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Multi-centric prospective evaluation of rk39 rapid test and direct agglutination test for the diagnosis of visceral leishmaniasis in Brazil

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ABSTRACT

The diagnosis of visceral leishmaniasis (VL) is still a major problem in Brazil and several other countries where the disease is endemic. The use of an easy-to-use and interpret, sensitive, and specific method that requires no complex infrastructure or specialized professionals, such as direct agglutination test (DAT) and the rK39-based rapid immunochromatographic test may enhance the diagnosis of disease. This study evaluated the performance of a rapid test (DiaMed- IT-LEISH[®]) and the DAT for the diagnosis of VL in 213 parasitologically confirmed cases and 119 controls with clinical suspicion of VL and confirmation of another etiology. The sensitivities and specificities of the rapid test were 93% and 97%, respectively and those of the DAT were 90% and 96%, respectively. The positive predictive values of the rapid test and the DAT were 98% and 97%, respectively and the negative predictive values were 89% and 84%, respectively. The Kappa index showed agreement between both methods classified as substantial (0.77). This study showed that the DAT and the rapid test can be used to diagnose VL in Brazil, following a pilot study for implementation of the rapid test in the health services.

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1. Introduction

Visceral leishmaniasis (VL) affects up to 500 000 people yearly in approximately 65 countries.¹ In Brazil, where the disease is caused by *Leishmania (L.) chagasi*, syn. of *Leishmania (L.) infantum*, approximately 3500 cases have been reported annually since 2002, with a mean incidence of two

cases per 100 000 inhabitants and a case-fatality rate of 5.5% over the last 12 years.² If left untreated, VL has a case-fatality rate as high as 100%.³ Other diseases that manifest with hepatosplenic febrile syndrome can share the same presentation. Therefore, laboratory testing is necessary to confirm the VL diagnosis.⁴

The reference standard for VL diagnosis is the demonstration of the parasite in smears and/or cultures of the spleen, bone marrow, lymph nodes or liver. These parasitological techniques are highly specific, but their sensitivity varies depending on the tissue evaluated. The sensitivity

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of the stained smear of the splenic aspirate is high, above 95%, but because of the risk of bleeding, the use of a splenic aspirate is restricted.⁵ The bone marrow aspirate, with a sensitivity varying from 52% to 69%, is the most commonly used.⁶ These techniques require clinical and laboratory expertise, both of which are usually limited in endemic areas.

Serological tests have been developed to replace parasitological methods for the diagnosis of VL in the field. These methods present the advantages of being non-invasive and highly sensitive; however, anti-*Leishmania* antibodies may last for months after clinical cure and may be found in healthy persons who had been infected but remained asymptomatic.⁵ In Brazil, the test available for the Public Health System is the indirect fluorescence antibody test (IFAT), despite it depending on fluorescence microscopes and the requirement of well-equipped laboratories. Sensitivities varying from 88% to 92% and specificities ranging from 67% to 83%, depending on the antigen used, have been reported.^{7,8}

The direct agglutination test (DAT), developed in the 1980s, has been validated in several endemic areas and is being used in countries such as Sudan and Ethiopia.^{9,10} Sensitivity varying from 93.4–97% and specificity of 81–98% has been reported.^{10–12} A study by Oliveira et al.¹² in Brazil evaluated the performance of a locally produced DAT, reporting sensitivity of 93.4%, specificity of 96.9% and diagnostic efficiency of 95.5%. The authors concluded that DAT constituted a useful test and can replace IFAT as the routine diagnostic test used by the Brazilian Leishmaniasis Control Program.

A rapid test using the rk39 protein as antigen is easy to perform and provides a visual interpretation of the reactions.¹³ The test can be performed using blood or serum and the results are obtained within 20 minutes. A sensitivity varying from 67–100% and a specificity ranging from 71–100% has been reported.^{4,7,13–16} A study by Carvalho et al.¹⁶ evaluated the performance of a rk39-based rapid test in Brazil showing sensitivity of 90% and specificity of 100%. The authors concluded that the test could be used for the diagnosis of VL in Brazil.

The use of an easy-to-use and interpret, sensitive, and specific method that requires no complex infrastructure or specialized professionals, such as rapid test and the DAT, may enhance the diagnosis of VL. The purpose of this study was to compare the performance of DAT with the rk39-based immunochromatographic test for the diagnosis of human VL in Brazil.

