

Evaluation of the reference value for the Montenegro skin test

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ABSTRACT

Introduction: The Montenegro skin test (MST) has good clinical applicability and low cost for the diagnosis of American tegumentary leishmaniasis (ATL). However, no studies have validated the reference value (5mm) typically used to discriminate positive and negative results. We investigated MST results and evaluated its performance using different cut-off points. **Methods:** The results of laboratory tests for 4,256 patients with suspected ATL were analyzed, and 1,182 individuals were found to fulfill the established criteria. Two groups were formed. The positive cutaneous leishmaniasis (PCL) group included patients with skin lesions and positive direct search for parasites (DS) results. The negative cutaneous leishmaniasis (NCL) group included patients with skin lesions with evolution up to 2 months, negative DS results, and negative indirect immunofluorescence assay results who were residents of urban areas that were reported to be probable sites of infection at domiciles and peridomiciles. **Results:** The PCL and NCL groups included 769 and 413 individuals, respectively. The mean \pm standard deviation MST in the PCL group was 12.62 ± 5.91 mm [95% confidence interval (CI): 12.20-13.04], and that in the NCL group was 1.43 ± 2.17 mm (95% CI: 1.23-1.63). Receiver-operating characteristic curve analysis indicated 97.4% sensitivity and 93.9% specificity for a cut-off of 5mm and 95.8% sensitivity and 97.1% specificity for a cut-off of 6mm. **Conclusions:** Either 5mm or 6mm could be used as the cut-off value for diagnosing ATL, as both values had high sensitivity and specificity.

Keywords: Cutaneous leishmaniasis. Montenegro skin test. Cut-off value. Diagnosis.

INTRODUCTION

Leishmaniasis can affect the skin, mucous membranes, and internal organs in humans and other mammals. There are 310 million people in 6 countries at risk of infection, with 1 million cases of cutaneous leishmaniasis reported in the last 5 years, 300,000 estimated cases of visceral leishmaniasis, and over 20,000 deaths annually⁽¹⁾. In the Americas, cutaneous leishmaniasis occurs in the geographic area ranging from southern United States to northern Argentina, with the exception of Chile and Uruguay.

American tegumentary leishmaniasis (ATL) is widely distributed from northern to southern Brazil. From 1990 to 2013, 635,399 cases of ATL were reported in Brazil, 13,889 from the southern region and 95% from the State of Paraná, from

276 of the 399 municipal districts^{(2) (3)}. Deforestation, urbanization, and the consequent invasion of vectors in the peridomicile have increased the incidence of ATL⁽⁴⁾. The main species of *Leishmania* that cause ATL in Brazil are *Leishmania (Viannia) braziliensis*, *Leishmania (Viannia) guyanensis*, and *Leishmania (Leishmania) amazonensis*⁽⁴⁾.

The diagnosis of ATL is based on epidemiological, clinical, and laboratory data, including parasitological and immunological techniques^{(5) (6) (7)}. An association between two or more techniques provides a more valid diagnosis of ATL. The direct search (DS) for parasites is performed by identifying amastigote forms in smears of the lesion. However, sensitivity decreases with lesion progression. The immunological methods include the Montenegro skin test (MST) to detect delayed hypersensitivity and indirect immunofluorescence (IIF) and enzyme immunoassay (EIA) to search for antibodies. The antibody search tests are sensitive and specific, but they are associated with cross-reactivity with other diseases, mainly Chagas disease and visceral leishmaniasis^{(6) (7)}.

The MST was introduced in 1926 by Montenegro to diagnose ATL with good clinical applicability and low cost⁽⁸⁾. The antigen is inoculated in the forearm of the patient, and the reaction is read after 48h⁽⁵⁾. The reaction is characterized by induration at the inoculation site due to abundant infiltration of lymphocytes and macrophages⁽⁹⁾. A diameter of ≥ 5 mm is

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considered to indicate a positive reaction. The MST is useful in cases in which the parasites are scarce or absent (i.e., when the ATL lesion has undergone more than 2 months of evolution) and in epidemiological surveys in endemic areas⁽⁵⁾.

