Evaluation of the cross-reaction between *Leishmania* spp and *T. cruzi* by structural equation model

Avaliação da reação cruzada entre *Leishmania* spp e *T. cruzi* pelo modelo de equação estrutural

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ABSTRACT

Visceral Leishmaniasis and Chagas disease are characterized by parasitic diseases of wide worldwide distribution caused by protozoa of the genus *Leishmania* spp. and *Trypanosoma cruzi*, and that, due to phylogenetic homogeneity, share surface antibodies, which may cause cross-reactions in the face of serological tests used in the routine diagnosis of diseases. The present study aimed to analyze the occurrence of cross-seroreactivity between *Leishmania* spp. and *Trypanosoma cruzi* antigens in serum samples from dogs when submitted IFAT, DPP and Elisa serological tests, also verifying the possible correlations and associations between the multiple variables observables and risk factors on seroreactivity using mathematical modeling. Was concluded that there was cross-seroreactivity between the antigens of parasite. The Elisa test, the neighborhood and the clinical signs constituted risk factors for the presence of anti-*Leishmania* spp. and anti-*T. cruzi* antibodies. The latent variable had a greater influence for IFRS *T. cruzi* than for IFRS *Leishmania* spp. There was a correlation between the observable variable and the results' seroreactivity, and the structural equation model proved to be an excellent tool for data analysis.

Keywords: [Antibodies; Dogs; Visceral Leishmaniasis; Chagas Disease; Factor Risk]
RESUMO

Leishmaniose visceral e a doença de Chagas são doenças parasitárias de ampla distribuição mundial causadas por protozoários do gênero Leishmania spp. e Trypanosoma cruzi, e que, devido à homogeneidade filogenética, compartilham anticorpos de superfície, que podem causar reações cruzadas diante de testes sorológicos utilizados no diagnóstico rotineiro de doenças. O presente estudo teve como objetivo analisar a ocorrência de sororreatividade cruzada entre Leishmania spp. e Trypanosoma cruzi em amostras de soro de cães submetidos aos testes sorológicos IFAT, DPP e Elisa, verificando também as possíveis correlações e associações entre as múltiplas variáveis observáveis e fatores de risco na sororreatividade por meio de modelagem matemática. Concluiu-se que houve sororreatividade cruzada entre os antígenos do parasita. O teste Elisa, a vizinhança e os sinais clínicos constituiram fatores de risco para a presença de anti-Leishmania spp. e anti-T. cruzi anticorpos cruzi. A variável latente teve maior influência para IFRS T. cruzi do que para IFRS Leishmania spp. Houve correlação entre a variável observável e a sororreatividade dos resultados, e o modelo de equações estruturais mostrou-se uma excelente ferramenta para análise dos dados.

Palavras-chaves:[Anticorpos; cães; Leishmania visceral; Doenças de Chagas; Fatores de risco]

INTRODUCTION

Visceral Leishmaniasis and Chagas disease are zoonoses of great importance to public health. They are caused by protozoa of the genus Leishmania spp. and by Trypanosoma cruzi, and have a high rate of morbidity and lethality (Brazil, 2014). The transmission of Leishmania spp. occurs through the bite of a blood-sucking female mosquito, Lutzomyia spp., popularly known as straw mosquito, birigui or tatuquiras. The infection has three forms of presentation: visceral/kala-azar leishmaniasis, cutaneous leishmaniasis and mucocutaneous leishmaniasis.

Dogs are considered the main reservoirs of Leishmania spp.; they present intense cutaneous parasitism, close contact with humans and attract the presence of the vector (Costa, 2011; Snowden and Kjos, 2015; Troncarelli et al., 2009).

Triatomine insects known as kissing bugs vector Chagas disease. Transmission occurs when the insect’s urine and feces come into contact with the mucosa or skin lesion of the host, and may also occur through the ingestion of an infected vector or small hosts or food contaminated with triatomine feces (Messenger et al., 2015). In the state of São Paulo/SP, the authors Troncarelli et al. (2009) and Viol et al. (2012), detected positive dogs for Leishmania spp and T. cruzi in serological tests.

Serological tests, ie indirect immunofluorescence (IFAT) and enzyme-linked immunosorbent (ELISA) tests are used to detect anti-Leishmania spp. antibodies.
Serological reactions should not be used as an isolated criterion for diagnosis, as they may present a cross-reaction with other *Trypanosomatids* (Batista et al., 2010).

Structural Equation Modeling (SEM) is a set of techniques and procedures that approach an extension of other multivariate techniques, evaluating simultaneous relationships, that is, dependence and independence relationships between one or more variables (Hair J. F. et al., 2005).

