samples were previously shown to be suitable for PCR-based diagnosis of canine visceral leishmaniasis[13], although issues with DNA preparation and PCR failure have been reported[14]. In addition, parasite load in the blood tends to diminish over the course of infection[2]. Acceptable results were also obtained by PCR on skin samples[15], which are, however, not easy to collect, and only through procedures that may cause pain and injury. Here, we report that conjunctival swabs are also suitable for PCR-based detection of *L. infantum* infection in dogs. In addition, conjunctival swabs are easy to collect, and thus provide opportunities for further research that may help quickly, rationally, and effectively control visceral leishmaniasis in Brazil and other countries.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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