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Polymerase chain reaction and real-time PCR for diagnosing of *Leishmania infantum chagasi* in dogs

Reação em Cadeia da Polimerase e PCR em tempo real para diagnóstico de *Leishmania infantum chagasi* em cães

Rafael Antonio do Nascimento Ramos[1]*; Carlos Alberto do Nascimento Ramos[2]; Márcia Mariza Gomes Jusi[3]; Flábio Ribeiro de Araújo[2]; Rosangela Zacarias Machado[3]; Maria Aparecida da Glória Faustino[1]; Leucio Câmara Alves[1]

[1]Laboratório de Doenças Parasitárias dos Animais Domésticos, Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco – UFRPE, Recife, PE, Brasil

[2]Laboratório de Imunologia, Área de Sanidade Animal, Embrapa Gado de Corte, Campo Grande, MS, Brasil

[3]Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Universidade Estadual Paulista – UNESP, Jaboticabal, SP, Brasil

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Abstract

The importance of dogs as a reservoir for *Leishmania infantum chagasi* in urban environments has stimulated numerous studies assessing diagnostic techniques. When performed properly, such procedures are an important step in preventing leishmaniasis in humans. Molecular methods have become prominent for this purpose. The aim of the present study was to determine the performance of the polymerase chain reaction (PCR) and real-time PCR (qPCR) for diagnosing of canine visceral leishmaniasis (CVL) using different biological samples. For this, 35 dogs from an area endemic for CVL were used. Bone marrow aspirate and lymph node and spleen fragments from these dogs were used for the molecular diagnosis. In the present study, qPCR was able to detect a greater number of positive animals than seen with PCR. Among the different biological samples used, there was no significant difference in *L. infantum chagasi* DNA detection between PCR and qPCR. However, considering that lymph nodes are easy to acquire, these can be considered to be the best samples for making molecular diagnoses of *L. infantum chagasi* infection.

Keywords: Leishmaniasis, diagnosis, molecular, *L. infantum chagasi*.

Resumo

A importância do cão como reservatório de *L. infantum chagasi* no meio urbano tem estimulado a realização de inúmeros trabalhos de avaliação de técnicas de diagnóstico, uma vez que este procedimento, quando realizado corretamente, torna-se um importante passo na prevenção da doença em humanos. Dentre os métodos de diagnóstico, as técnicas moleculares têm adquirido destaque. Objetivou-se neste trabalho verificar o desempenho da Reação em Cadeia da Polimerase (PCR) e da PCR em tempo real (qPCR) para diagnóstico da Leishmaniose Visceral Canina (LVC) utilizando diferentes amostras biológicas. Para tanto foram utilizados 35 cães provenientes de uma área endêmica para LVC, onde foram utilizados para o diagnóstico molecular, aspirado de medula óssea, fragmentos de linfonodo e baço. Neste estudo a qPCR foi capaz de detectar um maior número de animais positivos quando comparada com a PCR. Já entre as diferentes amostras biológicas utilizadas não foi observada diferença significativa na detecção de DNA de *L. infantum chagasi* por meio da PCR e qPCR. Mesmo assim, considerando a facilidade de obtenção, o linfonodo pode ser considerada como a melhor amostra para diagnóstico molecular da infecção por *L. infantum chagasi.*

Palavras-chave: Leishmaniose, diagnóstico, molecular, *L. infantum chagasi*.

*__Corresponding author:__ Rafael Antonio do Nascimento Ramos
Laboratório de Doenças Parasitárias dos Animais Domésticos,
Programa de Pós-Graduação em Biociência Animal,
Universidade Federal Rural de Pernambuco – UFRPE,
Av. Dom Manoel de Medeiros, s/n, Dois Irmãos, CEP 52171-900, Recife, PE, Brasil
e-mail: rafaelanramos@yahoo.com.br

Introduction

Correctly diagnosing for canine visceral leishmaniasis (CVL) in dogs is a challenge in routine veterinary practice. Depending on the stage of the disease and immunological conditions, animals may be asymptomatic (TASCA et al., 2009), which makes the correct diagnosis more difficult. Parasitological techniques are used for diagnosing this disease (BARROUIN-MELO et al., 2006), but these methods have some limitations, such as, a low degree of sensitivity (PIARROUX et al., 1994).

In some parts of Brazil, where CVL is endemic, the diagnosis of this disease is done by serological tests, such as the indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA). However, false-negative results from asymptomatic dogs and cross-reactions with other organisms may occur. Culturing of *Leishmania* cells and xenodiagnosis, are also used. However, these techniques are time-consuming, has low sensitivity, and the latter is little used in routine diagnosis (PIARROUX et al., 1994).