The criteria for defining a positive result on skin tests for delayed hypersensitivity vary among authors, but a positive test result is considered when the induration diameter is $\geq 5\text{mm}$ ⁽¹⁰⁾. Similarly, with the MST, authors have considered diameters $\geq 5\text{mm}$ as a positive test result^{(8) (9) (11) (12) (13) (14) (15) (16) (17)}. Reaction diameters $\geq 6\text{mm}$ have also been reported^{(18) (19)}. However, no studies have validated the reference value (5mm) that is typically used to discriminate positive and negative MST results for ATL. The objective of the present study was to analyze MST results and evaluate different cut-off values for the diagnosis of ATL.

METHODS

In this retrospective, descriptive, analytical study, we analyzed laboratory test results of patients with suspected American tegumentary leishmaniasis (ATL) who were referred by the 15th Regional Health Division of the State of Paraná and attended the *Laboratório de Leishmanioses* of the *Laboratório de Ensino e Pesquisa em Análises Clínicas, Universidade Estadual de Maringá* (LEPAC/UEM) between June 1999 and February 2014. The available epidemiologic data included sex, age, place of residence and work (rural or urban), lesion characteristics, number of lesions, and duration of lesion evolution. The tests used for laboratory diagnosis included DS for parasites by microscopy, IIF, and the MST.

The DS was performed with tissue obtained by scarification of the edge of the lesion, which was smeared on a slide and stained with Giemsa. Indirect immunofluorescence was performed with *L. (V.) braziliensis* promastigotes and anti-human immunoglobulin G conjugated to fluorescein, with positive titers considered as ≥ 40 . The MST used dead *L. (L.) amazonensis* promastigotes that contained 40 μg protein nitrogen/mL supplied by the *Centro de Produção e Pesquisa em Imunobiológicos* (CPPI), State of Paraná, Brazil. Briefly, 0.1mL of the antigen was intradermally injected into the anterior surface of the forearm. The induration diameter at the injection site was measured 48h later using a millimeter-graduated ruler and ballpoint pen⁽⁶⁾.

Statistical analysis

For analysis, patients who underwent the MST were selected based on the presence of skin lesions. To evaluate the cut-off point for the MST, two groups were formed based on laboratory results and clinical and epidemiological data according to criteria that define the epidemiologic characteristics of ATL in Northwestern Paraná⁽²⁰⁾. The positive ATL [positive cutaneous leishmaniasis (PCL)] group included patients with skin lesions and positive DS results. The negative ATL [negative cutaneous leishmaniasis (NCL)] group included patients with skin lesions of an evolution up to 2 months, negative DS results, and negative IIF results who were residents in urban areas and reported to be probable sites of infection at domiciles and peridomiciles.

The data were plotted in Microsoft Excel 2010 spreadsheets. Quantitative data are described using mean \pm standard deviation.

For comparisons of means, Student's *t*-tests were used. The Z test was used to compare proportions. Sensitivity and specificity values were used to construct receiver-operating-characteristic (ROC) curves to analyze different reference values (cut-off values) for all of the patients. Statistical analysis was performed using Statistica 8.0 software, with a 5% level of significance ($p < 0.05$).

Ethical considerations

The procedures followed the Resolution 466/2012 of the National Health Council of the Brazilian Ministry of Health, and ethical approval was obtained from the *Comitê Permanente de Ética em Pesquisa Envolvendo Seres Humanos* of the *Universidade Estadual de Maringá* (Report 744.556, July 14, 2014). The procedures followed were in keeping with the Helsinki Declaration of 1964, as revised in 1975, 1983, 1989, 1996, and 2000.

RESULTS

Of the 4,256 patients initially evaluated, 1,182 were selected for inclusion (**Figure 1**) based on the epidemiological data and laboratory results. These patients had a mean age of 40.34 ± 17.80 years and mean MST induration diameter of $8.71 \pm 7.27\text{mm}$ (**Table 1**). Patient age in the PCL group ($n = 769$) was 39.58 ± 16.38 years (range, 1-86 years) and that in the NCL group ($n = 413$) was 41.76 ± 20.12 years (range, 1-90 years). The MST induration diameters of the PCL and NCL groups were $12.62 \pm 5.91\text{mm}$ (range, 0-44mm) and $1.43 \pm 2.17\text{mm}$ (range, 0-15mm), respectively ($p < 0.01$).

