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PARASITOLOGY

In-house serological ELISA as a leishmaniosis diagnostic test: development and applications in canines from the western border of Brazil

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ABSTRACT: *Leishmaniosis is a great public health problem affecting both humans and animals. The disease is caused by the protozoan Leishmania spp., which has a complex cycle involving a phlebotomine vector. The ELISA test (Enzyme-Linked Immunosorbent Assay) along with a chromatographic immunoassay was defined by the Brazil Health Ministry as the confirmatory screening protocol in 2011. Uruguaiana city is 630 km away from Porto Alegre, which makes it difficult to send samples and diagnose leishmaniasis, as well as receive quick results. In view of this, the present study evaluated an in-house indirect ELISA method compared to indirect immunofluorescence assay (IFA) and dual-path platform chromatographic immunoassay (DPP-BioManguinhos®) for the detection of an immune response to Leishmania spp. in canine species. The serological evaluation included 48 canines from the western border of Brazil (Uruguaiana and Barra do Quaraí city). Among the 48 canine samples tested, 18 were positive when using the ELISA technique, 19 were positive with IFA, and 17 were positive with rapid test DPP®. The ELISA technique showed a sensitivity/specificity of 83.3%/86.7% when compared to IFA and 100%/96.8% compared to DPP®. The present study showed a prevalence of 37.5%, demonstrating that the infection circulates in the studied population. It can be concluded that the ELISA technique was valuable for use in field conditions when performing screening tests in endemic areas.*
Key words: *Leishmania sp., diagnostic, public health.*

ELISA "in house" para o diagnóstico de leishmaniose: desenvolvimento e aplicação em caninos da fronteira Oeste do Brasil

RESUMO: *A leishmaniose é um grande problema de saúde pública que afeta tanto humanos quanto animais. A doença é causada pelo protozoário Leishmania spp., que possui um ciclo complexo envolvendo um vetor flebotomíneo. O teste ELISA (Enzyme-Linked Immunosorbent Assay), juntamente com um imunoensaio cromatográfico, foi definido pelo Ministério da Saúde do Brasil como protocolo de triagem confirmatória em 2011. A cidade de Uruguaiana fica a 630 km de Porto Alegre, o que dificulta o envio de amostras e o diagnóstico da leishmaniose, além da demora para obter os resultados. Diante disso, o presente estudo teve como objetivo avaliar um método de ELISA indireto in-house comparado ao ensaio de imunofluorescência indireta (IFA) e imunoensaio cromatográfico de plataforma de dupla via (DPP-BioManguinhos®) para a detecção de uma resposta imune contra Leishmania spp. na espécie canina. A avaliação sorológica incluiu 48 caninos da fronteira oeste do Brasil (cidade de Uruguaiana e Barra do Quaraí). Das 48 amostras caninas testadas, 18 foram positivas na técnica de ELISA, 19 foram positivas com IFA e 17 foram positivas com o teste rápido DPP®. A técnica de ELISA apresentou sensibilidade/especificidade de 83,3%/86,7% quando comparada ao IFA e 100%/96,8% em relação ao DPP®. O presente estudo apresentou prevalência de 37,5%, demonstrando que a infecção circula na população estudada. Pode-se concluir que a técnica de ELISA foi valiosa para uso em condições de campo na realização de testes de triagem em áreas endêmicas.*
Palavras-chave: *Leishmania sp., diagnóstico, saúde pública.*

INTRODUCTION

Leishmaniosis is a zoonotic disease that is at the top of the list of globally neglected diseases. Annually, 12 million people are infected, and 20,000 to 30,000 die from it worldwide. Most cases in 2017 occurred in seven countries, including Brazil (World Health Organization [WHO], 2018). The domestic dog (*Canis familiaris*) is the main reservoir of different protozoan species, playing an essential role in its maintenance. Its participation in the zoonotic transmission of *Leishmania infantum* has been well defined (DANTAS-TORRES, et al., 2019).

