Serological reactivity of different antigenic preparations of *Leishmania (Leishmania) amazonensis* and the *Leishmania braziliensis* complex

Reatividade sorológica frente a diferentes preparações antigênicas de *Leishmania (Leishmania) amazonensis* e do complexo *Leishmania braziliensis*

Adriano Gomes-Silva[1], Maria Aparecida Souza[1], Sandra Regina Afonso-Cardoso[1], Lívia Resende Andrade[1], Reynaldo Dietze[3], Elenice Lemos[3], Alejandro Belli[4], Silvio Favoreto Júnior[5] and Marcelo Simão Ferreira[2]

ABSTRACT

Total antigen from *Leishmania (Leishmania) amazonensis* and isolates from the *Leishmania braziliensis* complex, along with their respective antigenic fractions obtained by affinity chromatography on concanavalin-A-Sepharose and jacalin-agarose columns evaluated using immunoenzymatic ELISA assay. For this, serum samples from 229 patients were used, grouped as American tegmental leishmaniasis (nº=58), visceral leishmaniasis (nº=28), Chagas disease (nº=49), malaria (nº=32), tuberculosis (nº=13) and healthy volunteers (nº=49). Samples from American tegmentary leishmaniasis showed higher reactivity with antigens isolated from the *Leishmania braziliensis* complex than with antigens from *Leishmania amazonensis* ($p<0.001$). ELISA assays showed a sensitivity range from 60% to 95% with antigens isolated from the *Leishmania braziliensis* complex. There was marked nonspecific reactivity among serum samples with the use of antigenic fractions binding with concanavalin-A and jacalin from both *Leishmania* complexes, in comparison with other antigens ($p<0.001$). The results presented in this study suggest that the use of homologous antigens increases the efficiency of anti-*Leishmania* immunoglobulin detection, which may be very valuable for diagnostic purposes.

Key-words: American tegmentary leishmaniasis. Antigens from *Leishmania braziliensis* isolates. *Leishmania amazonensis* antigens. Concanavalin-A. Jacalin.

RESUMO

Antígeno total de *Leishmania (Leishmania) amazonensis* e isolado do complexo *Leishmania brazilienis,* assim como suas respectivas frações antigênicas obtidas por cromatografia de afinidade em coluna de concanavalina-A ligada a sepharose e Jacalina ligada a agarose foram avaliadas por ensaio imunoenzimático ELISA. Para tanto, foram utilizadas amostras de soros de 229 pacientes agrupadas em leishmaniose tegumentar americana (nº=58), leishmaniose visceral (nº=28), doença de Chagas (nº=49), malaria (nº=32), tuberculose (nº=13) e voluntários saudáveis (nº=49). Houve maior reatividade das amostras de leishmaniose tegumentar americana com a utilização dos antígenos obtidos do isolado do complexo *Leishmania braziliensis* quando comparado com antígenos de *Leishmania amazonensis* ($p<0,001$). Observou-se ainda que a sensibilidade do teste ELISA variou de 60 a 95% entre os antígenos obtidos do isolado do complexo *Leishmania braziliensis*. Houve acentuada reatividade inespecífica das amostras de soros com a utilização das frações antigênicas ligantes de Concanavalina-A e Jacalina de ambos os complexos *Leishmania* em comparação aos demais antígenos ($p<0,001$). Os resultados apresentados no presente trabalho sugerem que a utilização de antígenos homólogos aumentam a eficiência de detecção de imunoglobulina anti-*Leishmania* o que pode ser de grande valia para o propósito de diagnóstico.

Palavras-chaves: Leishmaniose tegumentar americana. Antígenos de isolados *Leishmania braziliensis*. Antígenos *Leishmania amazonensis*. Concanavalina-A. Jacalina.

Leishmaniasis is a complex of zoonotic diseases found worldwide that has a high impact on public health. It is recognized by the World Health Organization as one of the tropical diseases targeted for special research and training programs[30]. American tegmental leishmaniasis (ATL) is the most frequent manifestation of infection and represents about 75% of all recorded cases. The national health surveillance program in Brazil has indicated that the prevalence of ATL in this country ranged from 11.9 to 22.9 cases per 100,000 inhabitants between 1990 and 2006[21].

