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Comparative Study of Amplification Systems in Immunoenzyme Assays for the Diagnosis of American Tegumentary Leishmaniasis

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We compared the accuracy and reliability of three amplification systems for enzyme immunoassays in the detection of specific IgG antibodies for the diagnosis of cutaneous leishmaniasis caused by *Leishmania* (*Viannia*) *braziliensis* in patients from an endemic area in Rio de Janeiro, Brazil. Partially soluble antigens obtained from the promastigote forms of *L.* (*V.*) *braziliensis* were used. For development of the reaction, two chromogens, 1,2-orthophenylenediamine (OPD) and 3,3′,5,5′-tetramethylbenzidine (TMB), and a fluorogen, 4-methylumbelliferylphosphate (MUP), were tested. The performance of each system was compared using the following parameters: accuracy, intraclass correlation coefficient (ICC), and area under the receiver operating characteristic (ROC) curve. Sensitivity was the same (97.4%) for all systems. The reliability was excellent (ICC = 98.6, 98.7, and 99.1%) and specificity was 93.7, 95.8, and 97.4% for OPD, MUP, and TMB, respectively, showing no statistical significance. Despite the absence of differences in the performance of the three systems, the use of TMB is suggested because of its operational advantages, such as low cost compared with fluorogens, easy manipulation, greater stability, and lower toxicity. J. Clin. Lab. Anal. 23:152–156, 2009.

Key words: American tegumentary leishmaniasis; *Leishmania (Viannia) braziliensis*; immunoassay; amplification systems

INTRODUCTION

Leishmaniasis is a zoonosis caused by flagellate protozoans of the genus *Leishmania*. It is estimated that 350 million people worldwide are at risk of contracting the disease and 12 million are infected (1). The cutaneous form is endemic in 88 countries. However, 90% of cases occur in only seven countries (Afghanistan, Saudi Arabia, Brazil, Iran, Peru, Saudi Arabia and Syria), with an annual incidence of 2 million new cases (1). In the New World, 12 *Leishmania* species have been recognized. In Brazil, the three main dermotropic species are *Leishmania* (*Vianna*) *braziliensis*, which shows a broad geographic distribution and is responsible for most cases that occur outside the Amazon region, *L.* (*V.*) *guyanensis*, which predominates in the Amazon region, and *L.* (*Leishmania*) *amazonensis*, whose occurrence is rare (2). In Brazil, the number of

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cases of American tegumentary leishmaniasis (ATL) has been increasing in practically all states over the last 20 years, with more than 30,000 annual cases (3).

The clinical presentation of ATL is related in part to the *Leishmania* species involved and ranges from self-resolving or persistent skin lesions to disfiguring and disabling mucosal lesions (4,5).

The definitive diagnosis of ATL is based on the isolation of the parasite in culture, its visualization upon histopathological analysis or in imprints (3), or on the detection of parasite DNA by the polymerase chain reaction (6,7). However, these techniques are expensive, require invasive procedures for sample collection, and are relatively time consuming.

The humoral response in ATL is characterized by low serum antibody levels and cross-reactions with other infectious diseases (8,9). However, serological techniques such as indirect immunofluorescence and enzyme immunoassays for the detection of anti-*Leishmania* antibodies have been shown to be useful in epidemiological studies owing to their easy and rapid execution, automatization, and low cost (10–12). The performance of enzyme immunoassays is similar to that of indirect immunofluorescence (13,14) when soluble *Leishmania* extracts are used as antigen (10,15). However, immunoassays need to be improved in terms of different aspects, such as the ideal antigen preparation, positivity thresholds of the samples, and the amplification system used (16).

The detection threshold of the amplification system can affect the performance of immunoenzymatic methods. Generally, chromogens are sufficiently sensitive. In ATL, procedures able to amplify the enzymatic signal need to be improved, such as reagents that generate fluorescent or luminescent products, which can be detected even in extremely small quantities (17–19).

In this study, we compared the accuracy of three amplification systems for enzyme immunoassays in the detection of specific IgG antibodies for the diagnosis of ATL: two chromogens, 1,2-orthophenylenediamine (OPD) and 3,3′,5,5′-tetramethylbenzidine (TMB), and a fluorogen, 4-methylumbelliferylphosphate (MUP).