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Leishmaniosis has been disseminating and establishing itself in different ecosystems in South America, which is why it is considered an emerging infection in the outer south region of Brazil (Pan American Health Organization [PAHO]; World Health Organization [WHO], 2019). The first reported case of canine visceral leishmaniosis in Argentina-Brazil's border region was in the São Borja municipality in 2008 (SOUZA et al., 2009b). Prior to this, the Health State Secretary was notified of a human visceral leishmaniosis case. Uruguaiana city registered the first human case in 2011 (Centro Estadual De Vigilância Em Saúde [CEVS], 2017). Municipal health departments are responsible for investigating cases of suspected canine visceral leishmaniosis. These investigations are based on serological tests carried out using dual-path platform (DPP®) chromatographic immunoassays. Positive cases are sent to the Central Public Health Laboratory of the state of Rio Grande do Sul (LACEN), located in the state capital, Porto Alegre, to be tested using the confirmatory method, Enzyme-Linked Immunosorbent Assay (ELISA) (Government Of The State Of Rio Grande Do Sul, 2014). The estimated time for the result is 20 working days (LACEN/ CEVS/SES-RS, 2020). During 2010 to 2019, 2273 suspected dogs were surveilled, and the infection was present in at least 1478 (FARIAS et al., 2020).

As the confirmatory ELISA test is typically performed at the LACEN in Porto Alegre, it is difficult to quickly confirm cases, and diagnoses and infection control measures are delayed (DANTAS-TORRES et al., 2019).

In 2006, the Federal University of Pampa began promoting local development and enabled the implementation of different areas of research, thereby assisting local and regional research societies (ESCOBAR et al., 2020; ESCOBAR et al., 2018; PRADELLA et al., 2020), such that in the present study.

In order to carry out epidemiological surveys in the region and speed up diagnosis, this study's objective was to evaluate an in-house indirect ELISA *Leishmaniasis* detection method on canine samples from the western border of Rio Grande do Sul, Brazil. This was done using crude *L. infantum* antigens, and the method was compared with an indirect immunofluorescence assay (IFA) and a rapid test dual-path platform chromatographic immunoassay (DPP-BioManguinhos®) of canine samples.

MATERIALS AND METHODS

Leishmania infantum cell culture

The *L. infantum* (2906) strain was made available from FRIOCRUZ/RJ, and the culture was maintained and cultivated according to their manual, with modifications (MINISTRY OF HEALTH et al., 2018). The species was chosen by taking into account the prevalence of the disease in the region of the study (ESCOBAR et al., 2020; PRADELLA et al., 2020).

Antigen preparation

The antigen preparation was done according to established methods, with slight modifications (SOARES, 2012). Solid medium (Blood BHI) was aliquoted to cell culture flasks (T-25 cm^2). The liquid phase (Schneider) was added, and the medium was stabilized at 25 °C in the BOD (biological oxygen demand) incubator. The *Leishmania* strains (*L. infantum* 2906) were transferred from the culture tubes to a bottle, and their viability was evaluated using an optical microscope (Zeizz® primo star). Incubation was performed for 72 hours until the cells reached the stationary phase. After this period, the liquid phase was transferred to a 15 mL tube. The sample was evaluated using an optical microscope to determine the cell viability and the absence of contamination. The liquid phase was centrifuged at 2885 xg 4 °C for 10 minutes, and three washes with saline phosphate buffer (PBS) were performed to remove the residues from the medium. The cells were inactivated by thermal shock using a water bath at 60 °C (10 minutes) and liquid nitrogen (10 minutes). The procedure was performed twice. Subsequently, microscopic inspection was performed to ensure that there were no viable cells and the material obtained was submitted to protein quantification by the Bradford method (LABORATORIES, 2017).

Antigen concentration and coating on ELISA plates

The antigen was diluted in coating buffer (bicarbonate carbonate pH 9.6). The concentrations used in the tests were 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL. The antigen was added to ELISA plates at 100 µL/well. Subsequently, the plates were packed with plastic film and incubated overnight at 4 °C to fix the antigen. After this, the plates were washed with washing solution (0.05% polysorbate/Tween 20 and 0.9% sodium chloride [NaCl] in distilled water) four times in the microplate washer. The methodology was conducted according to previous studies, with modifications (SOARES, 2012).

Blocking

The blocking was performed differently by testing the best solution, volume, and blocking time (Table 1). After the blocking, the plate was washed twice with a solution in a microplate washer. Samples

were packed in aluminum foil and stored at -20 °C until use.

Between the blocking tests applied, the most effective was 300 µL/well of 1% nonfat dried milk (Molico®); and subsequent incubation at 37 °C for 1 hour. Results were similar with casein 2% and 5%; however, nonfat dried milk resulted in more homogeneous solutions.

