Detection of *Leishmania spp.* in canine serum fixed in card

Detecção de *Leishmania spp.* em soro de caninos fixados em cartões

Detección de *Leishmania spp.* en suero de caninos fijados en tarjetas

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Abstract
Visceral leishmaniasis is a serious zoonosis, and its molecular diagnosis is an important strategy. The detection of *Leishmania spp.* DNA from biological samples fixed in cards could solve problems related to the collect and transport of biological samples. The purpose of this study was to detect *Leishmania spp.* by PCR, using canine serum fixed in cards (CSFC). For evaluation, the PCR with DNA extracted from CSFC (PCR/card) was compared to the PCR with DNA from the same serum extracted using the Qiagen kit (PCR/Qiagen) and the immunochromatography test (Rapid test - RT). The results showed that out of the 112 analyzed samples, 12 (10.71%) were positive in the PCR/Qiagen, and of those, only two failed to amplify with DNA extracted from the CSFC. The RT was positive in 11 (9.8%) samples; however, only 3 (2.75%) samples of the total agreed with the detection of *Leishmania spp.* through PCR. Considering the PCR/Qiagen test as the reference standard for DNA detection, the agreement with the PCR/card was nearly perfect (K = 0.8). The concentration and purity of DNA in the two extractions were not significantly different. The use of PCR with DNA from CSFC showed that it can be an interesting alternative for the diagnosis of leishmaniasis.
Keywords: FTA ELUTE CARDS®; Molecular diagnosis; Leishmaniasis; Leishmania.

Resumo
A leishmaniose visceral é uma zoonose grave, e o diagnóstico molecular é uma estratégia importante. A detecção de DNA de *Leishmania spp.* a partir de amostras biológicas fixadas em cartões comerciais pode resolver problemas relacionados à coleta e transporte de amostras biológicas. O objetivo do estudo foi detectar *Leishmania spp.* por PCR utilizando DNA de soro canino fixado cartão (SCFC). Para avaliação, o PCR com DNA extraído do SCFC (PCR/cartão) foi comparado com o PCR com DNA extraído com kit Qiagen (PCR/Qiagen) do mesmo soro e com teste de imunocromatografia. Os resultados mostraram que, de 112 amostras analisadas, 12 (10,71%) foram positivas quando o DNA era extraído com Kit Qiagen e dessas, apenas duas não amplificaram com DNAs extraído do SCFC. A imunocromatografia (teste rápido) foi positiva em 12 (10,71%) das amostras. Porém, apenas três (2,75%) do total de amostras concordaram com a detecção de *Leishmania spp.* por PCR. Considerando o teste da PCR/Qiagen como um padrão de referência para detecção de DNA, a concordância com o PCR/cartão foi quase perfeita (K=0,8). A concentração e o grau de pureza de DNA nas duas extrações não tiveram diferença significativa. O PCR a partir de SCFC mostrou pode ser uma alternativa interessante para o diagnóstico de leishmaniose.
Palavras-chave: FTA ELUTE CARDS®; Diagnóstico molecular; Leishmaniose.

Resumen

La leishmaniasis visceral es una zoonosis grave y el diagnóstico molecular es una estrategia importante. La detección de Leishmania spp. a partir de muestras biológicas fijadas en tarjetas comerciales puede resolver problemas relacionados con la recogida y transporte de muestras biológicas. El objetivo del estudio fue detectar Leishmania spp. por PCR utilizando ADN de suero canino fijado en tarjeta (SCFC). Para la evaluación, se comparó PCR con ADN extraído de SCFC (PCR/tarjeta) con PCR con ADN extraído con kit Qiagen (PCR/Qiagen) del mismo suero y con prueba inmunocromatográfica. Los resultados mostraron que, de 112 muestras analizadas, 12 (10,71%) resultaron positivas cuando se extrajo el ADN con el Qiagen Kit y de estas, solo dos no amplificaron con los ADN extraídos de SCFC. La inmunocromatográfica (test rápido) fue positiva en 12 (10,71%) de las muestras. Sin embargo, solo tres (2,75%) del total de muestras coincidieron con la detección de Leishmania spp. por PCR. Considerando la prueba PCR/Qiagen como estándar de referencia para la detección de ADN, la concordancia con la PCR/tarjeta fue casi perfecta (K=0,8). La concentración y el grado de pureza del ADN en las dos extracciones no difirieron significativamente. La PCR de SCFC mostró que puede ser una alternativa interesante para el diagnóstico de leishmaniasis.