The PCL group had more men (632/769) than women (137/769; $p < 0.01$). The mean age of the men (39.82 ± 15.83 years) was similar to that of women (38.50 ± 18.76 years; $p = 0.32$). The mean induration diameter in men ($12.51 \pm 5.73\text{mm}$) was also similar to that of women ($13.11 \pm 6.67\text{mm}$; $p = 0.28$). The numbers of men and women in the NCL group were similar (210/413 and 203/413, respectively; $p = 0.68$), as were the mean ages of men and women (42.05 ± 20.20 years and 41.45 ± 20.10 years; $p = 0.76$). The mean induration diameter in men ($1.65 \pm 2.57\text{mm}$) was significantly different from that in women ($1.20 \pm 1.64\text{mm}$; $p = 0.03$).

Based on ROC curve analysis (**Figure 2**), the sensitivity and specificity for a 5mm cut-off value were 97.4% and 93.9%, respectively. The sensitivity and specificity for a 6mm cut-off value were 95.8% and 97.1%, respectively.

Receiver-operating-characteristic curves are shown according to sex in **Figure 3**. The best curve was found for women who had higher specificities for the two cut-off points. **Figure 4** shows the ROC curves according to age (0-14 years, 15-29 years, 30-49 years, and ≥ 50 years). The best curve was observed for patients who were 0-14 years old.

DISCUSSION

Since June 1999, LEPAC/UEM has performed the MST with an antigen from the Brazilian Ministry of Health that is produced in large scale according to good-practice standards of the *Centro de Produção e Pesquisa em Imunobiológicos*⁽²¹⁾