Biological samples

Biological sample collection was carried out in accordance with animal use guidelines. The biological samples tested are part of the PPSUS project (Research Program for the Health Unic System: Shared Health Management). A total of 48 canines were collected from the urban and rural areas of the municipalities of Uruguaiana/RS and Barra do Quaraí/RS. Blood samples were collected by puncture of the external jugular or cephalic veins and stored in glass tubes with EDTA and without anticoagulants. The blood was centrifuged at 406 xg for 20 minutes. The serum was stored in 2 mL microtubules and frozen at -20 ºC.

The 1st antibody dilution tests included 1/100, 1/400, and 1/800 dilutions, according to previously studies (REITHINGER et al., 2002). All whole blood serum samples were submitted to a rapid test dual-path platform chromatographic immunoassay (DPP-BioManguinhos®; Oswaldo Cruz foundation) for canine visceral leishmaniosis, according to the manufacturer's instructions.

The positive controls were serum of three positive animals (immunochromatography and PCR) from endemic areas. The negative controls were two dogs from non-endemic regions tested using immunochromatographic methods and PCR. The blank controls for the primary and secondary antibodies were included to identify the presence of an unspecific reaction.

Cross check

After the primary tests, the following conditions were applied for the in-house ELISA: antigen concentration, 10 µg/mL; blocking conditions, 300 µL/well of 1% nonfat dried milk (Molico®), and incubation at 37 °C for 1 hour; and 1st antibody dilution, 1/800. The best conditions were defined based on the greatest differentiation results from the controls (positive, negative, and blank).

ELISA (Enzyme-Linked Immunosorbent Assay): indirect assay

Initially, the first antibody dilution (serum of the animal to be tested) was diluted in phosphate-saline dilution buffer (PBS) supplemented with 0.05% polysorbate 20 (PBS Tween) and 0.25% casein, at a dilution of 1/800, as previously defined. Next, 100 µL/well of the diluted sample was added to the plate with the desired concentration. The plate was gently shaken and incubated for 45 minutes at 37 ºC (Thermo-Shaker Agimaxx®). After that, washing in a microplate washer (Wellwash, Thermo Fisher Scientific®) with PBS tween was performed using four cycles. Next, we added 100 µL/well of the 2º antibody (species specific (Goat Anti-Dog IgG H&L [HRP] ab112852) in PBS Tween). The concentration used was 1/10000. The plate was again gently stirred and incubated for 45 minutes at 37 ºC.

Table 1 - Blocking techniques used for antigen fixation, including solutions, volume/well, temperatures and times tested.

Blocking solution	Volume/well	Temperature (°C)	Time (hours)
Casein 2%	150 µL	37	0.5
Casein 5%	150 µL	37	0.5
NFDM* 1%	150 µL	37	0.5
NFDM*10%	150 µL	37	0.5
NFDM* 1%	300 µL	37	0.5
Inactivate serum 2%	300 µL	37	0.5
NFDM* 1%	300 µL	37	1
Inactivate serum 2%	300 µL	37	1
Bovine albumin 2%	300 µL	37	1.5
NFDM*1%	300 µL	37	1.5

*NFDM – Nonfat Dried Milk (Molico®, Nestle)

The last wash was performed in a microplate washer with PBS tween using four cycles, and 100 µL/well of the substrate solution (Tetramethylbenzidine, TMB) was added and incubated for 15 minutes in the dark. The reaction was finalized with the addition of 25 µL/well of the stop solution (sulfuric acid 2M). Finally, readings were performed according to the manufacturer's instructions using the development solution (TMB) with an ELISA reader (Multiskan FC- Thermo Fisher Scientific®) at 450 nm.

Cut-off determination

The ideal conditions for the immunodiagnostic assay's performance were obtained from the mean absorbance of the positive and negative controls (signal-to-noise). The cut-off was determined using the mean absorbance of the negative controls plus three times the standard deviation of the negative controls (RAJASEKARIAH et al., 2001). Samples were established as negative and positive samples if the absorbance value was 10% below or above the cut-off, respectively. Samples with values within the cut-off determination were considered suspected cases.