MATERIALS AND METHODS

A cross-sectional masked diagnostic study was conducted with independent repetitions for comparison of the three amplification systems in enzyme immunoassays for the diagnosis of ATL using a random sample of patients seen at the outpatient clinic of Reference Center of Leishmaniasis, Evandro Chagas Clinical Resarch Institute (IPEC/FIOCRUZ), between January 2003 and July 2005. The patients signed a free informed consent form and the study was approved by the Ethics Committee of IPEC/FIOCRUZ (protocol 0015.0.009.000-06).

The size of the sample was 378 subjects divided into two groups of 189 each (cases vs. controls), considering an expected sensitivity of 84%, a 5% limit of equivalence difference between methods, a level of significance of 5%, and a power of 80% using a sample size formula for the equivalence of two proportions.

For the study, only the first serum sample collected from patients with ATL originating from an area of occurrence of *L.* (*V.*) *braziliensis* in the State of Rio de Janeiro, whose parasitological diagnosis was positive by isolation in culture, imprint, or histopathology, was considered. The control group consisted of serum samples from patients with other skin diseases that require a differential diagnosis from ATL, according to the investigation protocol (20): sporotrichosis ($n = 158$), pyodermitis ($n = 14$), vascular ulcer ($n = 11$), neoplasm ($n = 3$), dermatophytosis ($n = 1$), syphilis ($n = 1$), and paracoccidioidomycosis ($n = 1$).

For the analysis of reliability, the same examiner performed two measurements in each group (189 cases and 189 controls) at different times for the three amplification systems.

Antigens

Partially soluble antigens obtained by the culture of promastigote forms of *L.* (*V.*) braziliensis (MHOM/BR/75/M2903) in brain heart infusion broth (Difco, Detroit, MI), supplemented with 10% fetal bovine serum, 200 U/mL of penicillin, 20 μg/mL of streptomycin, and 1% human urine (21), were used. Applying an initial inoculum of 1×10^6 parasites/mL, promastigotes were collected in the logarithmic phase and washed three times in phosphate-buffered saline (PBS), pH 7.2. For complete rupture of the parasites, the sediment was resuspended in lysis buffer containing protease inhibitors (1 mM iodoacetamine, 1 mM phenylmethylsulphonyl fluoride, and 1 mM phenanthroline) and then submitted to 30 freeze–thaw cycles on dry ice and in a water bath at 60°C and 2 hr of ultrasound (50/60 Hz; Transsonic 310, Elma, Singen, Germany). The sample was centrifuged at 10,000*g* for 5 min at 4°C and the protein content of the supernatant was determined by the Folin–Lowry method (micro-Lowry Total Protein Determination kit, Peterson's modification; Sigma Chemical Co., St. Louis, MO).

Enzyme Immunoassay

The method described by Voller et al. (14) was used. Briefly, 96-well polystyrene plates (catalog No. 436110 for the fluorogen and catalog No. 439454 for the chromogen, Nunc Maxisorp, Nalgene Nunc

International, Rochester, MN) were sensitized with 100 μL of partially soluble total antigen at a protein concentration of 5 μg/mL diluted in carbonate–bicarbonate buffer, pH 9.6, and then incubated overnight (18 hr) at 4°C. After four washes in PBS, pH 7.2, and 0.05% Tween 20 (PBS-T), 100 μL of the serum samples diluted 1:40 in PBS-T containing 1% skim milk (PBS-TM) was added to each well and the plates were incubated for 45 min at 37°C in a humid chamber. After four additional washes, 100 μL of peroxidase-conjugated anti-IgG (γ-specific) (A3187, Sigma, St. Louis, MO), diluted 1:4,000 for OPD and 1:12,000 for TMB in PBS-TM, or alkaline phosphataseconjugated anti-IgG (A3150, Sigma), diluted 1:150,000 for MUP in PBS-TM was added. After incubation at 37°C for 45 min in a humid chamber and four new washes, 100 μL of the developing solution was added: OPD (P6912, Sigma) (10 mg OPD+10 μL of hydrogen peroxide+25 mL of citrate–phosphate buffer, pH 5.0); TMB (T0440, Sigma), and MUP (M6491, Invitrogen-Molecular Probes, Carlsbad, CA) (0.1 mM MUP in diethanolamine buffer, pH 9.8).