Thus, alternative techniques are needed for diagnosing in dogs naturally infected by *Leishmania infantum chagasi*. A number of studies have proposed that the polymerase chain reaction (PCR) could be used for diagnosing of this disease (HU et al., 2000; LEONTIDES et al., 2002; OSHAGHI et al., 2009). Real-time PCR (qPCR) is also a good molecular tool for diagnosing CVL and makes it possible to determine the parasite load in different tissues (FRANCINO et al., 2006; ROLÃO et al., 2004; VITALE et al., 2004; WORTMANN et al., 2004). High sensitivity and specificity values for detection of protozoa have made molecular tools important for correctly diagnosing CVL. Moreover, these are fast, practical methods that can be used with different biological samples (IKONOMOPOULOS et al., 2003).

The aim of the present study was to determine the performance of PCR and qPCR assays for making molecular diagnoses of *L. infantum chagasi* infection using different biological samples.

Materials and Methods

1. Animals

A total of 35 dogs naturally infected by *L. infantum chagasi* were used. The animals were obtained from the Zoonotic Disease Control Center of the city of Petrolina (Brazil). The infection was confirmed by means of the indirect fluorescent antibody test (IFAT ≥ 40).

According to their clinical signs of leishmaniasis, the dogs were divided into three groups: polysymptomatic, i.e. more than three clinical signs; oligosymptomatic, one to three clinical signs; and asymptomatic, when they were not presenting any clinical sign.

The dogs were anesthetized with sodium thiopental (25 mg/kg) and euthanatized by means of an intravenous injection of 19.1% potassium chloride, at the Zoonotic Disease Control Center of the city of Petrolina.

2. Biological samples

Initially, bone marrow biopsy was performed, directly from the manubrium of the sternum. After the dogs had been put down, lymph node and spleen fragments were collected.

3. Molecular diagnosis

3.1 DNA extraction

Genomic DNA from blood and bone marrow was extracted using the QIAamp DNA Blood mini-kit (Qiagen), while genomic DNA from lymph nodes and the spleen was extracted using the QIAamp DNA mini-kit (Qiagen), following the manufacturer's instructions.

3.2 Polymerase chain reaction (PCR)

The reactions were performed in a final volume of 25 μL, containing 2.5 μL of PCR buffer: 20 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase (Invitrogen), 10 pmol of each primer and 100 ηg of genomic DNA. The amplification profile consisted of one cycle of denaturation at 94 °C for 5 minutes, annealing at 65 °C for 1 minute and extension at 72 °C for 1 minute, followed by 29 cycles of denaturation at 94 °C for 1 minute, annealing at 65 °C for 1 minute and extension at 72 °C for 1 minute, with a final extension at 72 °C for 5 minute.

The primers utilized (MC1 and MC2) were described by Cortes et al. (2004): MC1: (5' – GTT AGC CGA TGG TGG TCT TG – 3') and MC2: (5' – CAC CCA TTT TTC CGA TTT TG – 3'). By using these primers, amplification of a fragment of 447 base pairs was enabled.

The amplification products were viewed under ultraviolet light following electrophoresis on agarose gel (2%) stained with ethidium bromide.

3.3 Real-time PCR

Real-time PCR for detecting and quantifying kinetoplast minicircle DNA was performed using the primers LEISH-1 (5'AACTTTTCTGGTCCTCCGGGTAG-3') and LEISH-2 (5'-ACCCCCAGTTTCCCGCC-3'), and the TaqMan-MGB probe (FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent quencher-MGB), as described by Francino et al. (2006).

The reaction mixture (12.5 μL) contained 6.25 μL of Taqman® Universal PCR Master Mix, each primer at a concentration of 900 nM, the probe at a concentration of 200 nM and 50 ηg of template DNA.

The run consisted of a hot start at 95 °C for 3 minutes and 42 cycles of denaturation (95 °C for 10 seconds) and annealing-extension (60 °C for 30 seconds). All assays were carried out in triplicate, with a negative control (DNA from a dog from a non-endemic area) and a positive control included in each run.

4. Statistical analysis

Differences between the frequencies of positive results were evaluated using Fisher's exact test and the chi-square test, with a significance level of 5%.

Results

1. Clinical evaluation

Out of the 35 dogs studied, 14.28% (5/35) were classified as asymptomatic, 68.87% (24/35) as oligosymptomatic and 17.14% (6/35) as polysymptomatic for canine visceral leishmaniasis. The clinical signs most commonly present were: dermatopathy, weight loss and onychogryphosis.

2. PCR and qPCR

From the PCR tests, DNA of *L. infantum chagasi* was detected in 40% (14/35) of the animals. In these animals, 42.85% (6/14) were positive in only one biological sample and 57.15% (8/14) were positive in three analyzed samples.

Agarose gel electrophoresis revealed amplification of a 447 bp fragment of *L. infantum chagasi* in positive reactions. On the other hand, no amplification was observed in negative controls.

From the qPCR tests, DNA of *L. infantum chagasi* was detected in 100% of the animals. In these animals, 2.85% (1/35) were positive in only one sample, 2.85% (1/35) were positive in two samples and 94.30% (33/35) were positive in three analyzed samples. No amplification was observed in negative controls.