2. Methods

2.1. Study site and patients

This was a prospective multi-centre study conducted between May 2005 and May 2007 in four states of Brazil: Maranhão, in the Federal University of Maranhão, Piauí, in the Federal University of Piauí, Bahia, in the Gonçalo Moniz Research Center and Minas Gerais, in the René Rachou Research Center. We evaluated 213 cases and 119 non-cases. Informed consent was obtained from all the adults and from minors' parents or legal guardians. The patients

underwent a clinical examination and bone marrow aspiration, and peripheral blood was collected for the serological tests.

2.2. Sample size

The sample was calculated based on data from a pilot study conducted at the René Rachou Research Center, considering sensitivity, specificity, a 95% confidence level and 4% accuracy. The aim was to enroll at least 115 true cases of VL and 115 true non-cases in the study. Patients were enrolled consecutively until the required sample size was achieved.

2.3. Inclusion and exclusion criteria

Patients with a history of fever accompanied by at least one of several clinical signs (splenomegaly, hepatomegaly, anemia, leucopenia or trombocitopenia) were included. The exclusion criteria were patients with a known immunodeficiency, use of an immunosuppressive agent and past history of VL. A patient was considered as a case when he or she presented the above mentioned symptoms and signs and had a diagnosis of VL confirmed by parasitological methods. A non-case was defined as a patient presenting the suggestive clinical picture, with a negative parasitological diagnosis and a firm diagnosis of another disease.

2.4. Direct parasite identification

In order to detect active VL, bone marrow smears were stained with Giemsa and evaluated under a 1000× oil immersion lens on an optic microscope. At least two bone marrow smears were evaluated for each patient.

2.5. Direct agglutination test

The antigen was prepared as previously described by Pedras et al.⁸ Sera were diluted in a saline citrate solution containing 1% 2-ME. Two-fold dilution series of the serum samples were made, from 1:100 to 1:102,400. Fifty microliters of the DAT antigen solution (concentration of 5×10^7 parasites/mL) were added to each well of the V-shaped microtiter plates (Nunc-Immuno Plate Brand Products, Roskilde, Denmark) containing 50 μ L of diluted serum. After the samples were incubated for 18 h at room temperature, the end titer was read as the dilution immediately before the well containing a clear, sharp-edged blue spot identical to the negative control. Appropriate control samples with known DAT titers were included as controls. The cut-off value was determined by analyzing the receiver-operating characteristic curve. Based on this analysis, 1:1600 was established as optimal cut-off for interpreting positive and negative results without compromising the sensitivity and the specificity (data not shown).

2.6. Rapid test

The kit IT-LEISH[®] was provided by the Diamed Latino-America S. A. (Cressier sur Morat, Switzerland). The test kit

Table 1

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of serological diagnostic methods for visceral leishmaniasis evaluated in four research centers in Brazil.

Methods	Sensitivity (%) ^a (CI: 95%)	Specificity (%) ^b (CI: 95%)	PPV (%) ^c (CI: 95%)	NPV (%) ^d (CI: 95%)
DAT	90 (85.3–94.0)	96 (90.5–99.0)	97 (94.2–99.2)	84 (77.2–90.1)
Rapid test	93 (89.2–96.4)	97 (92.0–99.1)	98 (95.0–99.5)	89 (82.5–94.0)

^aRapid test vs. DAT: $P=0.29$; ^bRapid test vs. DAT: $P=1.00$; ^cRapid test vs. DAT: $P=0.52$

^dRapid test vs. DAT: $P=0.25$. CI: Confidence interval

is composed of a membrane coated with a line of recombinant antigen K39 across the strip. Antibodies against the *Leishmania* present in a blood sample reacted with the K39 antigen, and their presence was evaluated with a mouse anti-human antibody conjugated to an indicator. A finger prick sample (10 μ L) of blood was added to a well and mixed with a drop of buffer. A test strip was placed vertically in the well, and the diluted blood migrated up the nitrocellulose strip. After the blood was completely wicked up, the strip was transferred to the next well, which contained a few drops of wash buffer, and was allowed to clear. The process took approximately 20 minutes, and the results were read visually. The test was positive when two red lines appeared in the middle of the nitrocellulose membrane, negative when only one red line appeared and invalid when no line was evident.