The plates were then incubated for 15 min (OPD) or 30 min (TMB and MUP) in the dark and the reaction was stopped by the addition of 50 μL of 1 N H_2SO_4 (OPD and TMB) or 100 μL of 3 N NaOH (MUP) to each well. The optical density (OD) and fluorescence (relative fluorescence units, RFU) of the wells were determined in a plate reader (Genius, Tecan, Salzburg, Austria). Filters with a wavelength of 492 and 450 nm were used for reading the plates containing the chromogens OPD and TMB, respectively, and filters with an emission at 465 nm and excitation at 360 nm were used for the fluorogen MUP.

Statistical Analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio (LR) and their respective 95% confidence intervals (CI) were calculated using the Statistical Package for the Social Science (SPSS), version 11.0 (SPSS Inc., Chicago, IL). The reliability of the enzyme immunoassay was analyzed using the intraclass correlation coefficient (ICC) with its 95% CI.

Cut-off values were established for each immunoassay according to the amplification system used. The results are expressed as OD and RFU and were treated as continuous variables using a receiver operating characteristic (ROC) curve. Analysis of variance was used for comparison of the performance of the different tests by means of their area under the ROC curve (AUC) using the MedCalc 8.3 software (MedCalc Software, Mariakerke, Belgium).

RESULTS

The cut-off points defined by the ROC curve for OPD and TMB, expressed as OD, were 0.203 and 0.239, respectively. For the fluorogen MUP, the cut-off expressed as RFU was 11,103.

The accuracy parameters indicated an identical sensitivity (97.4%) for the three systems and specificity of 93.7, 97.4, and 95.8% for OPD, TMB, and MUP, respectively. A slightly higher specificity was obtained with the use of TMB when compared with OPD ($p = 0.082$) and MUP ($p = 0.397$), with a PPV and NPV of 97.4%. The negative LR was the same for the three systems. For TMB, the LR for a positive test (LR+) was 36.8, i.e., there was a 36.8 higher chance of finding a positive result in the test in patients with the disease compared with those without the disease (Table 1).

The area under the curve (95% CI) was the same for OPD and TMB, i.e., 0.984 (0.966; 0.994 and 0.965; 0.994, respectively), and 0.986 for MUP (0.968; 0.995), demonstrating a small difference, which, however, was not statistically significant. As can be seen in Figure 1, the AUC overlap irrespective of the type of system used.

The ICC and its respective 95% CI was 0.993 (0.991; 0.994) for OPD, 0.996 (0.995; 0.996) for TMB, and 0.994 (0.992; 0.995) for MUP. The reliability of the three systems was considered to be excellent.

TABLE 1. Sensitivity, Specificity, PPV, NPV, LR+, and LR− and Their Respective 95% Confidence Intervals Obtained for Each Amplification System in the Enzyme Immunoassays

System	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	LR+	LR−
OPD	97.4 (93.9; 99.1)	93.7 (89.2; 96.7)	93.9 (89.3; 96.9)	97.3 (93.4; 98.9)	15.3	0.03
TMB	97.4 (93.9; 99.1)	97.4 (93.9; 99.1)	97.4 (92.9; 98.7)	97.4 (93.6; 99.0)	36.8	0.03
MUP	97.4 (93.9; 99.1)	95.8 (91.8; 98.1)	95.8 (91.7;98.0)	97.3 (93.5; 99.0)	23.0	0.03

PPV: positive predictive value; NPV: negative predictive value; LR+: positive likelihood ratio; LR−: negative likelihood ratio; CI: confidence interval.