Palabras clave: FTA ELUTE CARDS®; Diagnóstico molecular; Leishmaniasis.

1. Introduction

Visceral Leishmaniosis (VL) is a chronic systemic infectious-parasitic zoonosis caused by the protozoan *Leishmania spp.*, which contains the *L. donovani*, *L. chagasi*, and *L. infantum* species, which infect cells of the mononuclear phagocytic system of human beings, and domestic (dog and cat) and wild animals (Millán & Solano 2014; Maia-Elkhouri et al., 2019). The dogs, due to their proximity to humans, are considered parasite reservoirs (Brazil, 2014).

In 2019, 97% of the cases related to VL occurred in 10 countries (Brazil, Argentina, Bolivia, Colombia, Guatemala, Honduras, Mexico, Paraguay, Venezuela, and Uruguay) (Organização Pan-Americana, 2020). In Brazil, in the state of Santa Catarina (from 2010 to 2020), a region that until recently was considered of low risk for VL, 1,042 cases of canine visceral leishmaniosis (CVL) were reported (State Health Department of Santa Catarina, 2020), (Brazil, 2014).

CVL is classified according to its clinical presentation, with the period of incubation varying from three months to many years. As the relationship between the canine infection and VL has already been well-defined, the detection of infected dogs is essential in controlling the disease. The Brazilian Ministry of Health (MH) advocates diagnostic methods that are similar for the diagnosis of leishmaniosis in both humans and animals (Brazil, 2014).

The parasitological diagnosis is considered a definitive method due to its high specificity, given that it is possible to view and isolate the parasite in a culture medium. However, the disadvantage is the low sensitivity resulting from the variable parasite load and the distribution of the parasite in tissues (Antunes et al., 2018). The immunodiagnostic methods are the most used, such as immunochromatography, known as the rapid test (RT), which detects antibodies against *Leishmania* in only a few minutes. Nonetheless, it is impossible to distinguish between infected animals and those that had a spontaneous resolution of the infection or those that were immunized (Assis et al. 2018). The ELISA and the RIFA (indirect immunofluorescence) methods are confirmatory tests (Quinnel & Courtenay, 2009), but they also vary regarding the sensitivity and specificity (Tlamcani, 2016). Specific factors, such as a window period, antibody concentration, and crossed reaction, favor false positive and false negative results, which can contribute to the maintenance of the reservoirs (Souza et al., 2013, Paiva-Cavalcanti et al., 2015; Riboldi et al., 2018).

The limitations of the diagnostic methodologies in relation to the sensitivity and specificity reinforce the need to search for more precise methods to confirm CVL. The molecular methods, such as the polymerase chain reaction (PCR), can aid in resolving problems, as it detects the parasite directly through its DNA in biological samples, including blood, serum, plasma, biopsies, and lymph nodes (Almeida et al., 2013; Rolim et al., 2016; Silva et al., 2017; Riboldi et al., 2018). However, the difficulties faced with the transport and storage of samples, in addition to the laboratory structure, have been limiting the use of PCR (Palido-Landínez et al., 2012; Rolim et al., 2016; Gonzáles-Marcano et al., 2016) because of the need to send biological samples to the reference centers to carry out the molecular methodologies.

