Arquivos do Instituto Biológico
ISSN 1808-1657
https://doi.org/10.1590/1808-1657000232022

SCIENTIFIC ARTICLE
Animal Infectious Diseases

# Standardization and validation of an indirect ELISA based on the extraction of proteins from *Leptospira spp.* as an antigen for the diagnosis of canine leptospirosis

Thaise Alves[1] https://orcid.org/0000-0002-0934-530X
Bruno Fernandes[1,*] https://orcid.org/0000-0002-4465-6402
Vinícius Vieira[1] https://orcid.org/0000-0002-6163-0298
Alexandre Pinheiro[1] https://orcid.org/0000-0002-2475-3272
Robson Cerqueira[1] https://orcid.org/0000-0001-5054-0353

1. Universidade Federal do Recôncavo da Bahia – Laboratório de Doenças Infecciosas – Hospital Universitário do Recôncavo da Bahia – Cruz das Almas (BA), Brazil.

*Corresponding author: brunofernandesnv@gmail.com

**ABSTRACT**

Leptospirosis is an important zoonosis. The World Health Organization recommends the use of microscopic soroagglutination in the diagnosis of the disease. However, this is a time-consuming technique that offers a risk of contamination to the performer and requires a trained professional. The indirect enzyme-linked immunosorbent assay (ELISA) test presents less risk, is faster, and has lower cost benefit for high sampling. In view of the advantages of using the test, and the importance of the development of techniques that facilitate and make the diagnosis more efficient, the objective of this study was to standardize an indirect ELISA technique using an immunogenic protein of *Leptospira* spp. for the diagnosis of canine leptospirosis. The standardized test obtained a cut off of 0.314, 76% of sensitivity, 34% of specificity, 53.52% of positive predictive value, 58.62 of negative predictive value, and 55% of accuracy. The protein extraction technique was efficient for antigen production, and the standardized test can be used in the diagnosis of canine leptospirosis.

**Keywords:** leptospirosis; indirect ELISA; microscopic agglutination test; diagnostic immunology.

## INTRODUCTION

Leptospirosis is a zoonosis, and the diagnosis of infected animals is an important control of the disease. The etiologic agent is the bacterium *Leptospira spp.*, which is part of the order Spirochaetales, family Leptospiraceae, and has 20 serogroups consisting of 250 serovars.

The bacteria have cell wall and cytoplasmic membrane surrounded by the outer membrane containing lipoproteins (LigA, LigB, LenA, LenB, LcpA, Loa22, LipL32) and porins or transmembrane protein (OmpL1), and surface lipoproteins (FRAGA et al., 2016).

The outer membrane surface lipoproteins present in pathogenic strains play an important role as an immunogen, being expressed both in in-vivo and in-vitro cultures, and such characteristics confer potential in the development of diagnostic tests for leptospirosis (JOSE et al., 2018). Notwithstanding, MONARIS et al. (2015), when studying outer membrane proteins of *Leptospira interrogans*, found that LigA can provide protective immunity. CASTIBLANCO-VALENCIA et al. (2016) found that LigA and LigB proteins are also related to the pathogen's evasion mechanism from the immune system thanks to their ability to inactivate the complement system. FERNANDES (2012) highlights that the OmpL1 protein plays an important role in the hemorrhagic picture of leptospirosis infection, with competence in binding to plasminogen and fibronectin.

Received: Nov 2, 2022. Accepted: Aug 29, 2023
Associate Editor: Silvia Galleti
Peer Review History: Double-blind Peer Review.

Given the importance of studies on outer membrane proteins, tests that offer less risk in the execution and present good sensitivity and specificity efficiency, the aim of this study was to standardize an indirect enzyme-linked immunosorbent assay (ELISA) technique using an immunogenic protein from *Leptospira spp*. for diagnosis of leptospirosis in dogs.

