Full Article

Rev. Bras. Parasitol. Vet., Jaboticabal, v. 20, n. 1, p. 36-41, jan.-mar. 2011
ISSN 0103-846X (impresso) / ISSN 1984-2961 (eletrônico)

The use of conjunctival swab samples for PCR screening for visceral leishmaniasis in vaccinated dogs

O uso de amostras de *swab* conjuntival para triagem por PCR da leishmaniose visceral em cães vacinados

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Received September 13, 2010
Accepted November 29, 2010

Abstract

The polymerase chain reaction (PCR) has been shown to provide a rapid and sensitive technique for *Leishmania* detection. The aim of this study was to evaluate the technique of noninvasive conjunctival swabs (CS) as a sampling method for molecular screening for visceral leishmaniasis (VL) in a group of 42 police dogs, all of them vaccinated against VL, and to compare the results with those obtained by serological tests. The serological assays were performed independently by three laboratories. Laboratories 1 and 2 were private laboratories and laboratory 3 was the National Reference Laboratory. The first serological screening performed by laboratory 1 showed 15 reactive dogs and 4 indeterminate. Laboratory 2 confirmed only 3 reactive dogs and 2 indeterminate. Laboratory 3 confirmed 7 reactive dogs and 3 indeterminate. The PCR diagnosis using the CS procedure was performed on all 42 animals and was able to detect *Leishmania* DNA in 17 dogs. The PCR assay confirmed all the cases that were simultaneously reactive in the serological tests by two laboratories. The results showed that the CS technique was a sensitive and practical method for sample collection, thus allowing reliable diagnostic tests through PCR.

Keywords: Visceral leishmaniasis, dog, diagnosis, PCR, conjunctival swab.

Resumo

A PCR (do inglês *Polymerase Chain Reaction*) tem demonstrado ser uma técnica rápida e sensível para detecção de *Leishmania*. O objetivo deste estudo foi avaliar a técnica não invasiva do *swab* conjuntival na identificação por PCR de animais infectados em um grupo de 42 cães policiais, todos vacinados contra a Leishmaniose Visceral (VL), e comparar os resultados com aqueles obtidos pelos testes sorológicos. Os ensaios sorológicos foram realizados independentemente por três laboratórios. Os laboratórios 1 e 2 eram privados. O laboratório 3 era o Laboratório de Referência Nacional. A primeira triagem sorológica realizada pelo laboratório 1 apresentou 15 cães reativos e 4 indeterminados. O laboratório 2 confirmou apenas 3 cães reativos e 2 animais indeterminados. O laboratório 3 confirmou 7 cães reativos e 3 cães foram classificados como indeterminados. O diagnóstico pela PCR, utilizando o procedimento do *swab* conjuntival, foi realizado em todos os 42 animais e foi capaz de detectar DNA de *Leishmania* em 17 cães. A PCR confirmou todos os casos simultaneamente reativos nos testes sorológicos de dois laboratórios. Os resultados demonstraram que o *swab* conjuntival é um método sensível e prático para coleta de amostra, permitindo um diagnóstico consistente através da PCR.

Palavras-chave: Leishmaniose visceral, cão, diagnóstico, PCR, swab conjuntival.

Introduction

Visceral leishmaniasis (VL) is an infectious disease caused in Brazil by the protozoan *Leishmania* (*Leishmania*) *infantum* (syn. *Leishmania* (*Leishmania*) *chagasi*) (DANTAS-TORRES, 2009). Dogs are the major domestic reservoirs of *L. infantum*, and constitute part of the epidemiological cycle of human transmission (LAISON; SHAW, 1987). Epidemiological control for VL in Brazil involves elimination of infected dogs (TESH, 1995) in an attempt to reduce human disease. Therefore, reliable diagnostic tests are essential to avoid disease transmission or unnecessary culling of dogs. Dog removal is based on seropositivity, mainly using the enzyme-linked immunosorbent assay (ELISA) and the immunofluorescence antibody test (IFAT). However, these techniques present limitations (SILVA et al., 2006), since the sensitivity of antibody detection is generally lower in early or asymptomatic canine infections (LEONTIDES et al., 2002). In addition, the serological tests can present false positive results in

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dogs due to cross-reactions with other diseases like trypanosomiasis (BARBOSA DE DEUS et al., 2002).

