An assessment of serological techniques for the identification of asymptomatic visceral leishmaniasis in blood donors in Northeastern Brazil

Avaliação de técnicas sorológicas para identificação da leishmaniose visceral assintomática em doadores de sangue no Nordeste do Brasil

Una evaluación de las técnicas serológicas para la identificación de la leishmaniasis visceral asintomática en donantes de sangre en el noreste de Brasil

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Abstract
Background: Visceral leishmaniasis (VL) is a neglected disease caused by species of the *Leishmania donovani* complex and transmitted mainly by the bite of infected sandflies. A less frequent form of transmission is the transfusion route, important in countries lacking screening procedures for VL in blood banks. Methods: To identify asymptomatic infection by *Leishmania* sp, our goal here was to evaluate and compare the performance of three different serological techniques: Flow Cytometry (FC); Direct Agglutination Test (DAT); and Immunoenzymatic Test (ELISA). Thirty-one asymptomatic carriers of *L. infantum* were selected, from a single blood bank located in Recife, northeastern Brazil. Results : Foi avaliada a frequência de amostras positivas assintomáticas nos testes, no ELISA baseado no antígeno solúvel de Leishmania (25,8%), FC (22,8%), DAT (9,7%) e ELISA baseado em uma única proteína recombinante (12,9%). A sensibilidade dos testes também foi avaliada, o FC (22,58%) e o ELISA com o antígeno solúvel (22,58%) apresentaram os maiores valores de sensibilidade que o DAT (6,45%) e o ELISA recombinante (12,9%). Apenas a CF apresentou baixa especificidade (22,22%), em contraste com as demais técnicas (100% de especificidade). Conclusion:

Given the results presented, we highlight the low effectiveness of serological techniques for the identification of asymptomatic individuals, thus emphasizing the need to develop more accurate methods for this purpose.
Keywords: Flow cytometry; Direct agglutination test; ELISA; Recombinant ELISA.

Resumo
Introdução: A leishmaniose visceral (LV) é uma doença negligenciada causada por espécies do complexo Leishmania donovani e transmitida principalmente pela picada do vetor infectado. Uma forma menos frequente de transmissão é a via de transfusão, fato importante em países que carecem de procedimentos de rastreio da LV em bancos de sangue. Métodos: Para identificar a infecção assintomática por *Leishmania* spp., avaliamos e comparamos o desempenho de três técnicas serológicas diferentes: Citometria de fluxo (FC); Teste de aglutinação directa (DAT); e Teste Imunoenzimático (ELISA). Foram selecionados trinta e um portadores assintomáticos de *L. infantum*, de um único banco de sangue localizado em Recife, no nordeste do Brasil. Resultados: Foi avaliado a frequência de amostras positivas assintomáticas nos testes, no ELISA baseado com o antígeno solúvel de *Leishmania* (25,8%), CF (22,8%), DAT (9,7%) e ELISA baseado numa única proteína recombinante (12,9%). Foi avaliado também a sensibilidade dos testes, a CF (22,58%) e o ELISA com o antígeno solúvel (22,58%) apresentaram os maiores valores de sensibilidade do que o DAT (6,45%) e o ELISA recombinante (12,9%). Apenas a CF mostrou baixa especificidade (22,22%), contrastando com as outras técnicas (100% de especificidade). Conclusão: Dados os resultados apresentados, destacamos a baixa eficácia das técnicas serológicas para a identificação de indivíduos assintomáticos, enfatizando assim a necessidade de desenvolver métodos mais precisos para este fim.
Palavra-chave: Citometria de fluxo; Teste de aglutinação directa; ELISA; ELISA recombinante.