The biologist Sewell Wright (1918, 1921, 1934, 1960) was the pioneer in the study of models that include path analysis and showed how the correlations between variables constitute the parameters of the model represented by the path diagram (Amorim et al., 2010).

The Statistical Model (SEM), explains the relationships between multiple variables, and examines the structure of interrelationships expressed in a series of equations (Amorim et al., 2010). They often involve complex relationships of various types between variables, which can also be of various types. This makes the visualization and understanding of all existing relationships in a model quite difficult to understand.

Based on the above, the objective of this study was to evaluate the occurrence of cross-response between antigens and their relationships with multiple observable variables, which are considered risk factors, using structural equation modeling.

**MATERIALS AND METHODS**

A total of 266 serum samples were used. Samples were tested for *Leishmania* spp. and were stored in the Microbiology laboratory of the Faculty of Veterinary Medicine of Araçatuba/SP FMVA-Unesp from dogs from the FMVA- Veterinary Hospital, during the period from 2017 to early 2020 (Figure 1).
SEROLOGICAL TESTS

For Canine Visceral Leishmaniasis

The search for anti-Leishmania ssp antibodies was initially performed using the rapid qualitative technique, using the rapid screening test – “TR DPP® kit (Teste Rapid Dual Path Platform, Bio-Manguinhos) and later, they were submitted to the quantitative test of the Enzyme Immunoassay (ELISA), for confirmation of results. The ELISA was carried out at Instituto Adolfo Lutz - Araçatuba-SP, according to its own protocol.

For Chagas Disease

The samples were tested by the Indirect Immunofluorescence Reaction (IFAT) according to Camargo's method (1966). For FIAT was used slides for immunofluorescence composed of two rows of six holes, containing fixed antigens of Leishmania major and Trypanosoma cruzi, with the cut-off of 1:40 and 1:20.

STATISTICAL ANALYSIS

In the study, the concordance in the serological diagnoses was calculated using Cohen’s Kappa index, based on the contingency table, and the interpretation of the results according to the classification by Landis & Koch) and presented by Capp and Nienov (2020); k > 0.80-1.00 almost perfect agreement; 0.60-0.79 substantial; 0.40-0.59 moderate; 0.20-0.39 regular; 0.00-0.19 discrete and 0.00 no agreement.
The correlation analysis between the IFAT for *Leishmania* spp. and *T. cruzi* was evaluated using the Spearman correlation test (ρ). To verify the occurrence of association between the variables: sex, race, and clinical signs, in view of the serological tests (IFAT - *Leishmania* spp. and IFA - *T. cruzi*) the chi-square tests and continuity correction were applied. Multiple observable variables influencing the potential for error were also considered, such as: gender, age, coat, size, characteristics of the floor (earth and cement), access to the street, location of the residence (green area, periphery neighborhood, and/or central) as the possible risk factors analyzed by the Structural Equation Model (SEM), and the estimation method: WLSMV (Robust Weighted Least Squares).

The fit models were: CFI (comparative fit index) and TLI (Tucker Lewis index) calculate the fit of the observed model, presenting the values: above 0.95 indicate excellent fit, and those greater than 0.90 indicate adequate fit (Bentler, 1990; Hu and Bentler, 1999). The RMSEA (root mean square error of approximation), evaluates whether the model fits the population, with values lower than 0.05, but acceptable up to 0.08 (Amorim et al., 2010).

The statistical program used was Software Version 2.3 jamovi project (2022) (Epskamp, 2015; Galluci and Jentschke, 2021).

**RESULTS**

Of the 266 samples used for the study, 226 (85%) were reactive and 40 (15%) non-reactive against the DPP tests and the qualitative ELISA test, for *Leishmania* spp. (Table 1). In the Indirect Immunofluorescence Reaction (IFAT) for *Leishmania* spp and *T. cruzi*, 156(59%) reactive samples and 110(41%) non-reactive; 141 (53%) samples simultaneously presented anti-*Leishmania* spp. and anti-*T. cruzi* antibodies, with cross-reactivity, in both serological tests (Table 1).
To verify concordance of cross-reactions for anti-\textit{Leishmania} spp. and anti-\textit{T. cruzi} by the Indirect Immunofluorescence Reaction (IFAT), the Cohen's Kappa test statistical model was adopted, which revealed an agreement of 89% and a reliability of 0.7675, considered substantial, between the IFA for \textit{Leishmania} spp. and IFA for \textit{T. cruzi}.