The polymerase chain reaction (PCR) has been shown to provide a rapid, specific and sensitive technique for *Leishmania* detection (MAIA; CAMPINO, 2008; ASSIS et al., 2010; QUEIROZ et al., 2010). A broad range of clinical specimens (including blood, skin biopsies, lymph node, bone marrow and spleen) have been used for PCR detection of the parasite (SOLANO-GALLEGO et al., 2009). However, noninvasive samples are very desirable since they could be obtained outside of veterinary centers and could be applied to massive screenings of dogs. One useful method for sample collection is the conjunctival swab (CS) technique, which consists of using a sterile swab to sample the dogs' conjunctivas. This method has been shown to be highly sensitive for VL diagnosis using PCR, both among symptomatic dogs (STRAUSS-AYALI et al., 2004; FERREIRA et al., 2008; PILATTI et al., 2009) and among asymptomatic dogs (LEITE et al., 2010).

The aim of this study was to evaluate noninvasive CS for molecular screening of a group of police dogs (which were all vaccinated against VL), including seronegative and seropositive animals, and to compare the results with those obtained from serological tests. A group of vaccinated dogs was chosen in view of the potential use of CS as a confirmatory diagnosis for these animals, in which vaccination could render some dogs positive in the serological tests. Moreover, this group of dogs was continuously monitored and subjected to regular serological screenings.

Material and Methods

1. Dogs

Forty-two dogs belonging to the Military Police of the state of Minas Gerais (PMMG) were included in this study. The dogs were working and living in the area of the Fourth Company of Military Police/Command of Specialized Police, which is located in the city of Belo Horizonte, Minas Gerais, Brazil. All the dogs had been vaccinated against VL using the Leishmune® vaccine (Fort Dodge, Brazil), in accordance with the manufacturer's protocol. The animals were subjected to clinical examination and classified as symptomatic (presenting at least one symptom of VL) or asymptomatic (not presenting any clinical signs). The clinical signs evaluated were: alopecia, weight loss, onychogryphosis, skin lesions, lymphadenopathy and splenomegaly. The samples were collected between August and September 2009.

2. Samples

Exfoliative epithelial cells were collected from the right and left conjunctiva of each animal using sterile cotton swabs that had been manufactured for bacteriological isolation. The cotton tips were broken off and only the cotton parts were transferred to sterile tubes and stored at –20 °C until use. Peripheral blood (B) was collected from each dog (2.7 mL) in tubes containing EDTA and stored at –20 °C.

3. DNA extraction

DNA purification from CS was carried out as described by Strauss-Ayali et al. (2004), with minor modifications. Each cotton tip received 300 µL of lysis buffer (50 $mMol.L^{-1}$ of Tris, 50 $mMol.L^{-1}$ of NaCL and 10 mMol/l of EDTA; at pH 8.0) containing proteinase K (250 $\mu g.mL^{-1}$) and Triton X-100 (1%). After the incubation (2 hours at 56 °C) the solution was eluted from the cotton, transferred into phase-lock gel tubes (PLG-H; Eppendorf, Hamburg, Germany) and was mixed with 500 µL of 75% Tris-saturated phenol (Sigma-Aldrich, St. Louis, USA), 25% chloroform-isoamyl alcohol. The organic phase was separated from the aqueous phase by centrifugation at 12,000 g for 5 minutes, and the organic material was transferred to a new phase-lock gel tube. The extraction was repeated with 500 µL of 50% phenol, 50% chloroform-isoamyl alcohol and once with 100% chloroform-isoamyl alcohol. DNA precipitation was performed using a single volume of isopropanol-sodium acetate, followed by washing with 75% ethanol. The DNA pellet was suspended in 30 µL of Tris-EDTA buffer (10 $mMol.L^{-1}$ of Tris and 1 mMol/l of EDTA; at pH 8.0). After extraction, DNA preparations from the right and left conjunctivas of the same animal were mixed and kept at –20 °C until needed.

4. PCR

The samples were analyzed by means of internal transcribed spacer-1 nested PCR (ITS-1 nPCR). A positive control consisting of genomic DNA from *L.* (*L.*) *infantum* (strain MHOM/1973/BH46) was used at 1.0 $ng.\mu L^{-1}$. A negative control without DNA was included in all tests.