2.7. Data analysis

The database was constructed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA), and contained epidemiological and clinical characteristics of all patients and laboratory test results. Epi Info 6.04 software (Centers for Disease Control, Atlanta, GA, USA) was used to calculate the confidence intervals. Statistical analysis was performed using the χ^2 test for comparison of proportions. The significance level was fixed at $P<0.05$. Agreement between the serologic tests was evaluated with the Kappa test and interpreted according to Landis and Koch: 1.00–0.81 almost perfect, 0.80–0.61 substantial, 0.60–0.41 moderate, 0.40–0.21 fair and 0.20–0.0 slight.¹⁷

3. Results

A total of 332 patients (213 parasitologically confirmed VL cases and 119 non-cases with clinical suspicion of VL but with another confirmed etiology) were included in the analysis: 57 (17.2%) from Minas Gerais, 119 (35.8%) from Bahia, 121 (36.4%) from Piauí, and 35 (10.5%) from Maranhão. The median age of the patients was 13 years (one month to 76.8 years), and 58% were male. The median time for symptoms in the cases group was 53 days (5–360 days; DP=55.0), and for non-VL cases was 71 days (3–720 days, DP=136.0).

Patients with VL had the following signs and symptoms: weight loss (169/202; 83.6%), paleness (98/136; 72.1%), cough (85/206; 41.3%), diarrhea (57/205; 27.7%), abdominal pain (68/136; 49.6%), jaundice (28/204; 13.7%) and bleeding (31/204; 15.1%). The signs and symptoms presented by non-cases were as follows: weight loss (71/108; 65.7%), paleness (82/95; 86.3%), cough (45/109; 41.3%), diarrhea (24/109; 22.0%), abdominal pain (39/88; 44.3%),

Table 2

Kappa index between the direct agglutination test (DAT) and the rK39-based rapid immunochromatographic test.

Rapid test	DAT			Total
	Positive	Negative	Total	
Positive	182	21	203	
Negative	15	114	129	
Total	197	135	332	

Kappa: 0.77; Confidence interval 95%: 0.72–0.82

jaundice (25/112; 22.3%) and bleeding (25/108; 23.1%). A significantly higher frequency of weight loss was registered among the cases and of paleness among the non-cases ($P<0.05$).

The sensitivity of the DAT and the rapid test were 90% (CI: 85.3–94.0) and 93% (CI: 89.2–96.4), respectively, while the specificity of both tests were 96% (CI: 90.5–99.0) and 97% (CI: 92.0–99.1), respectively. Sensitivity and specificity were similar for both methods ($P>0.05$) (Table 1). The simulation of parallel testing showed sensitivity and specificity of 99.3% (CI: 95%: 97–99.9) and 93.1% (CI: 95%: 87.2–97), respectively. The simulation of sequential testing, first the rapid test and then the DAT, showed sensitivity and specificity of 84% (CI: 78.4–89) and 100% (CI: 98.2–100), respectively.

The positive predictive values (PPVs) of the DAT and the rapid test were 97% and 98%, respectively, and the negative predictive values (NPVs) were 84% and 89%, respectively. These results represent the totals for the four centers. There was no significant difference in PPVs and NPVs between both methods ($P>0.05$) (Table 1). The Kappa index demonstrated agreement classified as substantial (0.77) between the rapid test and the DAT (Table 2).

4. Discussion

The DAT is the serological method of choice for the diagnosis of VL in several countries. This method can be used in the field and requires minimal laboratory infrastructure. In the present study, the DAT showed sensitivity and specificity of 90% and of 96%, respectively. These results corroborate the data reported by Chappuis et al.,⁴ Pedras et al.,⁸ Sundar et al.¹¹ and Oliveira et al.,¹² who showed sensitivities ranging from 93.4–97% and specificities ranging from 81–98%.