Samples fixed in FTA ELUTE CARDS® represent an alternative to obtaining and transporting DNA. As the cards contain chelating and denaturant agents in their matrix, the DNA remains attached even after cellular lysis (Lipic & Fredericks, 2018), which also does away with refrigeration, in turn reducing the costs to send samples (Palido-Landínez et al., 2012; Gonzáles-Marcano et al., 2016). Moreover, the extraction of the genetic material attached to the fibrous matrix of FTA Elute Cards occurs in a simple manner, with the use of only distilled water and heat (Rolim et al., 2016).

In this context, the use of PCR techniques by means of samples fixed in commercial cards can help to improve the diagnosis of leishmaniosis, even in regions that are more distant and that have a poor infrastructure. Therefore, the present study aimed to evaluate the detection of *Leishmania spp.* through PCR by means of DNA extraction directly from canine serum fixed in card (CSFC) and compare this result with the PCR performed with DNA extracted using a commercial kit with the same serum. These results were also compared with the rapid test.

2. Methodology

2.1 Samples

This study used 112 canine serum samples (two aliquots of each), received from the Zoonosis Control Center, Municipal Health Department, Florianópolis, Santa Catarina (CCZ/SC).

This project was approved by the Ethics Committee on the Use of Animals (CEUA/UNISUL), logged under protocol number 16.050.2.13.1V on December 12, 2016.

2.2 Immunochromatography or Rapid test (RT)

The detection of antibodies was performed using the Bio-Manguinhos CVL Rapid test (TR DPP®) (Fiocruz, Rio de Janeiro, Brazil). The procedures followed the instructions established by the manufacturer.

2.3 DNA extraction using a commercial kit

The DNA of 200 μL of serum was isolated, using the QiAamp DNA commercial mini-kit (Qiagen™), following manufacturer recommendations, which included cellular lysis with proteinase K to release the DNA and silica resins for purification.

2.4 DNA extraction through canine serum fixed in card (CSFC)

In this study, 200 μL of serum was added to FTA ELUTE CARDS® (Whatman) and dried at room temperature for at least 4 hours (Figura 1A). The sample was stored in a location that was dry and without humidity. The DNA extraction was performed with two discs of 6 mm in diameter removed from the FTA ELUTE CARDS® using a puncher (Figure 1B). After washing and diluting the discs in sterile water and a dry bath of 95°C for 15 minutes, according to manufacturer recommendations, the discs were removed. The extracted DNA was stored at -20°C until use.

Figure 1 - Photograph of procedure of fixing sample to FTA ELUTE CARDS® (A) and after removing two discs of 6 mm to DNA extraction using a puncher (B).
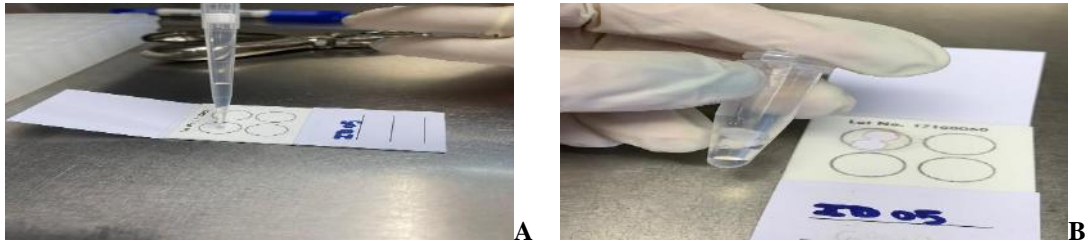
Source: Authors.

2.5 Quantity and quality analysis of DNA

The DNA extracted (2 µL) using two protocols was analyzed as regards the concentration and degree of purity, using the Eppendorf BioSpectrometer® spectrophotometer.