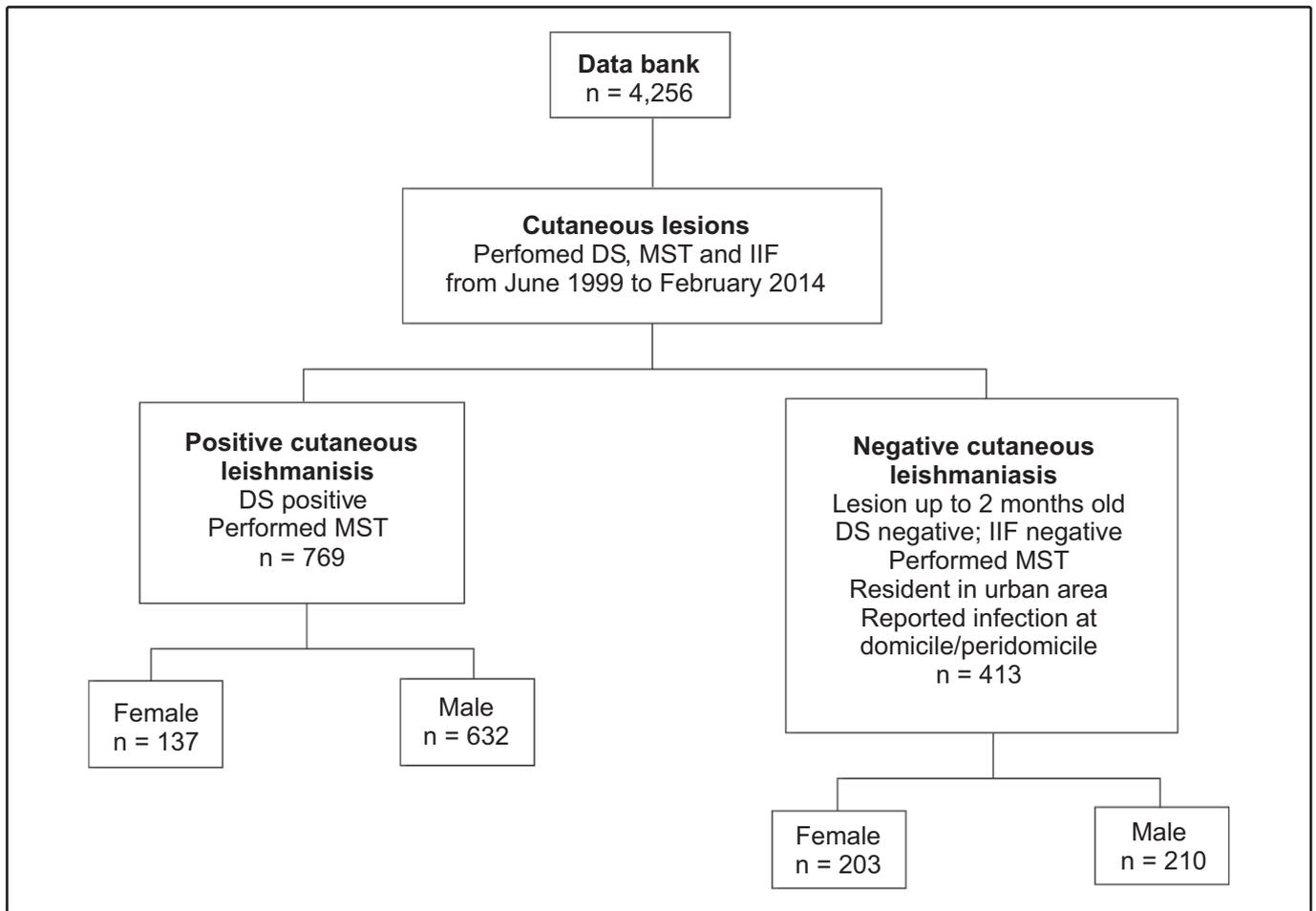


FIGURE 1 - Selection of groups after analysis from *Laboratório de Leishmanioses* of the LEPAC/UEM in Maringá, State of Paraná, Brazil, 1986 to 2014. DS: direct search of parasite; MST: Montenegro skin test; IIF: indirect immune fluorescence; LEPAC/UEM: *Laboratório de Ensino e Pesquisa em Análises Clínicas, Universidade Estadual de Maringá*.

of the *Secretaria de Estado da Saúde do Paraná*, Brazil. The importance of standardizing the application and reading of the MST has been highlighted by several authors. The test must be performed by a trained technician to ensure comparable results and avoid common causes of variation in the execution and interpretation of the tests⁽²²⁾ ⁽²³⁾. Patients who attend LEPAC/UEM always undergo a standard protocol that ensures the quality of test performance.

American tegumentary leishmaniasis is known to affect more individuals who live in rural areas. Ninety percent of patients have lesion times up to 2 months, during which time most patients show positive MST and DS results. Thus, the PCL group consisted of patients with positive DS results, and the NCL group consisted of patients with a lesion time up to 2 months and negative DS and IIF results who resided in urban areas. This strategy for the NCL group was employed because the MST, which applies *Leishmania* antigens, is not recommended for normal individuals; it could lead to sensitization and false-positive results if the MST is required in the future. In healthy subjects who underwent the MST and had tested negative in

a previous study, 50% had a positive test when the MST was performed again after 90 days, and γ -IFN produced by peripheral blood mononuclear cells stimulated with *Leishmania* antigens was detected in 78% of subjects 360 days after the initial MST⁽²⁴⁾. However, other authors observed no positivity with the MST with a ≥ 1 -year interval between two tests⁽²²⁾.

Comparisons between the PCL and NCL groups showed differences in the mean age of the individuals and the induration diameter. The latter was expected because the PCL group had a parasitological diagnosis of ATL. In addition, the PCL group consisted of more men than women, which is consistent with the literature that indicates that men engage in more rural leisure activities, such as fishing, than women⁽²⁰⁾ ⁽²⁵⁾. Within the PCL group, the mean age and mean induration diameter were not different between the sexes. Similarly, the number of men and women and their mean ages were similar within the NCL group; however, the mean induration diameter was higher in men.

The ROC curve that was constructed for the PCL and NCL groups indicated that the MST cut-off could be either 5mm (97.4% sensitivity and 93.9% specificity) or 6mm

TABLE 1 - Characteristics of 1,182 patients (PCL and NCL groups) according to sex, age, and diameter with the Montenegro skin test.

Groups	Age (years)			Induration (mm)		
	range	mean \pm SD	95% CI	range	mean \pm SD	95% CI
PCL (n = 769)	1-86	39.58 \pm 16.38	38.42-40.74	0-44	12.62 \pm 5.91	12.20-13.04
women (n = 137)	3-86	38.50 \pm 18.76	35.33-41.67	1-44	13.11 \pm 6.67	11.11-14.23
men (n = 632)	1-81	39.82 \pm 15.83	36.58-41.06	0-42	12.51 \pm 5.73	12.07-12.96
NCL (n = 413)	1-90	41.76 \pm 20.12	39.81-43.70	0-15	1.43 \pm 2.17	1.23-1.63
women (n = 203)	1-90	41.45 \pm 20.10	38.67-44.23	0-9	1.20 \pm 1.64	0.97-1.42
men (n = 210)	4-84	42.05 \pm 20.20	39.30-44.80	0-15	1.65 \pm 2.57	1.30-2.00
Total (n = 1,182)	1-90	40.34 \pm 17.80	39.33-41.36	0-44	8.71 \pm 7.27	8.3-9.12

PCL: positive cutaneous leishmaniasis; NCL: negative cutaneous leishmaniasis. SD: standard deviation; 95% CI: 95% confidence interval.