Clinical signs, histopathology and parasite detection in the lesion are generally used for ATL diagnosis. Although these tests are widely used, they nearly always present limitations[11]. The use of antigenic preparations of promastigote forms in indirect immunological assays has shown promising results for leishmaniasis diagnosis, with reported good sensitivity and specificity[3 17 27]. Previous papers have shown that some ATL patients develop low levels of anti-*Leishmania* antibodies during the infection. These cases can be explained by natural low production of specific antibodies or by the lack of reactivity against the antigenic preparations used in serological tests[7 29].

Several antigenic preparations of *Leishmania* parasites have been developed in attempts to increase the sensitivity and specificity of immunological assays. Low titers of specific immunoglobulins have been found in patients with cutaneous leishmaniasis, by using freeze-dried antigens[23]. However, soluble and insoluble antigens of *Leishmania (Viannia) braziliensis* used in immunoblot assays showed 91% sensitivity and 100% specificity[3] in similar groups of patients. High (92%) sensitivity levels were reported when soluble antigens from *Leishmania mexicana* promastigotes were used to detect specific immunoglobulins in human and canine serum samples[26].

Leishmania parasites express a variety of complex glycoconjugates in the cell membrane during all stages of their life cycle. Recent studies have suggested that glycoconjugates can be successfully used to improve the efficiency of indirect diagnostic techniques and evaluations of immunity induction[4 6 28]. Lectin affinity purification is one of the methods used for isolating different glycoconjugates from *Leishmania*[13 25]. One example of the diagnostic potential of these molecules is a glycoconjugate of 27-39kDa from *Leishmania (Leishmania) donovani* that binds to concanavalin-A (Con-A) and is recognized specifically by serum from patients with visceral leishmaniasis[2]. Moreover, a lipophosphoglycan from *Leishmania (Leishmania) mexicana* isolated by Con-A affinity chromatography was strongly recognized by rabbit IgG after immunization with *Leishmania (Leishmania) mexicana* promastigotes[12].

The carbohydrate residue profile of the cell membrane of *Leishmania* promastigotes has demonstrated that Jacalin lectin (Jaca) has an average of 3.8 x 10^{6} binding molecules on the surface of *Leishmania (Leishmania) amazonensis* promatigotes[1]. These Jaca-binding molecules have not yet been systematically evaluated regarding their diagnostic potential. Therefore, considering the importance of glycoconjugate immunogenic properties, their potential as diagnostic markers for *Leishmania* infection and the advantage of using lectin-binding properties for isolating

these molecules, we conducted the present study to evaluate the serological reactivity of different *Leishmania* antigen fractions obtained by Con-A and Jaca affinity chromatography.

MATERIAL AND METHODS

Human serum. A total of 229 serum specimens were used in the present study. The samples were obtained from patients attended at Hospital de Clínicas, Federal University of Uberlândia (HC-UFU), Uberlândia, Minas Gerais, and at the Infectious Diseases Center of the Federal University of Espírito Santo, Vitória, Espírito Santo, Brazil. Both areas are endemic for *Leishmania (Viannia) braziliensis*, while *Leishmania (Leishmania) chagasi* has also been reported in Vitória. The panel of samples tested in this study included serum from patients with American tegmental leishmaniasis (ATL, nº=58), visceral leishmaniasis (VL, nº=28), Chagas disease (CD, nº=49), malaria (M, nº=32) and tuberculosis (TB, nº=13). All the leishmaniasis patients had had their infection confirmed clinically, epidemiologically, immunologically and parasitologically. The control samples consisted of serum from healthy subjects (N, nº=49). The procedures and sample utilization were approved by the ethics committee for human research at the Federal University of Uberlândia (protocol number 109/2002).