The rapid test IT-LEISH® (Diamed Latino-America, Lagoa Santa, Minas Gerais, Brazil) was designed for the qualitative detection of antibodies against *Leishmania* spp. rK39 antigen. The rK39 contains a 39-amino acid repeat that is part of a 230-kDa protein predominant in members of the *L. donovani* complex.¹⁸ This commercial test can be performed

with blood obtained by a finger prick, does not require laboratory complex structure or expertise in its interpretation, and the results are available within 20 minutes. The rapid tests have also been validated in several endemic areas. In Uganda, the rapid test IT-LEISH® was introduced as a first-line test for patients presenting clinical suspicion of VL.¹⁹

In the current study, the rapid test showed a sensitivity of 93% and a specificity of 97%; these results corroborate the data reported by Carvalho et al.¹⁶ in a study in Brazil, the rapid test Kala-azar Detect® (InBios International, Seattle, WA, USA) demonstrated 90% sensitivity and 100% specificity. In another study performed in Brazil, Amato-Neto et al.¹⁵ evaluated the incidence of false-positive results using the rapid test IT-LEISH® in patients with confirmed Chagas disease and healthy volunteers, reporting sensitivity and specificity of 100%. The authors concluded that the rapid test is a useful diagnostic assay, pointing out that a false-positive result rarely occurs in patients with a serological diagnosis of Chagas disease.

Both tests showed satisfactory performance and could be used for the diagnosis of VL in Brazil. The DAT has the advantage of being a quantitative test, but it has drawbacks that make it less suitable as a test in the field. It requires more pipetting and several hours of incubation. The price of the rapid test ranges from about US\$2.00 per test, comparable to that of the DAT, to US\$8.00 per test (Arruda M, personal information, Ministry of Health of Brazil). According to Sundar et al.,¹¹ the rapid test currently appears to be the best available option for VL diagnosis under field conditions. In regions where laboratories are equipped to perform DAT successfully, this test can also be used. Either test, if positive in a person with clinical suspicion of VL, warrants specific treatment or other parasitological investigation, depending on the epidemiological and clinical presentation.

When analyzing the performance of both methods simultaneously, there was a gain in sensitivity (99%) and a loss in specificity (93%) compared with the performance of the methods individually. In endemic settings where the two methods can be performed, the simultaneous assessment would be recommended. This strategy would provide high reliability in treatment of the suspect patient. When the methods were evaluated sequentially, there was a loss in sensitivity (84%) and a gain in specificity (100%) compared with the performance of the methods individually. Thus, the evaluation of the methods in sequence would be useful to increase reliability to exclude the diagnosis of VL, even in the presence of suggestive clinical picture, when other etiologies need to be further investigated. Undoubtedly, the cost-effectiveness of those combined strategies needs evaluation.

In our previous study we evaluated the enzyme-linked immunosorbent assay (ELISA) using the rK39 antigen in the same group of patients included in the present study; the sensitivity of the ELISA was 97%, and the specificity was 84%.⁷ Analysis of the performance of the rapid test and the rK39-ELISA in sequence revealed sensitivity of 91% and specificity of 99%. There was a loss in sensitivity and improvement of specificity, which could represent a gain for the diagnosis of VL.

The high PPV found in this study is due to the high frequency of VL among patients attending the specialized services. Therefore, the data must be interpreted with caution and should not be extrapolated to situations of low prevalence scenarios or epidemiological surveys. Present data are not valid for patients who do not show clinical signs consistent with VL.

The present work was conducted taking into account the Standards for Reporting of Diagnostic Accuracy.²⁰ This study showed that both the DAT and the rapid test can be used for the diagnosis of VL in Brazil. The implementation of these tests in the health services should be carried out cautiously. As with any diagnostic test, a laboratory result is a part of the diagnostic decision made by an experienced and well-trained medical doctor. Therefore, the introduction of one of these methods requires an algorithm and should take into account the effectiveness, costs and their potential sustainability.

Authors' contributions: AR, GASR and TSMA conceived and designed the study; TSMA, ASCB, MJP, AB, ICS, CHNC, DLC, TAH, VYRS, MB, AJMC and EO carried out the clinical management and the laboratorial assays; TSMA, GASR and AR carried out the analysis and interpretation of the data and drafted the manuscript. All authors read and approved the final manuscript. AR is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: The study was conducted in agreement with the principles of the Helsinki Declaration and the Resolution 196/96 of the National Health Council of the Ministry of Health that regulates research involving human subjects in Brazil. The Research Ethics Committee of Centro de Pesquisas René Rachou - Fiocruz, Belo Horizonte, Minas Gerais, Brazil, Centro de Pesquisas Gonçalo Moniz - Fiocruz, Salvador, Bahia, Brazil, Universidade Federal do Piauí, Teresina, Piauí, Brazil and Universidade Federal do Maranhão, São Luís, Maranhão, Brazil, previously approved the informed consent forms and procedures.

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