This PCR protocol was adapted from Schönian et al. (2003). Primers targeting 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 of deoxynucleoside triphosphate, 2 mM of $MgCl_2$, 5 mM of KCl, 75 mM of Tris-HCl (pH 9.0), 2.0 mM of $(NH_4)SO_4$, and 1.4 U of Taq DNA polymerase (Ludwig Biotec, Porto Alegre, Brazil). The cycling conditions were 94 °C for 5 minutes followed by 30 cycles of 94 °C for 30 seconds, 53 °C for 30 seconds and extension at 72 °C for 30 seconds, followed by a final extension at 72 °C for 5 minutes. The amplification products were viewed on 2% agarose gel stained with ethidium bromide. The PCR product size was 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 of between 280 and 330 bp.

5. Serological tests

The tests were performed one year after vaccination, independently by three different laboratories. Laboratories 1 and 2

(Lab 1 and Lab 2) were private laboratories. Laboratory 3 (Lab 3) was the National Reference Laboratory for Human and Canine Visceral Leishmaniasis Diagnosis of the Ezequiel Dias Foundation (Funed). The three laboratories used the same diagnostic kits, produced by the Oswaldo Cruz Foundation (Fiocruz) and validated by Funed. Two serological tests were used: enzyme-linked immunosorbent assay (ELISA – *EIE* - Canine Visceral Leishmaniasis produced by Bio-manguinhos/Fiocruz, Brazil) and the immunofluorescence antibody test (IFAT – *IFI* – Canine Visceral Leishmaniasis produced by Bio-manguinhos/Fiocruz, Brazil). In cases of divergence between the two techniques, the IFAT was considered to be confirmatory.

For ELISA, samples that showed optical density greater than or equal to the cutoff were considered to be reactive. Non-reactive samples presented optical density lower than the cut-off. Indeterminate samples had optical density between the cutoff and the cutoff value multiplied by 1.2. In the IFAT test, samples that showed fluorescence at serum dilution of 1:40 were considered reactive. Non-reactive samples did not present fluorescence. Indeterminate samples presented little fluorescence in the first dilution (1:40), but it was not possible to affirm that the reading was not reactive.

The consolidated serologic result was considered positive when ELISA and IFAT were simultaneously reagents or ELISA was non reagent and IFAT showed fluorescence at sera dilution of 1:80. The results were considered indeterminate when ELISA was non reagent and IFAT showed fluorescence at sera dilution of 1:40 or ELISA was reagent and IFAT was non reagent.

Results

The serological screening performed by Lab 1 showed 15 reactive dogs, and 4 dogs were classified as indeterminate. Only three reactive dogs (1, 3 and 4) and one indeterminate dog (8) were symptomatic (Tables 1 and 2). Because of the high positivity found in this assay, reactive and indeterminate animals diagnosed by Lab 1 were subjected to a new serological test by Lab 2. Lab 2 found only three reactive dogs, and two animals were indeterminate. Because of the low agreement between the results from Lab 1 and Lab 2, all the reactive and indeterminate animals as determined according to Lab 1 were again reanalyzed by Lab 3 (the National Reference Laboratory). Lab 3 confirmed that there were 7 reactive dogs, and 3 dogs were classified as indeterminate. The dogs that were confirmed as reactive in the serological diagnosis from Lab 3 were put down. The autopsies on the animals that were put down showed morphological changes relating to visceral leishmaniasis, in organs and tissues, except for dog 5 (Table 1).

Molecular diagnosis by means of PCR using the CS procedure was performed on all 42 animals and was able to detect *Leishmania* DNA in 17 dogs (Tables 1 and 2). Comparing the PCR results with those obtained from serological assays by Lab 1, PCR was positive for 10 reactive and one indeterminate case, but was negative for 5 reactive and 3 indeterminate cases. In addition, PCR was positive for 5 non-reactive cases (dogs 17, 18, 21, 24 and 26). The reactive cases according to Lab 1 that were PCR-negative (dogs 13,14,15,25 and 29)

Table 1. Comparison of serological and molecular tests on dogs that were put down.