Parasites and antigens. *Leishmania (Leishmania) amazonensis* (IFLA/BR/67/PH8 strain) was obtained from the Zoonosis Control Center of the municipal authority of São Paulo, State of São Paulo. A strain of the *Leishmania braziliensis* complex was isolated from a patient who had become infected in the region of Uberlândia, Minas Gerais. This newly isolated strain was characterized by means of Multiplex-PCR[15] and Lopez-PCR[19]. The promastigotes were maintained in brain heart infusion (BHI) medium (Oxoid, Basingstoke, England) containing 10% fetal calf serum (Cultilab, Campinas, Brazil), 2mM L-glutamine, and 100µg/ml gentamicin (Sigma, St Louis, USA). The parasites were harvested at the stationary growth phase, washed three times in Hanks' saline (pH 7.4) at 4ºC and centrifuged at 3,000g for 10 minutes. The parasites were then resuspended at a concentration of 10[9] parasites/ml in Con-A buffer (10mM Tris-HCl, pH 7.4, 0.5M NaCl, 1mM $CaCl_2$ and 1mM $MnCl_2$) or Jaca buffer (0.1M Na_2HPO_4 and 0.1M NaH_2PO_4, pH 8), both containing 0.2% n-octyl β-D-glucopyranoside (Sigma) and protease inhibitors: 1.6mM phenylmethylsulfonyl fluoride (PMSF) and 1mM benzamidine (Sigma). Membrane disruption and antigen release was effected by six cycles of freezing and thawing and one cycle of ultrasonication. The preparation was then centrifuged at 20,000 g at 4ºC for 60 min, and the supernatant was collected. The protein concentration was assessed by colorimetric assay[20].

Affinity chromatography of *Leishmania* promastigote antigens. Total antigen was loaded separately for affinity chromatography on concanavalin-A (Con-A)-Sepharose 4B or jacalin (Jaca)-agarose columns (1 X 10cm column, Pearce, Rockford, Illinois, USA). The columns were prewashed with their respective buffers (Con-A or Jaca) containing 0.1% n-octyl β-D-glucopyranoside, and were then loaded with 2mg of total antigen promastigotes. This was followed by 30 minutes of

incubation at 4°C under mild agitation. Removal of unbound antigen was achieved by washing the columns with Con-A buffer or Jaca buffer until no protein could be detected by absorbance at 280nm. Specifically bound proteins were eluted by 0.2M methyl α-D-mannopyranoside (Sigma) in Con-A buffer or 0.4M D-galactose (Mallinckrodt Baker, Inc. Paris) in Jaca buffer. Fractions with peak absorbance at 280nm were pooled, concentrated and desalted using Amicon columns (molecular weight cutoff of 10kDa, Stirred Ultrafiltration Cell, Millipore, USA). The protein concentrations of the fractions were determined by colorimetric assays. Total antigen and all the antigen fractions were analyzed by 12% polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions[18].

ELISA. Optimization of antigen concentrations and conjugated antibodies was performed using a panel of serum from healthy and ATL patients. Values were standardized based on discrimination between positive and negative results. Serological polystyrene plates (Montegrotto Terme, Padua, Italy), were coating with 50µl of each antigen preparation (1µg/well) diluted with carbonate buffer. The plates were then washed with phosphate buffer saline (PBS) containing 0.05% Tween-20 (PBS-T) and human serum samples were added in duplicates (50µl/well) diluted 1:40 (previously determined) in PBS-T containing 10% normal goat serum. The plates were incubated at 37ºC for 45 min. After incubation, they were washed with PBS-T six times. Goat anti-human IgG conjugated with horseradish peroxidase (Zymed Laboratories, Inc., San Francisco, USA) was added at 1:2,000 dilution and incubated at 37ºC for 45 min followed by six washes with PBS-T. A substrate solution containing 0.5mg/ml of OPD in sodium citrate buffer pH 5.0 and 0.03% H_2O_2 was used to develop the colorimetric reaction. The optical density was measured at 492nm in an ELISA microreader (Titertek Multiskan Plus, Flow Laboratories, Switzerland). The lower limit of positivity (cutoff) was determined as the mean of the negative controls plus three standard deviations. Serological parameters of sensitivity, specificity, positive predictive values and negative predictive values were calculated following standard laboratory procedures[13].

Statistical analysis. The reactivity of the serum samples against different antigenic preparations was evaluated by means of the one-way ANOVA non-parametric test (Kruskal-Wallis, GraphPad Prism version 3 for Windows). Evaluations of sensitivity, specificity, positive predictive value and negative predictive value between antigenic preparations were performed by comparison tests between two percentages (Statistica for Windows version 4.5). The results were considered statistically significant when p < 0.05.