Indirect immunofluorescence assay (IFA)

Multispot slides were coated with promastigote forms of *L. infantum* then fixed and permeabilized with cold acetone (100%). Serum samples were diluted in PBS at 1:40 (Department Of Health Surveillance Department Of Epidemiological Surveillance, 2006) and positive and negative serum samples were used as controls. Commercial fluorescein-labeled anti-Dog IgG© (Rabbit Anti-Dog IgG FITC®, F7884, Sigma-Aldrich, San Luis, Missouri, USA) was used as the secondary antibody. Slides were observed at 400x magnification under a fluorescence microscope (Optiphase® INV403F). A titer of 1:40 was considered positive (HEALTH, 2014).

Statistical analysis

The different diagnostic methods for visceral canine leishmaniosis were compared using the Kappa index and through sensitivity/specificity determination using the IBM SPSS statistics program.

RESULTS

The protocol used for the *L. infantum* cell culture and antigen preparation enabled the detection of a serological response against *Leishmania sp.* in dogs.

ELISA standardization

Through the different antigen concentrations used for the ELISA (5 µg/mL; 10 µg/mL; 20 µg/mL, and 40 µg/mL) and by observing the controls, 10 µg/mL had the best differentiation between the negative and positive controls (signal to noise value, 7.27; cut off value, 0.749; negative control mean, 0.232; positive control mean, 1.688). In addition, the blank control showed a low OD count (0.040–0.100) compared to the other plates. For the first antibody dilution (dog serum), a 1/800 dilution demonstrated the best results. Furthermore, it was possible to visualize the importance of casein in the dilution by observing the blank controls. Additionally, the best 2º antibody dilution for the canine species was determined to be 1/10,000.

The agreement between the different diagnostic methods for visceral canine leishmaniosis, including the DPP® test and ELISA, showed excellent results (Kappa index, 0.955; P-value < 0.001). The ELISA showed a sensitivity/specificity of 83.3%/86.7% when compared to IFA and 100%/96.8% compared to DPP® (Table 2).

Tests on biological samples

Among the 48 canine samples tested, 18 were positive when using the ELISA technique, 19 were positive with IFA, and 17 were positive with rapid test DPP®. Among the tested sera, 14 samples were positive in the three techniques, three were reacted with only two techniques (IFA and DPP® or ELISA and DPP®) and six samples reacted in only one test (IFA or ELISA). The results are shown in figure 1.

DISCUSSION

The standardization of an in-house indirect ELISA as a leishmaniosis diagnostic using crude antigens (*L. infantum*) was successful. It enabled the evaluation of canine samples from Uruguaiana and Barra do Quaraí, Argentina-Brazil's border region municipalities that are far away from the state's center, which typically lack access to ELISA tests.

In 2011, the Brazilian Ministry of Health defined the rapid test (dual-path platform chromatographic immunoassay) as the screening test for leishmaniosis and ELISA as the confirmatory test (MINISTRY OF HEALTH [MS], 2011). In this study, it was possible to evidence the strong correlation between these techniques.

The ELISA applied by the Central Public Health Laboratory of the State of Rio Grande do Sul (LACEN) was developed by the Bio-manguinhos

Table 2 - Sensitivity/specificity of each test (Enzyme-Linked Immunosorbent Assay [ELISA] and dual-path platform chromatographic immunoassay [DPP®]) compared to the gold standard indirect immunofluorescence assay (IFA).

	ELISA x IFA	DPP® x IFA	ELISA x DPP®
Sensitivity	83.3%	88.2%	100%
Specificity	86.7%	87.1%	96.8%
Kappa	0.692	0.734	0.955

laboratory, through the use of soluble and purified antigens from *Leishmania in vitro* cultivation (NASCIMENTO, 2012). A study showed that the sensitivity was 72.0% (CI 95%: 50.4–87.1%) and the specificity was 87.5% (CI 95%: 60.4–97.8%), with a Kappa index value of 0.975 (LIRA et al., 2006). These values demonstrated that the standardized tests in the present study reached adequate indexes of specificity (83.3%) and sensitivity (86.7%).

The DPP® had a sensitivity of 93.33% and specificity of 98.33% (BARBOSA, 2015). This test was used to compare the ELISA results obtained in the present study, and when the ELISA results were compared with those of the DPP®, we observed a specificity of 98.6% and a sensitivity of 100%. In the same way, the IFA test was applied as a gold standard in order to increase the test reliability, reaching a specificity of 83.3% and sensitivity of 86.7%.