Resumen
Introducción: La leishmaniosis visceral (LV) es una enfermedad desatendida causada por especies del complejo Leishmania donovani y transmitida principalmente por la picadura de un vector infectado. Una forma menos frecuente de transmisión es por transfusión, un hecho importante en los países que carecen de procedimientos de detección de la LV en los bancos de sangre. Métodos: Para identificar la infección asintomática por Leishmania spp., evaluamos y comparamos el rendimiento de tres técnicas serológicas diferentes: Citometría de flujo (FC); prueba de aglutinación directa (DAT); y ensayo inmunoenzimático (ELISA). Se seleccionaron 31 portadores asintomáticos de L. infantum de un único banco de sangre situado en Recife, al noreste de Brasil. Resultados: Se evaluó la frecuencia de muestras positivas asintomáticas en las pruebas, el ELISA basado en el antígeno soluble de Leishmania (25,8%), el FC (22,8%), el DAT (9,7%) y el ELISA basado en una sola proteína recombinante (12,9%). También se evaluó la sensibilidad de las pruebas, el FC (22,58%) y el ELISA con el antígeno soluble (22,58%) mostraron los valores de sensibilidad más altos que el DAT (6,45%) y el ELISA recombinante (12,9%). Sólo la FC mostró una baja especificidad (22,22%), que contrasta con las demás técnicas (100% de especificidad). Conclusión: A la vista de los resultados presentados, destacamos la escasa eficacia de las técnicas serológicas para la identificación de individuos asintomáticos, por lo que destacamos la necesidad de desarrollar métodos más precisos para este fin.
Palabra clave: Citometría de flujo; Prueba de aglutinación directa; ELISA; ELISA recombinante.

1. Introduction

Visceral Leishmaniasis (VL) is a neglected human disease, caused by protozoa belonging to the *Leishmania donovani* complex (*L. donovani* and *L. infantum*), whose main form of transmission is the bite of infected female sandflies (Michel *et al*., 2011) . VL diagnosis is based on the observation of clinical symptoms aligned with the results of laboratory tests (Chappuis *et al*., 2006). It is characterized by the classic triad: fever, hepatosplenomegaly and pancytopenia. The asymptomatic condition, where the individual does not show symptoms, remains an important epidemiological challenge. Afflicted individuals in most cases are characterized by a low parasitemia (Brazil, 2009), but reactivation of the disease may occur during the course of the infection. In addition, asymptomatic individuals can act as reservoirs for the parasite and facilitate VL transmission through different means, such as the blood transfusion route (Michel *et al*., 2011; Ferreira-Silva *et al*., 2018). Therefore, laboratory screening for VL detection in blood banks is necessary to ensure the safety of the blood donation process and the health of recipients, especially in VL endemic regions (França *et al*., 2031; Fukutani *et al*., 2014).

So far, there is still no gold standard method for the identification of asymptomatic VL cases. One of the standard methods more generally used for VL diagnosis is the Enzymatic Immunoassay Test (ELISA). Its sensitivity, however, varies depending on the antigen used (Liew e O'donnell et al., 1993; Riera et al., 2008) and cross-reactivity is common in methods using non-purified antigens, such as crude soluble *Leishmania* antigen. As an alternative, the Direct Agglutination Test (DAT)

uses lyophilized antigen to visualize agglutination in positive cases and is considered a promising tool for the VL diagnosis, especially in VL-HIV coinfections and immunosuppressed individuals (Cota *et al.*, 2013; Assis *et al.*, 2016; Kühne *et al.*, 2020). Alternatively, recombinant proteins have been identified and evaluated as potential antigens for the detection/capture of antibodies against *Leishmania* proteins. They can be applied in Cytometry (FC), used for the quantification of anti-*Leishmania* antibodies (Gomes et al., 2010; Pedro-sampaio et al., 2016; Ker et al., 2019)

In summary, there is a clear need for precise tools to be used for the VL screening in blood banks. However, few studies have compared the performance of available techniques for VL diagnosis in asymptomatic blood donors (Silvestre *et al.*, 2008; Carvalho *et al.*, 2018; Thakur *et al.*, 2020). In this context, the present study aims to evaluate different serological techniques to identify asymptomatic *Leishmania* infection in blood donors.