2.6 PCR amplification

The amplification by conventional PCR occurred by means of initial denaturation at 95°C for 15 min, followed by 48 cycles of 95°C for 15 s, 57°C for 30 s, 72°C for 30 s, and the final extension at 72°C for 7 min. LS-Fw (AGCTGGATCATTTTCCGATG) and LS-Rev (TCGCACTTTACTGCGTTCTT) primers were used, as described by Sagi et al. (2018), which amplified a region of 328 base pairs of *Leishmania spp*. The reaction of the amplification (mix) of the DNA occurred in a final volume of 25 µL, containing a mixture of 1 µL (50 Mm) of $MgCl_2$, 2.5 µL (2.5 mM) of dNTP, 0,5 µL (20 pmol/µL) of each primer, 10 µL of sample, and 0.25 U of the polymerase taq DNA enzyme. The PCR result was analyzed by 2% agarose gel electrophoresis, containing 0.05% of ethidium bromide and viewed under ultraviolet light. In each reaction, a positive control (DNA de *Leishmania spp.*) and a negative control (ultrapure water) were used.

To verify the presence of inhibitors, all of the negative samples were amplified with β-globin primers after adding human DNA (Saiki et al. 1985).

2.7 Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS), version 21.0, and Microsoft Excel 2016. The evaluation of agreements among the tests for the detection of the DNA of the protozoan *Leishmania spp.* and the immunochromatography method was carried out by calculating the Kappa index, with the following interpretation: K=<0 (absence of agreement), K=0-0.019 (poor agreement), K=0.20-0.39 (mild agreement), K=0.40-0.59 (moderate agreement), K=0.60-0.79 (substantial agreement), and K=0.80-1.00 (nearly perfect agreement) (Silva et al. 2012).

3. Results

Of a total of 112 canine serum samples analyzed by PCR with DNA extraction by the Qiagen kit (PCR/Qiagen) and with extraction of CSFC (PCR/card). It was possible to perform the amplification of the *Leishmania* DNA in 12 (10.71%) samples, using the PCR/Qiagen, and in 10 (8.92%) samples, using the PCR/card. The RT was positive in 10.71% (12/112) of the samples. However, agreement was only found in only three (2.72%) samples (5, 7, and 10) of the total (Table 1). Amplification was not verified in the PCR when using the β-globin primers, performed with negative samples.

Table 1 - Results of the positive samples by PCR and immunochromatography.

Samples	PCR/Qiagen	PCR/card	Immunochromotography
1	-	-	+
2	-	-	+
3	-	-	+
4	-	-	+
5	+	+	+
6	+	-	-
7	+	+	+
8	+	+	-
9	+	+	-
10	+	+	+
11	+	-	-
12	-	-	+
13	-	-	+
14	+	+	-
15	-	-	+
16	+	+	-
17	-	-	+
18	+	+	-
19	+	+	-
20	+	+	-

Source: Authors.

One comparison of the PCR analyses for the detection of the DNA of *Leishmania spp.* after having conducted the two procedures of DNA extraction, considering the PCR/Qiagen test as a reference standard for DNA detection. When the DNA was detected with the PCR/card, the sensitivity and specificity were 85% and 100%, respectively.

When analyzing the agreement of the results from the Kappa index methods, a nearly perfect agreement was found (K=0.8) for PCR/Qiagen versus PCR/card, as compared to a poor agreement (K=0.1) when both DNA extraction techniques were compared using the RT.

The average DNA concentrations of canine serum proved to be similar in both methods; however, the DNA obtained by Qiagen extractions was greater than that of CSFC (Table 2).

Table 2 - Results obtained through average values of DNA concentrations from the extractions performed with canine serum.

Extractions	A260/280 (≥1.8)	DNA ng/µL	Intensity of amplification in agarose gel electrophoresis
PCR/Qiagen	1.39±0,35	43.1±18.08	+
PCR/card	1.24±0,16	42.5±25.18	+
p-value	0.364	0.708	

Source: Authors.

4. Discussion

VL is a zoonosis disease that must be notified to the proper authorities, and infected dogs play a major role in the maintenance of this epidemiology. Although the immunological techniques are the most used in the diagnosis of CVL, the difficulties to detect infected dogs, primarily asymptomatic dogs, makes the diagnosis especially difficult (Carvalho et al., 2018).