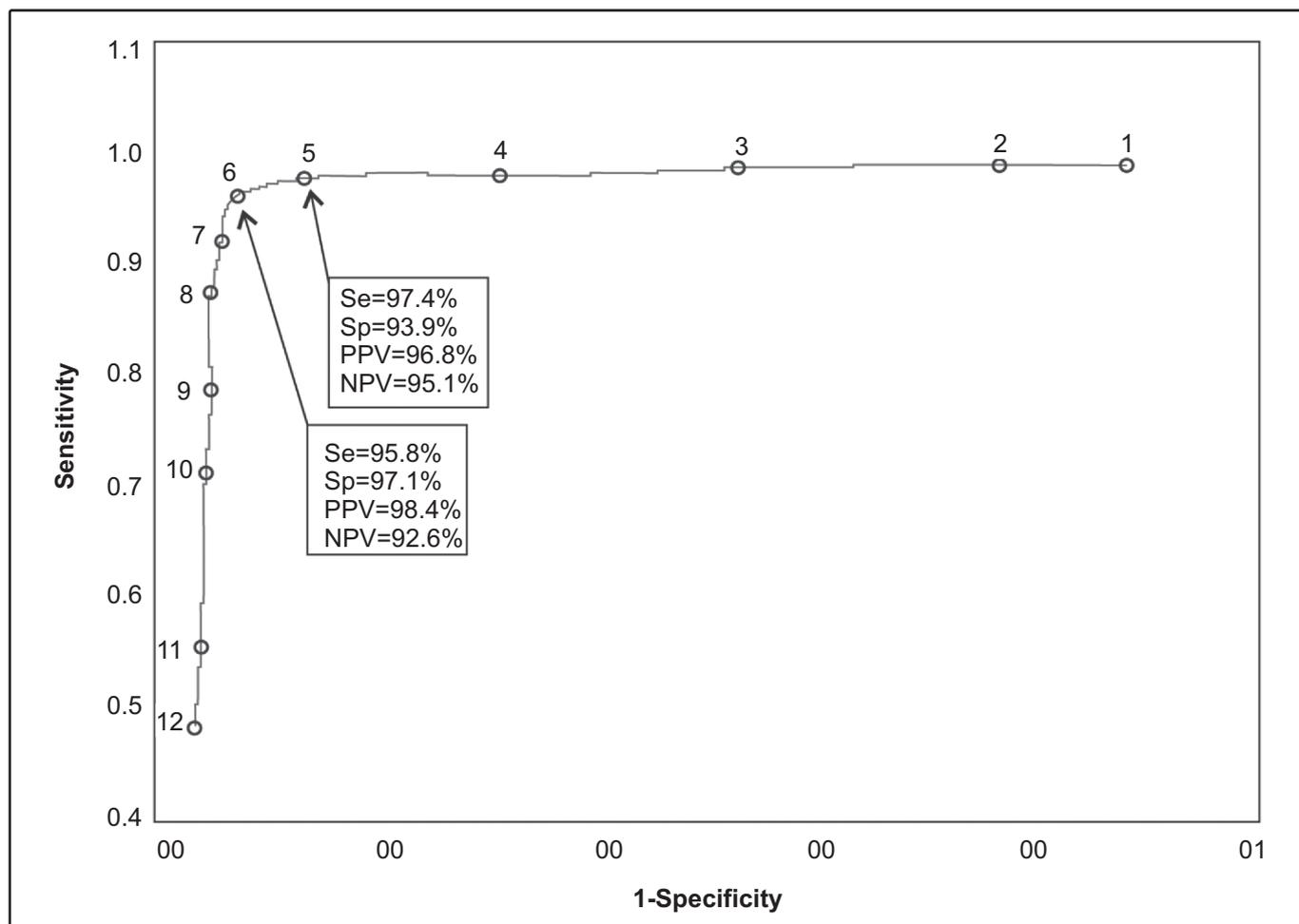


FIGURE 2 - Receiver-operating-characteristic curve for 1,182 patients in the positive cutaneous leishmaniasis and negative cutaneous leishmaniasis groups who attended LEPAC/UEM between June 1999 and February 2014. Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LEPAC/UEM: *Laboratório de Ensino e Pesquisa em Análises Clínicas, Universidade Estadual de Maringá.*