2. Methodology

Study population and serum samples

The asymptomatic donors whose sera were evaluated in the present study are all voluntary donors at the Pernambuco Foundation for Hematology and Hemotherapy (HEMOPE), located in the city of Recife, state of Pernambuco, Brazil. Blood samples from all *Leishmania*-positive individuals were previously screened and found to be positive for *Leishmania* spp. infection using a qPCR test, as previously reported (Silva *et al.*, 2020). All 31 samples were previously tested and found to be negative for Hepatitis, Syphilis, HIV, HTLV and Chagas Disease. The control group (n = 18) consisted of sera from healthy individuals, negative for leishmaniasis (ELISA, DAT, IIF) and/or PCR, residing in non-endemic areas for VL, from the biorepository of the Laboratory of Immunopathology and Molecular Biology (LIBM) of Instituto Aggeu Magalhães (IAM) - Fiocruz. To calculate the cutoff point, 45 sera, negatives for VL were selected from the same biorepository mentioned above. Ethics and research of participating institutions (HEMOPE and IAM/FIOCRUZ), sob CAEE 65770317.9.0000.5190.

Enzymatic immunoassay test (ELISA)

The ELISA tests were performed based on the protocol described by Oliveira *et al.* (2011). Where microplates (NUNC, Maxisorp, Roskilde, Denmark) were sensitized with 600 ng of soluble *L. infantum* antigen and chimeric protein Q5 by Santos et al. (2020) and peroxidase-conjugated anti-human IgG antibody (Sigma, St. Louis, MO) was used at the dilution of 1:10000. The conventional ELISA were those in which the soluble antigen was applied in the assay, whereas in the recombinant ELISA the Q5 protein was used.

Production of the L. infantum soluble antigen extract

Promastigote forms of *L. infantum*, from the MHOM/BR/BH46 strain, were maintained and expanded in Schneider's medium until reaching exponential phase. The parasites were harvested by centrifugation at 400 g, 10 min, washed with PBS and resuspended in (50mM NaCl, 10mM EDTA, 50mM Tris–HCl, pH 8.0) supplemented with 1mM of the protease inhibitors N-ethylmaleimide (NEM) and phenylmethylsulfonyl fluoride (PMSF). Cell lysis was performed through successive steps of freeze/thawing in liquid nitrogen and water bath (36°C). Cellular lysis was monitored in a light microscope followed by centrifugation at 10,000 g at 4ºC, for 15 min, with the supernatant (the soluble fraction) quantified with Bradford's reagent, aliquoted and frozen at -80ºC until use. The pellet (consisting of insoluble antigens) was resuspended in PBS and stored.

Q5 production

The expression and purification of the chimeric Q5 was performed as previously described by Santos et al. (2020). Briefly, the recombinant plasmid with the Q5 gene was transformed into *E. coli* RosettaTM 2 DE3 bacteria (from Novagen), with the transformed bacteria cultured in LB medium supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at 37ºC. Recombinant protein expression was induced with 0.5 mM IPTG at 30°C. Bacterial cells were resuspended in denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, 20 mM imidazole, pH 8.0) and lysed by ultrasonication. Proteins were purified with Ni-NTA agarose beads (Qiagen, catalog #30210), with washes and elution carried out with the same lysis buffer supplemented with different concentrations of imidazole (100 mM for washes and 1 M for elution).

**Direct Agglutination Test (*DAT)*

The test was performed on 96-well plates with V-bottom, with serial dilutions from 1:50 to 1:51200, according to the manufacturer's specifications (Royal Tropical Institute, Amsterdam, Netherlands). The plaque was read by visualizing the agglutination, where those with a titration equal to or above 1:6400 were considered positive. The values referring to the dilutions and the cut-off were performed according to Harith *et al.*(1986).