# MATERIAL AND METHODS

## Antigen production

Cultivation was carried out in EMJH medium (Ellinghausen-McCullough modified by Johnson and Harris / Difco) enriched with 10% rabbit serum from 19 serovars of *Leptospira spp.*, including the species *L. interrogans*, *L. kirschneri*, *L. santarosai*, *L. borgpetersenii*, *L. biflexa*, and *L. noguchii*; serovars: Icterohaemorrhagiae, Copenhageni, Canicola, Grippothyphosa, Pomona, Bataviae, Castellonis, Cynopteri, Javanica, Pyrogenes, Hardjo, Patoc, Tarassovi, Autumnalis, Hebdomadis, Wolfi, Panama, Australis, and Sejroe; from the collection kept at the Infectious Diseases Laboratory and kindly donated by the Oswaldo Cruz Foundation, from Rio de Janeiro, RJ, Brazil. The peaks were kept in an oven at 29°C for 20 days, weekly evaluating the occurrence of growth ring formation or contamination; growth confirmation was performed using darkfield microscopy with the presence of motility. After growth confirmation, the cultures were homogenized, and the spectrophotometer was used to verify the initial optical density of the cultures, before the antigen preparation treatment for later evaluation.

Cultures of each strain were homogenized and aliquoted. Adapted from the methodology carried out by SOUZA et al. (2012), the culture was centrifuged at 12,000 rpm at 18°C for 12 minutes. Fifty µ of TE-10X/TrisEDTA pH 8.0 buffer was added to buffer the solution and prepare the cells for further steps; the tubes were then kept overnight at 7°C. On the next day, the solution from each tube was collected in a container in which 5 mL of triton-X 5% was added, and homogenized for 12 hours at 10°C. After this period, the solution was centrifuged at 12,000 rpm, for 12 minutes at 18°C; the supernatant corresponding to the antigenic fraction was removed.

## Standardization of the dilution of the antigenic fraction to be used in indirect ELISA

Initially, the antigenic fraction obtained was verified as the best dilution. A 96-well polyethylene plate was sensitized with the antigenic fraction diluted in 0.5M carbonate bicarbonate buffer pH 9.6, in the proportion 1:50, 1:100, 1:150 and 1:200, in duplicate, and 12 repetitions for each control. The plate sensitized with the dilutions was kept overnight in a humid chamber at 7°C and then went through the blocking step. There was the addition of the serum to be tested, the conjugate, and then the revealing solution, and the reaction was interrupted using $H_2SO_4$, followed by reading in plate spectrophotometer.

For each dilution, the exact same process was carried out and the positive, negative, and blank controls were tested for each one. The positive control consisted of a positively positive serum from a sick mixed breed (SRD) dog that was seroreactive to microscopic seroagglutination (SAM). The negative control originated from a negative sample from an animal, SRD, neonate, unvaccinated mother, unvaccinated animal and clinically healthy. As a contamination test, the control called white was also tested, which was equivalent to not adding canine serum. Each 10 µ of control serum was diluted in 1,000 µ of the 0.5-g skimmed milk solution for each 50 mL of PBS-t.

With the optical density values obtained from each dilution, an analysis of variance (ANOVA) with blocking factor for positive, negative, and blank was applied, since decreasing values were expected from the positive, followed by the negative and blank. Since these values did not influence the significant difference between the tested dilutions, these factors were blocked considering $P < 0.10$. After ANOVA, it was found that there was no significant difference ($P = 0.647$) between the dilutions. Therefore, there is no higher dilution and none of the dilutions can be used.

## Standardization of indirect ELISA

For standardization of the test, a 1:50 dilution was used. One hundred samples of canine serum, both sexes, unvaccinated and from the following districts were used: Inocoop, Areal, Railroad and Table, in the city of Cruz das Almas, BA, Brazil. Fifty samples came from sick animals and positive in SAM with titers above 1:100, and the other 50 from healthy and SAM negative animals. SAM was considered the gold standard, as it is the diagnostic test recommended by the World Health Organization.