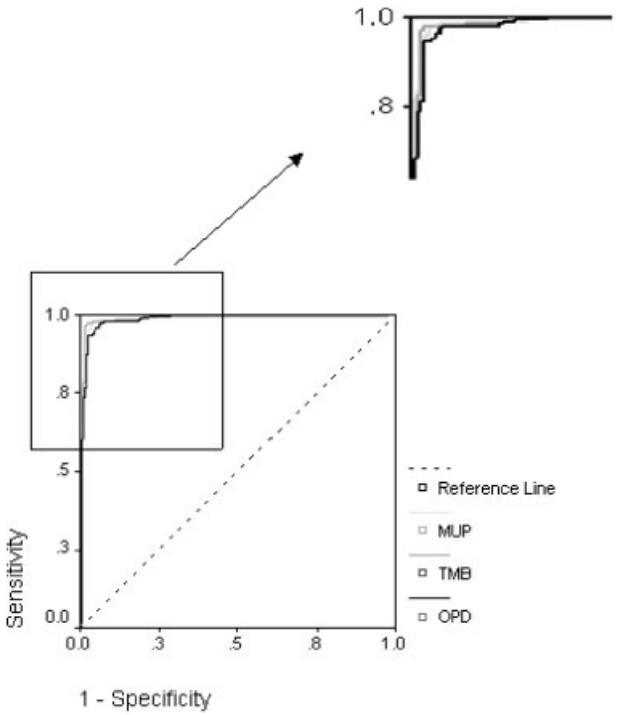

Fig. 1. Area under the ROC curve obtained for the three amplification systems.

DISCUSSION

Although developing solutions are an essential tool for enzyme immunoassays and numerous types are available on the market, studies evaluating these reagents are rare (22–24). We found no studies with this design in the literature investigating anti-*Leishmania* antibodies for the diagnosis of infection caused by this agent.

Using an antigen preparation of *L.* (*V.*) *braziliensis*, the predominant species in Rio de Janeiro, in our immunoassays we obtained optimal accuracy parameters, irrespective of the reagent used. This result agrees with previous studies that also demonstrated an increased sensitivity of the assays when homologous instead of heterologous antigens were used (25–27).

Sensitivity was the same for the three amplification systems used (OPD, TMB, and MUP). Similar results have been reported by Crowther et al. (23) who used the same reagents for the detection of foot-and-mouth disease virus, and by Shekarchi et al. (22) who used the chromogen *p*-nitrophenyl phosphate and the fluorogen MUP for the detection of rubella virus antibody and herpes simplex virus antigen. However, our results are in contrast to the findings of Shalev et al. (28) and Avrameas (17) who demonstrated an increased sensitivity with the use of fluorogens, and of Roberts et al. (24) who used the fluorogen 3-*p*-hydroxyphenyl propionic acid for the detection of anti-HIV antibodies compared with TMB and MUP.

Despite a similar accuracy, Shekarchi et al. (22) recommended the use of a fluorogen for the detection of rubella virus antibody and herpes simplex virus antigen based on the shorter incubation time necessary for development of the reaction with MUP in both cases and on the use of a lower antigen concentration for the detection of anti-rubella antibody. We observed no relevant differences in the incubation time or *L.* (*V.*) *braziliensis* antigen concentration (5 μg/mL) between the systems tested (data not shown).

Disadvantages of the use of the fluorogen MUP are the rapid decline in the emission of the fluorescence signal, requiring immediate reading, and the high cost of the appropriate equipment for the measurement of fluorescence intensity (23).

Interestingly, we observed small differences in specificity between the three reagents, with the highest specificity being obtained for TMB, although the difference was not significant.

Aspects of the robustness of this study design were randomization of the samples, masking of the assays, and maximization of internal representativeness by ensuring that the control samples were obtained from the same outpatient clinic as the cases and therefore shared the initial clinical suspicion of ATL. One limitation of this study was the lack of evaluation of reproducibility, i.e., analysis of the same samples by another laboratory. However, we evaluated repeatability and obtained excellent rates similar to those reported by Barroso-Freitas et al. (27) for an ELISA using *L.* (*V.*) *braziliensis* antigen.

As no difference in the accuracy of the amplification systems was observed in the model proposed, other parameters are considered for the choice of the adequate reagent. In this respect, some investigators proposed the use of TMB because it is highly stable and not mutagenic and/or carcinogenic (29), is less expensive than fluorogens, is easily manipulated (30), and is commercially available in the form of kits (30,31). In addition, TMB is more sensitive than OPD whose mutagenic properties have been demonstrated in the Ames test (a test used to detect the cytotoxicity and mutagenicity of chemical compounds) (32,33).

In view of the above considerations and the similar accuracy of the three amplification systems, we suggest the use of TMB in enzyme immunoassays for the diagnosis of ATL in our laboratory.

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