For the correlation matrix analysis, Spearman's correlation coefficient ($\rho$) was applied, showing a strong correlation, with a coefficient of $\rho = 0.784$ and $p$–value = $< 0.001$. The significant $p$-value expressed by the Spearman correlation, evidencing a significant relationship between the tested variables.In figure 2, it is possible to observe the behavior of the waves, thus, it was observed that the serological titers of deaths for both parasites were similar.
Figure 2. Results of serum samples from dogs from the northwest region of the state of São Paulo from 2017 to 2020 against the correlation matrix that expressed anti-

*Leishmania* spp. and anti-*T. cruzi*

Thus, to verify what the antigens have in common, the structural equation model (SEM) was adopted, the samples evaluated by the statistical model were those from the period from 2018 to 2020, as they were the samples that contained more information about the animals. For the structural equation model (SEM), the Robust Weighted Least Squares (WLSMV) estimation method was used, promoting quality of fits and the p-value for the use model was 0.990, the RMSEA 0.000, the CFI 1.000 and the TLI 1.115, being considered satisfactory values.

The Venn Diagram illustrates the cross-reaction (CR) highlighting the common variance, that is, what the *Leishmania* spp. and *T. cruzi* share among them (Figure 3).
Figure 3. The Venn diagram: illustration of variances: *T. cruzi* (T) and *Leishmania* spp. (L) and its relations with a latent factor (Cross reaction (CR)).

In the path diagram, among the twelve observed variables analyzed, considering as the predictors of the cross-reaction (latent variable), the variables: location in the neighborhood, the Elisa test and clinical signs showed significant influence on p less than 0.05, while the other variables had p values above 0.05, not presenting significant results. The neighborhood variable negatively influenced (-0.1693 / -16%) the potential for error, and the p value was significant (p=0.034) (Figure 4).
Figure 4. Diagram of the Structural Equation Modeling (SEM) technique demonstrating the influence of variables on the occurrence of reactivity for *Leishmania* spp and *T. cruzi* for the period 2018 – 2020.

![Diagram of Structural Equation Modeling](image)

The Elisa test influenced (0.25743 / 25%) the cross-reaction and the p-value was significant (p<0.00), as well as the clinical signs it presented for the cross-reaction (0.32359 / 32%), being a value of (p< 0.01). Regarding the observable measurement variables, the latent variable had a greater influence for IFAT *T. cruzi* 0.81/81% (p<0.01) than for IIF *Leishmania* spp. 0.77/77% (p<001). The variables coat, size, neighborhood and place of residence, and presence of flooring, did not present statistically significant results when correlated with the presence of anti-*Leishmania* spp. and anti-*T. cruzi* antibodies (Figure 4).

**DISCUSSION AND CONCLUSION**

The study demonstrated the high prevalence of anti-*Leishmania* spp. and anti-*T. cruzi* in samples from dogs from the northwest region of the state of São Paulo, revealing the free circulation of these protozoa among the canine population. The high occurrence
of cross-reactivity is based on the phylogenetic proximity between *Leishmania* spp. and *T. cruzi*, leading to false-positive serology results; depending on the antigen(s) used, IFAT may have less cross-reactivity compared to ELISA (Troncarelli et al., 2009).

Cross-reactivity impairs the ability to identify whether a dog is actively experiencing a *Leishmania* spp. co-infection with *T. cruzi* if it has had prior exposure to any of the pathogens or cross-reactivity in a diagnostic test (Beasley et al., 2021) or even as a result of co-infections in dogs by multiple trypanosomatids (Porfirio et al., 2018), bacteria such as *Ehrlichia* spp., *Anaplasma* spp., *Borrelia* spp. and parasites such as *Babesia* spp., *Neospora* spp. and *Toxoplasma gondii* (Zanette et al., 2014) among others, including Dirofilaria immitis, Paracoccidioides brasiliensis (Beasley et al., 2021), as it has been described in the published literature in several regions of Brazil.

The effects of these co-infections on the epidemiology, immune responses, clinical presentation, and management of Canine Leishmaniasis (CL) are important for understanding the clinical and molecular and epidemiological, clinical and immunological consequences of co-infections during leishmaniasis dogs with other vector-borne diseases can accelerate the progression of CL (Beasley et al., 2021).