In the execution of the ELISA indirect test, the sensitization was carried out with the antigenic fraction diluted in a concentration of 1:50 in a carbonate bicarbonate buffer 0.5M pH 9.6, distributed in a 96-well polyethylene plate, kept overnight in a humid chamber at 7°C. After this period, the wells were washed twice with PBS-t and then blocked with the addition of 200 µ of the skimmed milk dilution (1 g in 20 mL of PBS-T), remaining for 1 hour in a humid chamber at 37°C, followed by washing and adding 10 µL of each canine serum diluted in 1,000 µL of skimmed milk solution (0.5 g + 50 mL of PBS-T) distributed in duplicate after 1 hour, and carrying out five washes. Fifty µL of the dilution in PBSt was added (1:10,000) in the conjugated canine immunoglobulin IgM conjugated to enzyme peroxidase. After 1 hour, the plate was washed five times, and followed for development, using 50 µL of the revealing solution (30 µL 30% $H_2O_2$ + 4 mg of o-phenylenediamine/OPD + 10 mL of citric buffer pH 5.1), the plate was stored for 5 minutes away from light and soon after the reaction it was stopped using 25 µL of $H_2SO_4$.

The process was over and culminated in the optical density reading on the 492 nm filter. The values obtained were evaluated using the Med Calc software, and the cut-off point determined from the Roc curve, with $p < 0.05$. Once the cutoff point was determined, the optical densities of the positive and negative sampling were evaluated in relation to the gold standard. After using formulas for the evaluation of serological tests, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were determined.

## RESULTS

When evaluating the best dilution for performing the ELISA indirect it was verified by the ANOVA that there was no statistically significant difference between the dilutions (Table 1).

**Table 1.** Analysis of variance for 1:50, 1:100, 1:150, 1:200 dilutions.

| | Degree of freedom | Sum of squares | Mean square | F calculated | P for significance |
|---|---|---|---|---|---|
| Dilutions | 3 | 0.020 | 0.007 | 0.584 | 0.647 |
| Block | 2 | 7.530 | 3.765 | 336.345 | $6.91e^{-07}$ |
| Waste | 6 | 0.067 | 0.011 | | |
| Total | 11 | 7.597 | | | |

Source: Elaborated by the authors.

Using the Med Calc software, the cutoff point of 0.314 was obtained from the Roc curve, with $p < 0.05$ (Fig. 1).

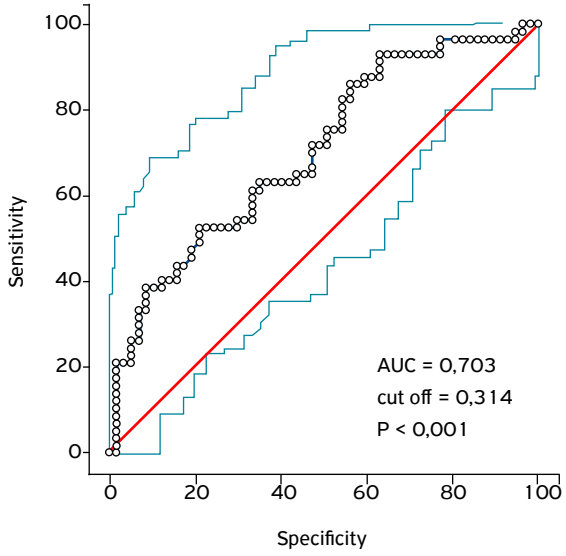


**Figure 1.** Roc curve for determining the specificity and sensitivity of the indirect ELISA test. Area under the ROC curve (AUC): 0.703.
Source: Elaborated by the authors.

Using the cutoff point determined by the Roc curve, the optical density values obtained in the test were contrasted to the gold standard in a contingency table for clinical test analysis (Table 2).

**Table 2.** Results of microscopic soroagglutination positive and negative samples submitted to indirect enzyme-linked immunosorbent assay (ELISA) with leptospira antigen.

| | | Golden pattern | | |
|---|---|---|---|---|
| | | Positive | Negative | Total |
| **Test** | Positive | 38 | 33 | 71 |
| | Negative | 12 | 17 | 29 |
| **Total** | | 50 | 50 | 100 |

Source: Elaborated by the authors.