Flow Cytometry

The flow cytometry (FC) assay for the detection of anti-promastigote antibodies of *L. infantum*, was carried out as previously described by Rocha *et al*. (2002). Briefly, 96-well plates were sensitized with sera samples diluted from 1:64 to 1: 32,768 in PBS plus 10% fetal calf serum, followed by incubations with a parasite suspension and anti-human IgG conjugated to fluorescein isothiocyanate-FITC (Sigma Chemical Corp., St. Louis, MO) at a 1: 200 dilution. Labeled parasites were washed, fixed and kept for 30 minutes at 4°C, protected from light, prior to reading on a FACScalibur flow cytometer (Becton Dickinson) using the Cell Quest Pro software. The labeled parasites were acquired in 20,000 events per sample and identified based on their specific frontal (FSC) and lateral (SSC) light scattering properties. The relative FITC fluorescence intensity of each event was analyzed in histograms, delimited by the FITC conjugate internal control histogram and applied to all samples, in order to determine the percentage of positive fluorescent parasites (PPFP) for each sample.

Statistical Analysis

All conclusions were taken at the 5% significance level. For the analysis of the results of the ELISA and FC test, we used version 7.0 of GraphPad Prism (GraphPad Prism Inc., San Diego, California, USA). The cutoff point for FC was defined using the PPFP, where we evaluated the positive and negative results for the test. For this, we built the Receiver Operating Characteristic (ROC) curve, from the values of specificity on the x axis and sensitivity on the y axis. Sensitivity and specificity values were calculated using the Open Source Epidemiologic Statistics for Public Health (OpenEpi) version 3.01 program. To assess the agreement of the tests performed, the Kapp identification measure was used, which helps to determine the concordant results of the tests. According to Landis JR & Koch GG (1977)

3. Results and Discussion

Performance of FC, DAT and ELISA in asymptomatic blood donors

When analyzing the performance of the tests with the 31 sera from the asymptomatic group previously classified as VL positive using a PCR based assay in the study by Silva et al. (2020). We observed a greater positivity using FC, although with

only 22.6% (7) of the sera tested having a VL positive result, while 77.4% (24) of those were negative, in the negative control group, 77.7% (14) of the samples were positive while 22.2% (4) remained negative. In contrast, the DAT test gave a positive test for only 9.7% (3) of the sera tested, with 90.3% (28) of those testing negative, in the negative control group none of the 100% samples (18) were positive. The ELISA assay using whole soluble *Leishmania* proteins performed similar to FC, with 22.6% (7) positive sera and 77.4% (24) negative, as in the DAT test, none of the samples from the negative control group were positive. Using only the Q5 recombinant protein decreased the efficiency of the assay with 12.1% (4) of the sera testing positive and 87.1% (27) negative. In addition, there was no positive sample from the negative control group. In table 1, the sensitivity and specificity values for the different test are shown. Noteworthy is the much-reduced specificity observed for FC in comparison with the other assays evaluated here. In the supplementary table we have the results of all tests.

Table 1. Sensitivity and specificity of FC, DAT, ELISA with soluble antigen and ELISA with Q5 protein in the 31 positive samples.

	FC	DAT	ELISA Soluble antigen	ELISA Q5
Sensitivity	22.58%	6.45%	22.58%	12.9%
Specificity	22.22%	100%	100%	100%

Source: Author's elaboration (2022)

Agreement between the tests used to identify the asymptomatic in the blood donor population.

After all tests were performed, an agreement assessment was performed using the Kappa measure. If the result less than zero means that there was no agreement between the tests. Said that, no agreement between the tests evaluated was observed when compared to qPCR (Kappa = 0).

Performing a routine laboratory screening for VL detection in blood banks is a major challenge for control of the disease (França *et al*., 2013; Fukutani *et al*., 2014). To this end, it is crucial the correct identification of asymptomatic individuals who may be potential blood donors and that, if unidentified, may transmit the disease through transfusion. Here, samples from donors known to be asymptomatic, diagnosed by qPCR (Silva *et al*., 2020), were subjected to different serological tests that included FC, DAT, and two different ELISA assays. Of the techniques used, the one that showed the greatest positivity and specificity was the ELISA based on soluble *Leishmania* proteins.