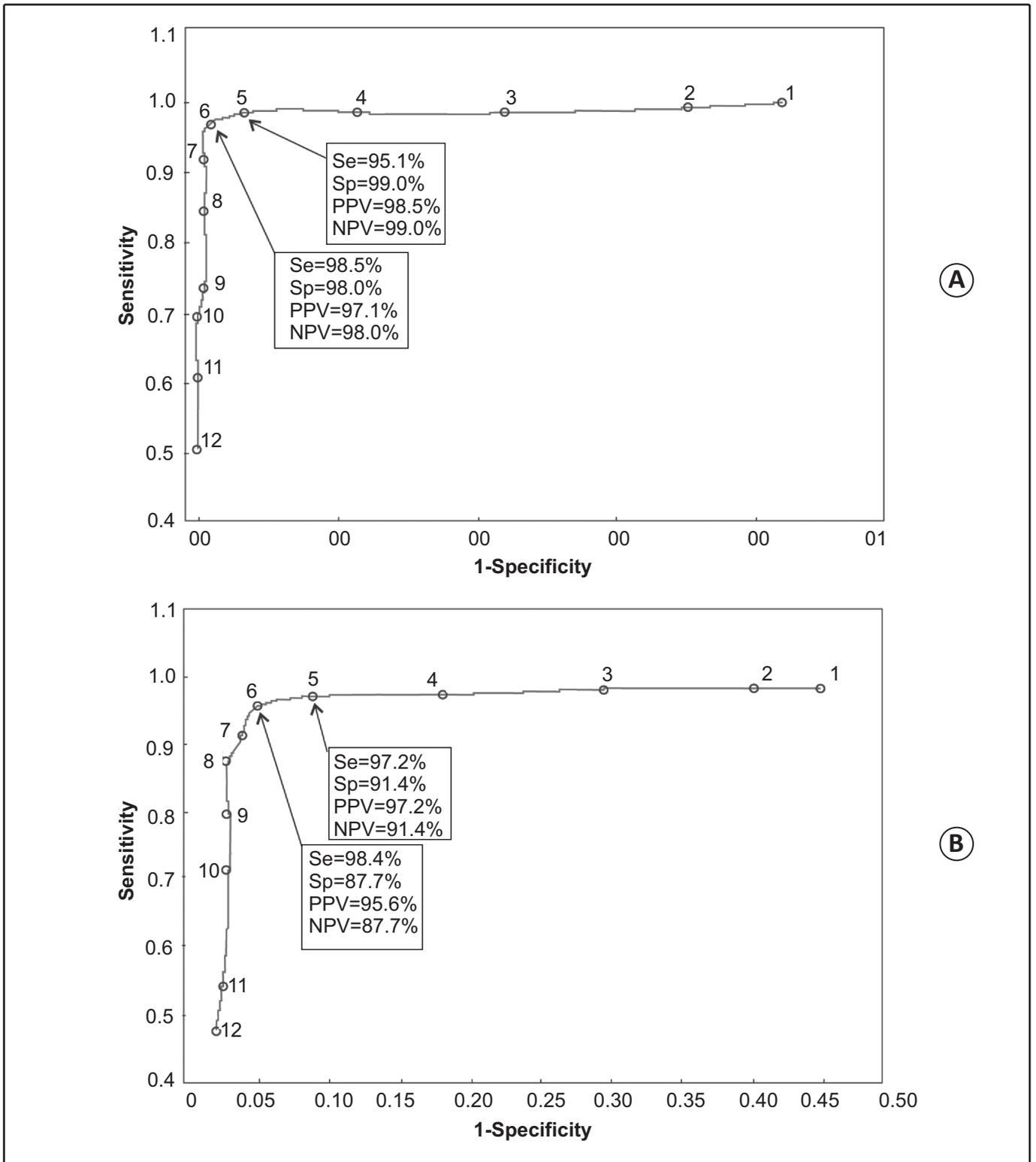


FIGURE 3 - Receiver-operating-characteristic curve for 1,182 patients in the positive cutaneous leishmaniasis and negative cutaneous leishmaniasis groups who attended LEPAC/UEM between June 1999 and February 2014 for (A) women (n = 340) and (B) men (n = 842). Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LEPAC/UEM: *Laboratório de Ensino e Pesquisa em Análises Clínicas, Universidade Estadual de Maringá*.