Through formulas used in epidemiology, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated; considering 38 true positives (VP), 33 false positives (FP), 12 false negatives (FN), and 17 true negatives (VN), among a sample of 100 animals tested in both tests (Eq. 1–5).

$$\text{Sensitivity} = \frac{38}{(38+12)} = 76 * 100 = 76\% \quad (1)$$

$$\text{Specificity} = \frac{17}{(17+33)} = 34 * 100 = 34\% \quad (2)$$

$$\text{Positive predictive value} = \frac{38}{(38+33)} = 53{,}52 * 100 = 53{,}52\% \quad (3)$$

$$\text{Negative predictive value} = \frac{17}{(17+12)} = 58{,}62 * 100 = 58{,}62\% \quad (4)$$

$$\text{Accuracy} = \frac{38+17}{(38+12+33+17)} = 55 * 100 = 55\% \quad (5)$$

## DISCUSSION

After antigen extraction process, there was a mechanical and chemical breakage of the bacterium's cell membrane, promoted by the centrifugation steps, and the use of Triton-X 5%, thus producing an antigenic fraction capable of becoming a capture antigen for an antibody against *Leptospira spp*. SOUZA et al. (2012), using Triton-X 5%, obtained outer membrane proteins from serovar Hardjo and also verified the efficiency of the antigen.

The cutoff value of a serological test directly influences the sensitivity and specificity values of the test, and this ultimately determines its use as a screening test, or confirmatory test. The greater the sensitivity, the greater the ability to identify false negatives. Therefore, tests with high sensitivity are more often used for screening, while tests with greater specificity better detect false positives and are used as confirmation (CARMINATI, 2005).

In standardizing the proposed test, the values obtained allowed us to infer that the ELISA indirect test, using the extracted antigenic fraction, obtained satisfactory sensitivity and reasonable specificity, being, therefore, more suitable for screening. Probably the variability of serovars used in the antigen production process contributed to the greater availability of epitopes in the antigenic molecule, favoring the sensitivity of the test. This data corroborate CHEN et al. (2018), who verified three recombinant antigens extracted from the membrane (rLipL32, rLipL41 and rLigA-Rep) for the diagnosis of leptospirosis in humans, obtained 79% sensitivity and 88% specificity; it was also verified that the mixture of antigens performed better than when used separately.

The ELISA indirect test has some advantages: speed of execution, low cost, and possibility of performing a large number of samples, consisting of an excellent seroepidemiological survey tool, which requires screening tests with good sensitivity

(SEABRA et al., 2016; NASCIMENTO et al., 2017; KIM et al., 2019). Positive and negative predictive values indicate that the standardized test described here is capable of detecting 53.52% of positives and 58.62% of negatives, offering a good safety margin for a screening test.

The use of ELISA tests in the diagnosis of human leptospirosis is commonplace, mainly as a screening test associated with SAM as confirmation. On the other hand, the use of this test in the diagnosis of animal leptospirosis is uncommon. An indirect ELISA test using serovar L. Hardjo for diagnosis of bovine leptospirosis was developed, which showed 100% sensitivity and 73% specificity (SOUZA et al., 2012). Using serovar from *Leptospira fainei*, PENNA et al. (2017) standardized an ELISA test for the diagnosis of canine leptospirosis with 95.6% sensitivity and 93% specificity, confirming the possibility and relevance of standardizing tests aimed at diagnosing the disease in animals. The production of the antigens mentioned before used only one serovar. However, it is also important to verify whether a more generic antigenic fraction is efficient as well, since infection in animals can occur by several serovars. The result of the standardized ELISA in this study could confirm the possibility of producing a more generic antigenic fraction with a greater variety of serovars and efficiently.