Co-infections are characterized by the presence of anti-trypanosomatid spp. antibodies, but without clinical manifestation, it constitutes a great challenge for the establishment of a diagnosis. The presence of serological reactivity for both trypanosomatids indicates the need for the association of more than one serological test for specific and real confirmation of the diagnosis. Thus, other diagnostic methods must be associated, such as the polymerase chain reaction (PCR) and/or simultaneous parasitological examination to establish the diagnosis of canine leishmaniasis (CL), as stated by Troncarelli et al. (2009) in a similar study carried out in the city of Bauru. However, if the parasite load of any organism is low in a relevant diagnostic sample such as whole blood, PCR may not be sensitive enough for detection depending on the target sequence used (Galluzzi et al., 2018; Ordóñez et al., 2020). Thus, the association between diagnostic, quantitative, and qualitative tests should be used in order to establish the specific diagnosis for the disease.

The analysis of the results refers to the concordance between the Elisa test and the IFAT for the detection of anti-*Leshmania* spp. antibodies were 71% and reliability (moderate) (Kappa = 0.350), which according to Pereira (2001) showed a good
concordance, as well as the results found by Zanette (2014), who found a Kappa value = 0.788, being considered substantial and Viol et al. (2012) who obtained a good agreement.

Simultaneous seropositivity for *Leishmania* spp. and for *T. cruzi*, in serum samples from 141 of the 266 dogs evaluated in the present study and these results corroborate those verified by (Troncarelli et al., 2009). It was also observed that cross-reactivity was higher in sera that showed high titers against both protozoa, with a Cohen's Kappa showing agreement of 89% and substantial reliability. The Spearman correlation coefficient ($\rho$) showed a strong correlation, Spearman = 0.784 and $p$–value = $< 0.001$, unlike (Mackinnon, 2012), who did not observe a significant Spearman correlation at 0.170 with $p$-value = 0.344.

The characterization of the ambiance and risk factors that have a direct impact on zoonosis has been described in the literature, opening up new possibilities for understanding the dynamics of the disease. In this context, it was found that variables using the structural equation model, such as neighborhood location, the Elisa test, and clinical signs, had a relevant influence on the presence of anti-Leishmania spp. and anti-*T. cruzi* antibodies.

The same occurs in the results found by Abrantes et al. (2018) who pointed to the location of households as an influencing factor and also found a higher prevalence of Leishmania infection in areas with greater coverage by sparse vegetation, contrary to the study, which did not observe influence the green area with the presence of anti-Leishmania spp. antibodies; however, the same author concluded that the age and sex of the animal were not associated with seropositivity, contrary to Fernandes et al. (2016) who concluded that age, sex, breed and coat of the dog were also risk factors and also inferred that the floor characteristic would also impact seroreactivity, differing from our findings. Corroborating our results, De Figueiredo et al. (2014) concluded that positive serology for CVL was not related to the variables sex and race; also observed an association between clinical signs and the presence of anti-Leishmania spp. antibodies; unlike our findings, the author observed that seropositivity is associated with age group.

In general, the results of the present study determined that the really impacting factors on the risks of reactivity against *Leishmania* spp. and *T. cruzi* were the accuracy of the serological test used for serology and the degree of impairment of the disease that may be directly associated with the degree of parasitism of the protozoa. Also analyzed
by SEM, variables directly related to host characteristics such as age, size, sex, breed, degree of nutrition, and coat did not influence the presence of antibodies. In this sense, the use of the structural equation model proved to be an excellent tool in the association of risk factors that really influenced the diseases.

The northwest region of the state of São Paulo has areas deforested for livestock and agriculture, suffering a strong anthropic action, thus corroborating other risk factors that should be investigated in the future and a recent study, which considered the socioecological vulnerability and the risk of the possibility of zoonoses in Brazil, pointed out that the state of São Paulo presents a medium risk based on the positive and negative effects of afforestation in the city, the richness of mammals, asphalting the city, natural vegetation cover and loss of vegetation. These characteristics influenced the average number of cases of zoonotic diseases per 100,000 inhabitants in the 27 Brazilian states, from 2001 to 2019 (Winck et al., 2022).

Araçatuba has been standing out in this sad scenario, as an endemic area for Leishmaniasis, since the first case described by (Luvizotto et al., 1999). In light of the above, other studies should be carried out to offer a better understanding of co-infections and co-exposures, in the face of intracellular pathogens.

Statistical modeling proved to be a great tool for the analyzed data. The presence of cross-seroreactivity between Leishmania spp and Trypanosoma cruzi antigens was observed. The Elisa test, the neighborhood, and clinical signs were risk factors for the presence of anti-Leishmania spp and anti-T. cruzi antibodies. The latent variable -Cross-reaction- had a greater influence on IIF T. cruzi than on IIF Leishmania spp, with an association between the observable variable and seroreactivity.

REFERENCES