Dog	Sex	Breed	Diagnosis												Autopsy report
			Clinical	Clinical signals	Serological									PCR	
					Lab 1			Lab 2			Lab 3				
					ELISA	IFAT	C	ELISA	IFAT	C	ELISA	IFAT	C		
1	M	GS	S	Recurrent dermatitis; Wound characteristics of vasculitis in syrup.	R	R	R	NR	NR	NR	R	R	R	+	Enlarged spleen, interstitial nephritis
2	M	LA	A	-	R	R	R	I	NR	NR	R	R	R	+	Enlarged spleen, interstitial nephritis
3	F	MB	S	Changes (bilateral) lymph nodes of the thoracic lymphatic chain; Presence of a hemorrhagic lymph node.	R	R	R	R	R	R	R	R	R	+	Enlarged spleen, interstitial nephritis
4	F	MB	S	Bilateral eye discharge; irritation lip; bald patches around the lips; dermatitis sacral.	R	R	R	NR	NR	NR	R	R	R	+	Enlarged spleen, interstitial nephritis
5	F	GS	A	-	R	R	R	NR	NR	NR	R	R	R	+	No change in the target organs (liver, spleen, kidney)
6	M	MB	A	-	R	R	R	NR	NR	NR	I	R	R	+	Enlarged spleen, interstitial nephritis
7	M	MB	A	-	R	R	R	R	R	R	R	R	R	+	Enlarged spleen, interstitial nephritis

M = Male, F = Female, S = Symptomatic, A = Asymptomatic, R = reactive, I = Indeterminate, NR = Non-reactive, C = consolidated result, + = Positive, - = Negative. To IFAT test, value > 1/40 was reactive. For breed: GS = German Shepherd; LA = Labrador; MB = Mallinois Belgian Shepherd.

Table 2. Comparison of serological and molecular tests on dogs that were not put down.

Dog	Sex	Breed	Diagnosis										
			Clinical	Serological									PCR
				Lab 1			Lab 2			Lab 3			
				ELISA	IFAT	C	ELISA	IFAT	C	ELISA	IFAT	C	
8	M	GS	S*	I	I	I	NR	NP	NR	NR	I	I	+
9	F	GS	A	R	R	R	R	R	R	R	NR	I	+
10	M	GS	A	R	R	R	NR	NP	NR	R	NR	I	+
11	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
12	M	MB	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
13	M	GS	A	R	R	R	NR	NP	NR	NR	NR	NR	-
14	F	GS	A	R	R	R	NR	NP	NR	NR	NR	NR	-
15	F	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
16	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
17	F	MB	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	+
18	F	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	+
19	F	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
20	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
21	M	LB	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	+
22	M	GS	A	R	R	R	NR	NP	NR	NR	NR	NR	+
23	M	GS	A	I	I	I	NR	NP	NR	NR	NR	NR	-
24	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	+
25	F	MB	A	R	R	R	NR	NP	NR	I	NR	NR	-
26	F	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	+
27	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
28	M	GS	A	I	I	I	NR	NP	NR	NR	NR	NR	-
29	F	GS	A	R	R	R	NR	NP	NR	NR	NR	NR	-
30	F	MB	A	I	I	I	NR	NP	NR	NR	NR	NR	-
31	M	GS	A	NR	NP	NR	NP	NP	NR	NP	NP	NP	-
32	M	LB	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
33	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
34	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
35	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
36	M	SP	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
37	M	LB	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
38	M	MB	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	+
39	F	LB	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
40	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
41	M	GS	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-
42	M	MO	A	NR	NP	NR	NP	NP	NP	NP	NP	NP	-

M = Male, F = Female, S = Symptomatic, A = Asymptomatic, R = reactive, NR = Non-reactive, I = Indeterminate, C = consolidated result, + = Positive, - = Negative, NP = Not performed, * = clinical signs: skin lesions, ear infections, alopecia on the chin and eschar sciatic. To IFAT test, value > 1/40 was reactive. For breed: GS = German Shepherd; LA = Labrador; MB = Mallinois Belgian Shepherd, SP = Springer, MO = Mongrel.

tested negative in the serological assays by Lab 2 and Lab 3, and may have been false positive cases. The same occurred with the three indeterminate cases from Lab 1 that were PCR-negative (dogs 23, 28 and 30). According to Lab 2, PCR confirmed all 3 reactive cases (dogs 3, 7 and 9) and was positive for the two indeterminate cases (dogs 2 and 4). In relation to the results obtained by Lab 3, PCR confirmed all the reactive cases (dogs 1, 2, 3, 4, 5, 6 and 7) and tested positive for the three indeterminate cases (dogs 8, 9 and 10). The PCR assay confirmed all the cases that were simultaneously reactive in the serological tests by two laboratories (dogs 1, 2, 3, 4, 5, 6, 7 and 9).