SAM requires maintenance of the collection of antigens to be used to ensure a test with good efficiency. It also demands time to perform, and the result can be influenced by the experience of the performer (SARMENTO et al., 2012). When only a serum sample is used to perform the SAM, this proves to be little viable, not detecting individuals outside the acute phase (BLANCO et al., 2016). In view of these factors, the indirect ELISA does not require the maintenance of a collection of bacteria, presents less risk to the person who works with antigens and not living cells, and is faster, as it is possible to perform 42 diagnoses at once, allowing for a contribution to the screening of animals suspected of leptospirosis, especially in an epidemiological survey, which uses large sampling.

There is also the possibility of alternating the use of the immunoglobulin conjugate in the indirect ELISA, which can detect both IgM and IgG. Antibody detection comes from vaccine response or natural infection.

## CONCLUSIONS

From the antigen extraction process, using rotation and 5% triton-x action for cell lysis, it was possible to extract surface membrane protein capable of responding to the indirect ELISA test, obtaining 76% sensitivity and 34% specificity, being able to confirm 53.52% of positives and 58.62% of negatives, with 55% accuracy. The antigen extraction technique is effective, and the standardized ELISA indirect can be used as a screening test. It is mainly indicated and relevant for the epidemiological survey of canine leptospirosis.

### AUTHORS' CONTRIBUTIONS

**Conceptualization:** Alves, T.M., Pinheiro, A.M. and Cerqueira, R.B.; **Investigation:** Alves, T.M. and Cerqueira, R.B.; **Methodology:** Alves, T.M., Pinheiro, A.M. and Cerqueira, R.B.; **Validation:** Alves, T.M., Vieira, V.P. and Pinheiro, A.M.; **Visualization:** Alves, T.M.; **Writing – original draft:** Alves, T.M.; **Formal analysis:** Fernandes, B.P., Vieira, V.P., Pinheiro, A.M. and Cerqueira, R.B.; **Writing – review & editing:** Fernandes, B.P. and Cerqueira, R.B.; **Data curation:** Vieira, V.P., Cerqueira, R.B. and Pinheiro, A.M.; **Supervision:** Pinheiro, A.M. and Vieira, V.P.

### AVAILABILITY OF DATA AND MATERIAL

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

### FUNDING

Not applicable.

### CONFLICTS OF INTEREST

Not applicable.

### ETHICAL APPROVAL

Not applicable.

### ACKNOWLEDGEMENTS

Not applicable.

## REFERENCES

BLANCO, R.M.; CASSIOLATO, A.P.; ROMERO, E.C. Avaliação do teste de aglutinação microscópica utilizando-se como antígeno leptospiras saprófitas para o diagnóstico da leptospirose humana. *Revista do Instituto Adolfo Lutz*, v.74, n.2, p.90-96, 2016. https://doi.org/10.53393/rial.2015.v74.33460

CARMINATI, R. *Estudo da sensibilidade e especificidade de quatro testes ELISA e utilização da técnica de PCR para o diagnóstico de Linfadenite Caseosa em caprinos*. 2005. Dissertação (Mestrado) – Universidade Federal da Bahia, Salvador, 2005.

CASTIBLANCO-VALENCIA, M.M.; FRAGA, T.R.; PAGOTTO, A.H.; DE TOLEDO SERRANO, S.M.; ABREU, P.A.E.; BARBOSA, A.S.; ISAAC, L. Plasmin cleaves fibrinogen and the human complement proteins C3b and C5 in the presence of Leptospira interrogans proteins: a new role of LigA and LigB in invasion and complement immune evasion. *Immunobiology*, v.221, n.5, p.679-689, 2016. https://doi.org/10.1016/j.imbio.2016.01.001

CHEN, H.W.; LUKAS, H.; BECKER, K.; WEISSENBERGER, G.; HALSEY, E.S.; GUEVARA, C.; KUO, L. An improved enzyme-linked immunoassay for the detection of leptospira-specific antibodies. *The American Journal of Tropical Medicine and Hygiene*, v.99, n.2, p.266-274, 2018. https://doi.org/10.4269%2Fajtmh.17-0057

FERNANDES, L.G.V. *Caracterização e avaliação imunológica de três proteínas de superfície de Leptospira interrogans obtidas em Escherichia coli*. 2012. Tese (Doutorado) – Universidade de São Paulo, São Paulo.