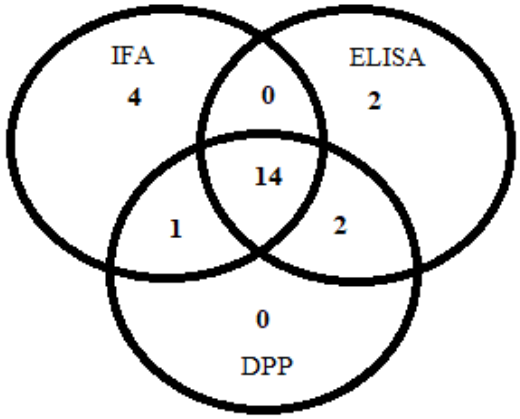

Figure 1 - Correlation between positive samples from the different techniques used: Enzyme-Linked Immunosorbent Assay (ELISA), indirect immunofluorescence assay (IFA), and dual-path platform chromatographic immunoassay (DPP®).

The use of promastigote antigens has been reported for the diagnosis of human visceral leishmaniosis through the use of *L. donovani* species (MARTIN et al., 1998). In this study, *L. infantum* was used due to its high prevalence in the region where the study was conducted (ESCOBAR et al., 2020; MASSIA et al., 2016).

There were notable differences between the assays in terms of plate antigen fixation, blocking, serum dilution, and incubation time (RAJASEKARIAH et al., 2001). This demonstrates the need for ELISA standardization for different species, and different laboratory conditions may be required depending on the best protocol.

Research comparing crude antigens with recombinant antigens (rK-39 and rK-26) for diagnosing canine visceral leishmaniosis noted that the sensitivity was similar in both groups (97% and 100%, respectively). However, the crude antigens demonstrated higher cross-reactivity than the recombinant antigens (DO ROSÁRIO et al., 2005), which could be related to their low specificity (COTA et al., 2013). In this study, the dog samples were submitted to different tests (IFA and DPP®) to confirm their *Leishmania* infection. Recombinant proteins produce the best ELISA results for diagnosing canine visceral leishmaniosis when compared with crude extracted antigens (FARIA et al., 2015; FONSECA et al., 2019). However, the ease and availability of obtaining crude antigens allow for the standardization of ELISA and its practical application in regions with fewer resources. This reinforces the need for stringent standardization and quality control of reagents. Another study used crude antigens from *L. amazonensis* and *L. chagasi (L. infantum* synonyms) as ELISA antigens and demonstrated its detection capabilities in most infected dogs. They also used recombinant antigens and observed that these were more sensitive for detecting dogs with clinical signs.

However, no statistically significant difference was observed between the two types of antigens (DO ROSÁRIO et al., 2005). With a *Leishmania* cell culture, it is possible to have a large volume of antigens with which to coat plates and develop an ELISA test. Our results showed that crude antigens can be used with ELISA to identify infected dogs.

CONCLUSION

Serodiagnosis using in-house ELISA with crude antigens was possible after standardization and definition of the best conditions. In addition, the comparison with IFA and DPP® allowed for validation of the method developed. A total of 37.5% (18/48) of the canine samples were positive. The ELISA technique showed a sensitivity/specificity of 83.3%/86.7% when compared to IFA and 100%/96.8% compared to DPP®, respectively. Therefore, ELISA with antigen extracts constitutes a fast, economical, and decentralized alternative for the diagnosis of canine visceral leishmaniosis in dogs from endemic regions.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The Ethics committee at the Federal University of Pampa (CEUA) approved the protocol for research animal use (approval number 014/2020).

DECLARATION OF CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

Gabriela Döwich Pradella: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Claudia Acosta Duarte: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing - review & editing. Luísa Zuravski: Conceptualization Investigation; Methodology; Validation; Visualization; Writing - review & editing. Taiane Acunha Escobar: Conceptualization; Investigation; Methodology; Validation; Visualization; Writing - review & editing. Roberto Thiesen: Visualization; Writing - review & editing. Mário Celso Sperotto Brum: Methodology; Supervision; Visualization; Writing - review & editing. Isac Junior Roman: Investigation; Methodology; Supervision; Validation; Visualization; Writing - review & editing. Fernanda Silveira Flôres Vogel: Supervision; Validation; Visualization; Writing - review & editing. Irina Lübeck: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - review & editing.

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