The PCR and qPCR results from each biological sample are described in Table 1.

No significant differences were observed between different biological samples according to each clinical group ($P > 0.05$).

Discussion

The clinical signs found in the animals in the present study were compatible with findings described in cases of CVL, and the most frequent of these were dermatopathy, cachexia and onychogryphosis.

Although the samples utilized were from euthanized dogs, these samples can also be obtained from live animals, especially samples from bone marrow and lymph node fragments. One of the drawbacks regarding the use of spleen fragments in this type of diagnosis relates to collection of the material, because of the possibility of hemorrhage (LÉVEILLÉ et al., 1993). However, this risk is considerably lowered with the aid of abdominal ultrasound (WATSON et al., 2011).

The sensitivity of PCR analysis can vary widely depending on the time of infection. Sensitivity is considered to be null immediately following infection and can reach values of 78 to 88%, 135 days after infection (QUINNEL et al., 2009). Moreover, the parasite load also affects the sensitivity of this technique, which, despite being capable of detecting small amounts of DNA, has its limitations.

Regarding the different biological samples analyzed using PCR, lymph node fragments proved to be the best option for detecting DNA from the protozoan, and these samples detected positive animals in all three clinical groups (A, O, P). According to Ikonomopoulus et al. (2003), lymph nodes are considered to be the preferred organ for making molecular diagnoses of *L. infantum chagasi* using PCR. In contrast, using bone marrow and spleen fragments, positive animals were only detected in Groups O and P. This difference in results may be related to the heterogeneous distribution of the parasites in each tissue, as well as to the parasite load and local immune response (MAIA et al., 2009).

Because of the limitations of PCR, *q*PCR has proven increasingly useful in detecting and quantifying protozoa, including *L. infantum chagasi* (ROLÃO et al., 2004). In the present study, qPCR was able to detect the presence of *L. infantum chagasi* DNA in all animals studied. From analysis on the different samples, high positivity values were found in the different clinical groups, with relative frequencies ranging from 83.3 to 100%. The samples from the spleen and lymph nodes achieved 95.8% positivity in Group O and 100% in Groups A and P. Considering ease of acquisition, lymph nodes once again proved to be the most viable organ for detecting *L. infantum chagasi* DNA in dogs.

Molecular diagnosis using qPCR proved reliable in detecting *L. infantum chagasi* DNA, regardless of the clinical state of the animal. This finding differs from what has been achieved with parasitological and serological methods, for which positivity values are greater in symptomatic animals (QUARESMA et al., 2009). Diagnosing asymptomatic animals is of considerable importance, since such animals constitute a reservoir for *L. infantum chagasi* (DANTAS-TORRES, 2006).

A large number of studies have reported that qPCR has greater sensitivity than PCR (FRANCINO et al., 2006). However, this is not always the case, since the variation depends on the primers used and the type of DNA to be amplified (BASTIEN et al., 2008). The sensitivity achieved in the present study may be related to the target DNA amplified, since detection of kinetoplast DNA is normally more sensitive due to the large number of copies per

Table 1. PCR and qPCR results from different biological samples from animals in different clinical groups: asymptomatic (A), oligosymptomatic (O) and polysymptomatic (P).

Clinical groups	Number of animals	PCR (%)			qPCR (%)		
		Bone marrow	Spleen	Lymph node	Bone marrow	Spleen	Lymph node
A	5	0 (00.0)	0 (00.0)	1 (20.0)	5 (100.0)	5 (100.0)	5 (100.0)
O	24	5 (20.8)	6 (25.0)	6 (25.0)	24 (100.0)	23 (95.8)	23 (95.8)
P	6	4 (66.6)	4 (66.6)	4 (66.6)	5 (83.3)	6 (100.0)	6 (100.0)

($P > 0.05$).

parasite (LACHAUD et al., 2002), in comparison with other target sequences, such as ribosomal DNA.

Studies addressing diagnostic methods for CVL are necessary in order to determine the best way to identify this infection in dogs in both endemic and non-endemic areas. Controlling CVL is particularly related to using diagnostic methods that provide reliable results, so that positive animals can be removed, thereby contributing towards non-propagation of the disease. The results from the present study demonstrate the importance of qPCR for diagnosing CVL: this test was able to detect positive animals regardless of the clinical state.

Conclusions

In the present study, qPCR was better diagnostic tool than PCR for detecting *L. infantum chagasi* infection. Among the biological samples analyzed, lymph nodes proved to be the most appropriate means of detecting *L. infantum chagasi* DNA using PCR and qPCR, since it was capable of detecting positive animals regardless of their clinical state. The ease of acquiring lymph node samples further demonstrates the greater viability of this biological material over spleen fragments for routinely diagnosing *L. infantum chagasi* infection.

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