In the absence of a gold standard diagnosis, qPCR can be considered one of the most promising techniques for the diagnosis of VL, as it has high values of sensitivity and specificity (Landis e Koch, 1977; Sakkas *et al*., 2016). Despite being the technique used as an initial screening to identify asymptomatic cases, the costs associated with its use still limit its use in the routine screening of blood banks, especially in endemic countries (Silva *et al*., 2020). This highlights the importance of studying the performance of different serological techniques for the identification of this population, as performed in this study.

DAT is a simple, semi-quantitative test that does not require the use of sophisticated equipment, in addition to being a low-cost method. In a meta-analysis developed by Chappuis et al. (2006), the sensitivity of the test for the diagnosis of VL reached 94.8%, being classified as an excellent test. However, in our study with asymptomatic patients, the sensitivity of the test was the lowest in comparison with the other techniques tested. Also evaluating asymptomatic patients, a previous study saw no positive results, reaching the conclusion that the test has a low sensitivity for detecting subclinical or past infections (Garcez *et al*., 1996). DAT has thus been advised to be performed in combination with the patient's clinical evaluation (Terán-Angel *et al*., 2007). However, there is no standard method for evaluating the test result and there is also no standard antigen (Srivastava *et al*., 2011). More validation studies would be needed for DAT to be applied for the identification of asymptomatic individuals.

Unlike DAT, the ELISA based on the soluble *Leishmania* antigens had the highest frequency of positive samples, however, published studies state that this test may cross-react with other endemic diseases (Caballero et al., 2007; Srividya et al., 2012). Our results corroborate a previous study, in which in a group of 115 asymptomatic individuals, 13% (15) of the samples were positive in a ELISA with soluble antigens (Porcino *et al*., 2019). Fukutami *et al*. (2014) reinforce that when the soluble antigen is used, the test will become more sensitive, but less specific. The results of these studies differ from the study by Lima et al. (2010), where the test had a sensitivity of 100% and a specificity of 91.2%. Similar results were also presented by Mettler *et al*. (2005). However, the differences in results between the studies, can be attributed to the origin of the antigen used (Mohammed *et al*., 1985;Reithinger *et al*., 2002; Singh *et al*., 2003). When comparing the ELISA with the soluble antigen with the recombinant Q5 protein (Santos *et al*., 2020), we observed a lower positivity for the ELISA Q5. One of the great advantages of this test, however is a much reduced chance of cross-reaction with sera from individuals exposed to other diseases.

Regarding the results shown here using FC, these are associated with a very low specificity, due to similar results seen for the tested sera and the negative control group. We emphasize that the use of FC has been an innovation for the diagnosis of parasitic diseases such as Leishmaniasis (Silva *et al*., 2019). Previous studies have shown that FC is a valuable diagnostic technique, with good sensitivity and specificity (Nolan *et al*., 2013; McKinnon *et al*., 2018; Ker *et al*., 2019). Silva *et al*. (2019) showed that when compared to other serological techniques, FC presented a better sensitivity for the diagnosis; however, the study was carried out with symptomatic patients. Regarding our results, we can take into account the work of Gama Ker *et al.* (2013), where they carried out a study involving FC and the antigens used. The authors state in the discussion that dilution can be one of the important points to avoid false negative and positive results; however, the study was carried out with canine samples.

When comparing the tests evaluated in the present study, we observed that each test performed differently. This variation in results can be justified by the methodological differences that are the basis for each approach (Elmahallawy *et al*., 2014. In addition, the evaluation of asymptomatic individuals, with reduced or even null humoral and cellular responses to the parasite (Riera *et al*.,2004; Gadisa *et al*., 2012; Sundar *et al*.,2018), might impact significantly on the efficiency of each test. This justifies the low agreement seen between the tests. Due to this difficulty, so far the best way to identify the asymptomatic population in the population is the combination of more than one diagnostic technique (Ibarra-Meneses *et al*., 2017).

4. Conclusion

Serological techniques have not been able to satisfactorily identify asymptomatic *L. infantum* infection. Further studies using more sensitive diagnostic approaches are needed for this grou. It would also be important to conduct a study to estimate the prevalence of asymptomatic *Leishmania* spp. infection in blood donors using different antigens.

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