FRAGA, T.R.; ISAAC, L.; BARBOSA, A.S. Complement evasion by pathogenic Leptospira. *Frontiers in Immunology*, v.7, p.623-635, 2016. https://doi.org/10.3389/fimmu.2016.00623

JOSE, L.R.; MAHADEVIAH, S.N.; BALAMURUGAN, V.; KINI, K.R. Evaluation of an in-house LipL32 polymerase chain reaction for diagnosis of leptospirosis and its correlation with various serological diagnostic techniques. *Indian Journal of Medical Microbiology*, v.36, n.3, p.385-392, 2018. https://doi.org/10.4103/ijmm.ijmm_17_355

KIM, P.C.P.; MELO, R.P.B.; ALMEIDA, J.C.; SILVA, J.G.; RIBEIRO-ANDRADE, M.P.; WAGNNER, J.N.; PINHEIRO JUNIOR, J.M.; RINALDO, A. Serological response to Neospora caninum infection in goats and agreement between three diagnostic techniques to detect caprine neosporosis. *Pesquisa Veterinária Brasileira*, v.39, n.1, p.25-31, 2019. https://doi.org/10.1590/1678-5150-PVB-5989

MONARIS, D.; SBROGIO-ALMEIDA, M.E.; DIB, C.C.; CANHAMERO, T.A.; SOUZA, G.O.; VASCONCELLOS, S.A.; FERREIRA, L.C.S.; ABREU, P.A.E. Protective immunity and reduced renal colonization induced by vaccines containing recombinant Leptospira interrogans outer membrane proteins and flagellin adjuvant. *Clinical and Vaccine Immunology*, v.22, n.8, p.965-973, 2015. https://doi.org/10.1128/cvi.00285-15

NASCIMENTO, A.K.Q.; LIMA, J.A.A.; BARBOSA, G.S. A simple kit of plate-trapped antigen enzyme-linked immunosorbent assay for identification of plant viruses. *Revista Ciência Agronômica*, v.48, n.1, p.216-220, 2017. https://doi.org/10.5935/1806-6690.20170025

PENNA, B.; MARASSI, C.D.; LIBONATI, H.; NARDUCHE, L.; LILENBAUM, W.; BOURHY, P. Diagnostic accuracy of an in-house ELISA using the intermediate species Leptospira fainei as antigen for diagnosis of acute leptospirosis in dogs. *Comparative Immunology, Microbiology and Infectious Diseases*, v.50, p.13-15, 2017. https://doi.org/10.1016/j.cimid.2016.11.004

SARMENTO, A.; AZEVEDO, S.S.; MORAIS, Z.M.; SOUZA, G.O.; OLIVEIRA, F.; GONÇALES, A.P.; MIRAGLIA, F.; VASCONCELLOS, S.A. Use of Leptospira spp. strains isolated in Brazil in the microscopic agglutination test applied to diagnosis of leptospirosis in cattle herds in eight Brazilian states. *Pesquisa Veterinária Brasileira*, v.32, n.7, p.601-606, 2012. https://doi.org/10.1590/S0100-736X2012000700003

SEABRA, M.; ALEIXO, A.L.; PEREIRA, P.; PINHEIRO, J.; AMENDOEIRA, M. Levantamento soroepidemiológico da frequência de Toxoplasma gondii em doadores de córnea do Banco de Olhos de Volta Redonda, RJ, Brasil. *Brazilian Journal of Veterinary Medicine*, v.38, Suppl.3, p.188-194, 2016.

SOUZA, M.A.; CASTRO, J.R.; TAVARES, T.C.F.; SOARES, P.M.; SANTOS, M.P.; SILVA, H.O.; LIMA-RIBEIRO, A.M.C. Padronização e validação de ELISA indireto para o diagnóstico da leptospirose bovina. *Bioscience Journal*, v.28, n.6, p.993-999, 2012.