Discussion

PCR assays have greatly improved the sensitivity and the specificity of diagnosing *Leishmania* infection in dogs. However, to make this technique achievable, the sampling methods need to be noninvasive, easy and painless. CS presented all these characteristics, together with high sensitivity. Using CS to diagnose symptomatic dogs through PCR, Strauss-Ayali et al. (2004) observed sensitivity of 92%; Ferreira et al. (2008) found 91.7% and Pilatti et al. (2009) obtained between 73.9 and 95.6%, depending on the PCR method used. Using asymptomatic infected animals, Leite et al.

(2010) obtained sensitivity of between 83.3 and 90% using CS, according to the PCR protocol used. In the present study, the CS sampling method was used to screen a group of police dogs that include seronegative and seropositive animals.

The PCR diagnosis using CS samples was positive for 17 dogs, and the positivity rate was higher than that of the serological test performed by Lab 1, which detected 15 reactive animals. PCR was positive for 5 seronegative dogs, which were all asymptomatic, thus demonstrating the higher sensitivity of this technique and its potential for identifying seronegative asymptomatic infected animals. Asymptomatic animals may account for a high percentage (up to 85%) of infected dogs in areas of endemicity (DANTAS-TORRES et al., 2006), and they serve as reservoirs for vector transmission (MICHALSKY et al., 2007). In this study, asymptomatic animals represented 70.6% (12/17 dogs) of the positive PCR dogs.

The 5 reactive and the 3 indeterminate cases obtained by Lab 1 that were PCR-negative were not confirmed by the serological tests of Lab 2 and Lab 3. In addition, all the reactive and indeterminate cases obtained by Lab 3 (the National Reference Laboratory) tested positive in the PCR diagnosis, thus indicating the high specificity of the PCR assay.

According to some studies, Leishmune® vaccinated dogs might test positive in conventional serological assays (SILVA et al., 2001; BORJA-CABRERA et al., 2002; OLIVEIRA MENDES et al., 2003), thus rendering these tests useless for surveillance or control programs involving vaccinated animals. The antigen in the ELISA assay that these studies used was the fucose-mannose ligand (FML) antigen, i.e. the same antigen as used in the Leishmune® vaccine formulation. However, a recent study demonstrated that by using the total promastigote lysate of *L. major* and *L. braziliensis*, which are the antigens of the official ELISA test recommended by the Brazilian Ministry of Health, only 1.3% of vaccinated dogs tested positive (PALATNIK-DE-SOUSA et al., 2009). Our results are in agreement with this study, since 15 of the 42 vaccinated animals tested positive in the assay performed by Lab 1 and 10 of them had the *Leishmania* infection confirmed by PCR. Five dogs could still be classified as false positive but, even so, they were not reactive in the serological assays performed by Lab 2 and Lab 3.

Although the three laboratories used the same official diagnostic kits to perform the serological assays, significant differences in the results were seen among them. For this reason, it was decided that only the cases confirmed by Lab 3 (the National Reference Laboratory) should be put down. These results highlight the need of molecular methods as complementary tools for accurately diagnosing canine visceral leishmaniasis, especially in Brazil, where infected dogs are put down. Another unexpected result was the high occurrence of serologically and molecularly positive animals, among which some were symptomatic, in this group of vaccinated dogs.

Restriction fragment length polymorphism (RFLP) performed on the ITS-1 nPCR products showed that all 17 PCR-positive dogs were infected with *L.* (*L.*) *infantum* (data not shown). This result disregards a case of *L.* (*Viannia*) *braziliensis* infection (in dog 5), in which no morphological changes relating to VL were seen in the autopsy. This animal was probably infected but completely asymptomatic, since it was PCR-positive and reactive in the serological tests by laboratories 1 and 3.

This was the first report on the use of CS for screening for VL by means of PCR in a group of vaccinated dogs. As a whole, our results showed that CS is a sensitive and practical method for sample collection, thus allowing reliable diagnostic tests using PCR. The CS procedure could be especially useful as a confirmatory diagnostic method for asymptomatic vaccinated dogs that test positive in serological assays.

Acknowledgements

This investigation was supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG) (CBB-APQ-00399-08), Pan-American Health Organization (PAHO) (SGP0911) and Centro de Desenvolvimento da Tecnologia Nuclear/Comissão Nacional de Energia Nuclear (CDTN/CNEN). We gratefully thank the Fourth Company of Military Police/Command of Specialized Police of Belo Horizonte, State of Minas Gerais.

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