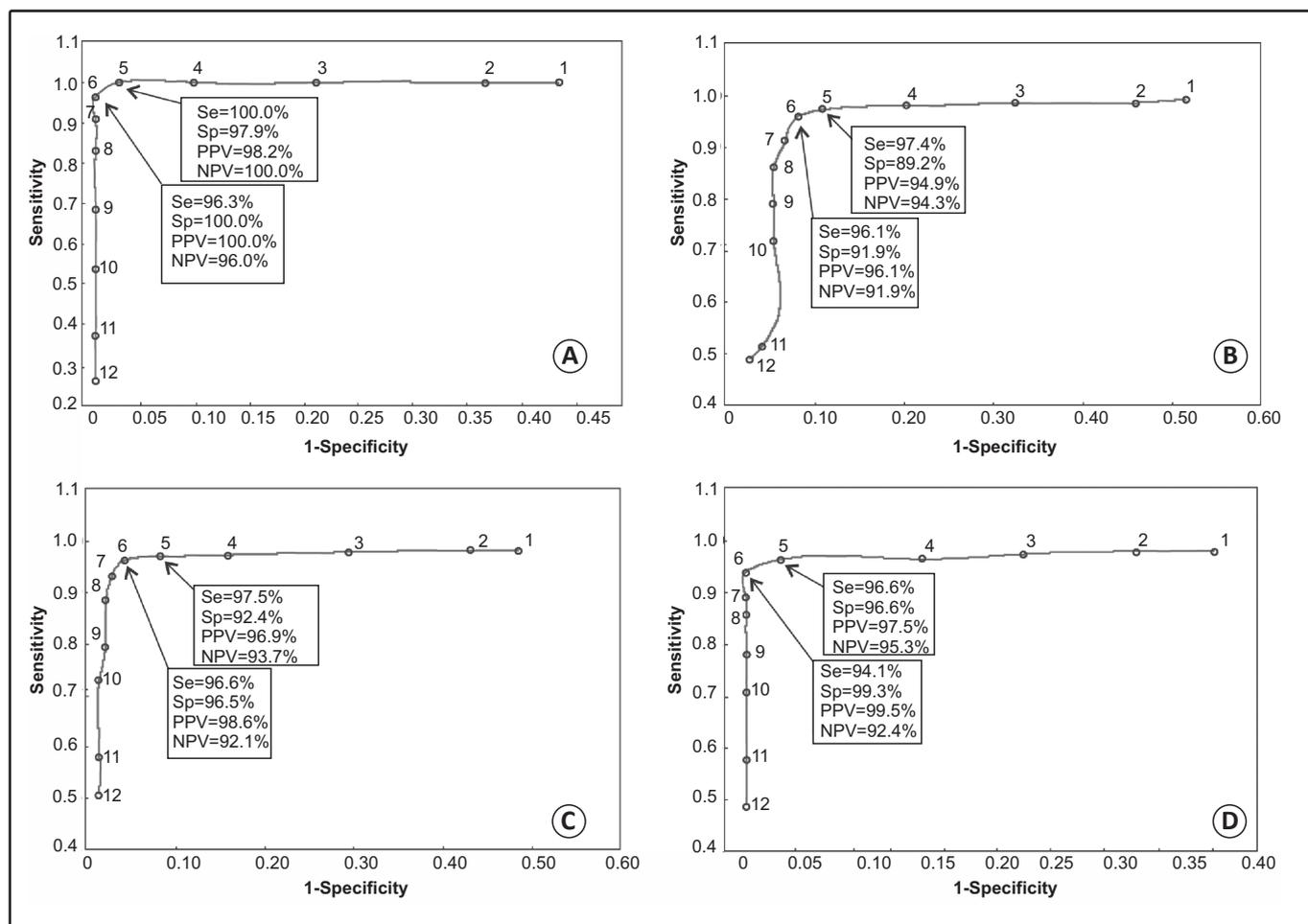


FIGURE 4 - Receiver-operating-characteristic curve for 1,182 patients in the positive cutaneous leishmaniasis and negative cutaneous leishmaniasis groups who attended LEPAC/UEM between June 1999 and February 2014 for patients aged: A. 0-14 years (n = 102); B. 15-29 years (n = 228); C. 30-49 years (n = 502), and D. ≥ 50 years (n = 350). Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; LEPAC/UEM: *Laboratório de Ensino e Pesquisa em Análises Clínicas, Universidade Estadual de Maringá*.