The PCR exam, despite the good results that have been reported, is still not consolidated in the diagnosis of CVL (Riboldi et al., 2018; de Brito et al., 2020). The difficulties include the collection and transport of the sample to the adequate infrastructure to conduct such techniques as the PCR exam, since combatting the disease, in most cases, occurs in rural zones

that are distant from the reference centers (Teixeira et al., 2016). The present work analyzes the obtaining of DNA through serum fixed in cards, comparing the DNA obtained by commercial kits (reference for DNA extraction) with the aim of facilitating the use of samples in molecular techniques.

The results show that the method chosen as the reference (Qiagen) for DNA extraction was what truly made it possible to detect the DNA of *Leishmania spp.* (12/112) using PCR. However, the DNA extractions by means of CSFC enabled the amplification of 83% of these samples (10/12), that is, two samples were not amplified by the PCR/card. The obtained results can be explained by the fact that, in the FTA ELUTE CARDS®, the DNA cannot be distributed in a uniform manner by the card in which the sample was fixed to the matrix (Cox et al., 2010), which can result in a false negative result. The samples fixed in cards have been described for the diagnosis of many diseases, including leishmaniosis, as an interesting alternative to obtain the DNA, facility in transport, in addition to the biological safety, since the fixed cells are completely lysed (Bankamp et al. 2019; Ali et al.,2020; Mota et al., 2020).

This study's results were also interesting when compared to the RT results. Of the 12 positive results in the RT, only three (25%) showed an amplification of DNA (PCR/Qiagen and PCR/card). The false negative result in the PCR for the other eight positive samples in the RT would be one possibility. However, the low parasite load was not evident, considering that the average DNA concentration in these samples was similar or higher than that of the positive samples. The presence of PCR inhibitors after extraction was also discarded due to the negative result of the inhibition test. Therefore, it is possible that these positive results may well be due to the presence of antibodies in animals with the spontaneous resolution and/or crossed reactions with other parasites. In relation to the nine negative samples in the RT, but with positive PCR, it is possible that this occurred due to a low sensitivity of the immunological tests, which has been well documented in other studies (Santos et al., 2010; Ribeiro et al., 2018; Riboldi et al., 2018).

The RT is a methodology recommended by the public health system in Brazil and is often used in the field and in veterinarian clinics to diagnose CVL, due mainly to its easy execution and quick results (Brazil, 2014). Nevertheless, the present study's findings highlight that despite the importance that this test has of screening in the handling of leishmaniosis, a detailed evaluation of its application is necessary, thus maintaining the confirmatory tests for the positive results and attention in the cases of negative results due to low sensitivity (Farahmand & Nahrevanian, 2016).

Another important point in DNA extraction for the molecular diagnosis to be successful is the possibility of obtaining a high-quality DNA (purity) and in adequate concentrations. The differences found between the methods in both parameters were not statistically significant. The fact that the quality of the DNA obtained by the FTA Elute Card is similar to the DNA extracted using the Qiagen kit, which is widely used and considered to be a reference, indicates that the card can be an interesting alternative to conduct DNA extraction (Lalani et al., 2018).

With a nearly perfect agreement among the methods (K=0.8), this strategy to collect, transport, and extract DNA from CSFC can be a viable alternative for the regions affected by CVL that have limited infrastructure and lack financial resources, which could confirm the screening tests through PCR (Mabayi & Ospina, 2018), thus contributing to the control of the disease.

5. Conclusion

Molecular diagnosis is important to detect infections caused by *Leishmania* and can contribute to the control of leishmaniosis. PCR using DNA from CSFC proved to be an interesting alternative, since it makes it possible to obtain DNA in adequate conditions and purity levels, much like that extracted from Qiagen kits, in turn facilitating the transport of samples for molecular tests.

Acknowledgements

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