(95.8% sensitivity and 97.1% specificity). Previous studies that were conducted by our research group using a 6mm cut-off value for MST found 96% sensitivity among patients with positive DS results and 95.1% sensitivity among patients with positive MST, DS, and IIF results⁽¹⁹⁾. Comparatively, 90.5% sensitivity was reported in patients with a positive DS⁽¹⁴⁾, and 78.2% sensitivity and 77.4% specificity were reported in patients who attended a hospital in Peru⁽²⁶⁾. Positive MST results were found in 93.3% of patients who had positive direct microscopic examination⁽²⁷⁾ and 84.4% of confirmed ATL cases from State of Paraná⁽²⁸⁾.

In the NCL group, six (1.5%) patients had induration diameters ≥ 10 mm with the MST. In endemic areas, a positive MST can be interpreted as prior leishmaniasis, prior injection of the antigen used in the test, exposure to the parasite without disease (infection), allergy to the test dilution agent, or cross-reactions with other diseases (e.g., Chagas disease, sporotrichosis, Virchowian leprosy, tuberculosis, chromomycosis, or others)⁽⁶⁾. Patients with sporotrichosis (12%)

had an induration diameter ≥ 10 mm with the MST⁽²⁹⁾. In a non-endemic region, almost 30% of the individuals had positive reactions with the MST⁽³⁰⁾.

For lesions lasting up to 1 month, the sensitivity was 97.5% (5mm cut-off) or 94.9% (6mm cut-off). For durations of 1 and 2 months, the sensitivity was 98.6% (5mm cut-off) or 97.1% (6mm cut-off). Previous studies have reported 78% sensitivity for cutaneous lesions with a duration of up to 2 weeks and 94% and 98% sensitivities for durations of 4 and 6 weeks, respectively, in patients with positive parasitological tests; however, the MST was repeated every 7 days until a positive reaction was obtained (5mm cut-off)⁽¹⁵⁾. Nine (1.2%) patients and 11 (1.4%) patients with lesion durations > 2 months presented negative MST results, depending on the cut-off (5mm and 6mm, respectively). A negative response with the MST after 2 months of disease evolution might be related with a defect of the host immune response⁽²³⁾.

The ROC curves that were constructed according to sex showed higher sensitivity for men, which supports the findings of a previous study⁽²⁶⁾, while higher specificity was found for

women for both cut-off points. Regarding the positive and negative predictive values, our results agree with this previous study, with higher predictive values in women. The superior specificity and positive predictive value in women is reported to be the result of fewer false-positive results⁽²⁶⁾. This is supported by the findings in the NCL group in the present study, in which 18 (8.6%) men and 7 (3.3%) women had induration diameters ≥ 5 mm with the MST.

The ROC curves for the different age groups showed that the 0-14 years old had a better curve, and both cut-off values (5 and 6mm) could be used. The individuals in this age group were likely not to have encountered many antigens yet, which would provide a more specific response with the MST. In a previous study, a 63.2% sensitivity for patients <12 years of age, 100% for patients 12-18 years of age, and 60% for patients ≥ 65 years of age have been reported⁽²⁶⁾. Although the age stratification was different, our results using a cut-off of 5mm showed higher sensitivities for the specific age groups (100% for patients 0-14 years old, 97.4% for patients 15-18 years old, 97.5% for patients 30-49 years old, and 96.6% for patients ≥ 50 years old).

The present study evaluated the response to the MST in a population from an ATL endemic area in the northern and northwestern regions of the State of Paraná, Brazil. The results showed 97.4% sensitivity and 93.9% specificity for the 5mm cut-off and 95.8% sensitivity and 97.1% specificity for the 6mm cut-off, with higher sensitivity in men, higher specificity and predictive values in women, and the best curves for both the 5 and 6mm cut-off values in 0-14 years old. Considering the need to balance sensitivity and specificity, both cut-off values (5 and 6mm) could be used in this population. It would also be interesting to investigate the cut-off values for the MST in populations from other ATL endemic areas.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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