RESULTS

***Leishmania* antigen analysis.** The *Leishmania* strain isolated from one of our patients was characterized as belonging to the *Leishmania braziliensis* complex. The determination of the complex was based on specific amplification of a DNA sequence of 146-149bp by means of multiplex-PCR (Figure 1A), and was confirmed by PCR-Lopez, which resulted in a 70bp amplicon that was characteristic for the *Leishmania braziliensis* complex

(Silva, AG, Afonso-Cardoso SR, Ferreira MS, Souza MA: unpublished data). The chromatographic profile and SDS-PAGE analyses of *Leishmania* antigenic preparations are shown in Figures 1B and 1C. The protein profile analysis using SDS-PAGE showed that the Con-A-bound fraction of *Leishmania amazonensis* antigen presented bands with apparent molecular weights of 14, 19 and 27kDa, and a protein complex ranging from 55 to 80kDa (Figure 1B, Con-A, lane E, arrowhead). Jaca-bound fraction analysis showed a large band with apparent molecular weight between 7.5 and 18kDa and another band at 66kDa (Figure 1B, Jaca, lane F, arrowhead). Con-A-unbound and Jaca-unbound fractions had electrophoretic profiles similar to total antigen without defined bands (Figure 1B, Con-A, lanes A and C; Jaca, lanes B and D). The Con-A-bound fractions obtained from the isolated *Leishmania braziliensis* complex showed bands with apparent molecular weights of 14, 19, 25, 36, 48 and 55kDa (Figure 1C, Con-A, lane E, arrowhead). Diffuse bands with apparent molecular weights of 14-23kDa, and 35-68kDa were detected in the Jaca-bound fraction (Figure 1C, Jaca, lane F). Comparisons between unbound fractions of both lectins showed similar electrophoretic profiles (Figure 1C, Con-A, lanes A and C; and Jaca, lanes B and D).

ELISA analysis. Total antigen and antigenic fractions obtained from *Leishmania amazonensis* and *Leishmania braziliensis* promastigotes were tested for reactivity with human serum by means of ELISA. Serum samples from ATL patients presented higher reactivity with antigens isolated from *Leishmania braziliensis* than with similar antigenic preparations obtained from *Leishmania amazonensis* (Figures 2A and 2B, p<0.001). The Con-A-unbound fraction of *Leishmania amazonensis* (C) showed higher serum reactivity than did the Jaca-unbound fraction (D) (Figure 2A, C and D, *p*<0.001).

Serum reactivity was significantly higher with Con-A bound fraction antigen (E) when compared to control total antigen (A) (Figure 2A, E and A, p<0.001). Similar increase in serum reactivity was observed with Jaca bound fraction (F) in comparison with control total antigen (B) (Figure 2A, F and B, p<0.001). Sera from ATL patients strongly recognize total antigen from *Leishmania braziliensis* in Con-A buffer (A) and Con-A bound fraction (E) when compared with Con-A unbound fraction (C) (Figure 2B, A and C, p<0.01; E and C, p<0.001). The Jaca unbound fraction (D) and Jaca bound fraction (F) were more reactive than total antigen in Jaca buffer (B) (Figure 2B, D and B, p<0.05; F and B, p<0.001). In addition, both the Con-A-bound fraction (E) and the Jaca-bound fraction (F) of *Leishmania amazonensis* and *Leishmania braziliensis* showed nonspecific reactivity, revealed by increased mean absorbance values for healthy volunteer (N) serum samples (Figures 2C and 2D, respectively, p<0.001).

Serological parameter analysis. We evaluated sensitivity, specificity, positive predictive value and negative predictive value for antigenic preparations of *Leishmania braziliensis* and *Leishmania amazonensis.* The antigens were tested against a panel of human sera. Our results indicated that *Leishmania braziliensis* total antigen in Jaca buffer (B) and in Con-A buffer (A), as well as in Con-A-unbound (C) and in Jaca-unbound (D) fractions presented the best sensitivity and negative predictive
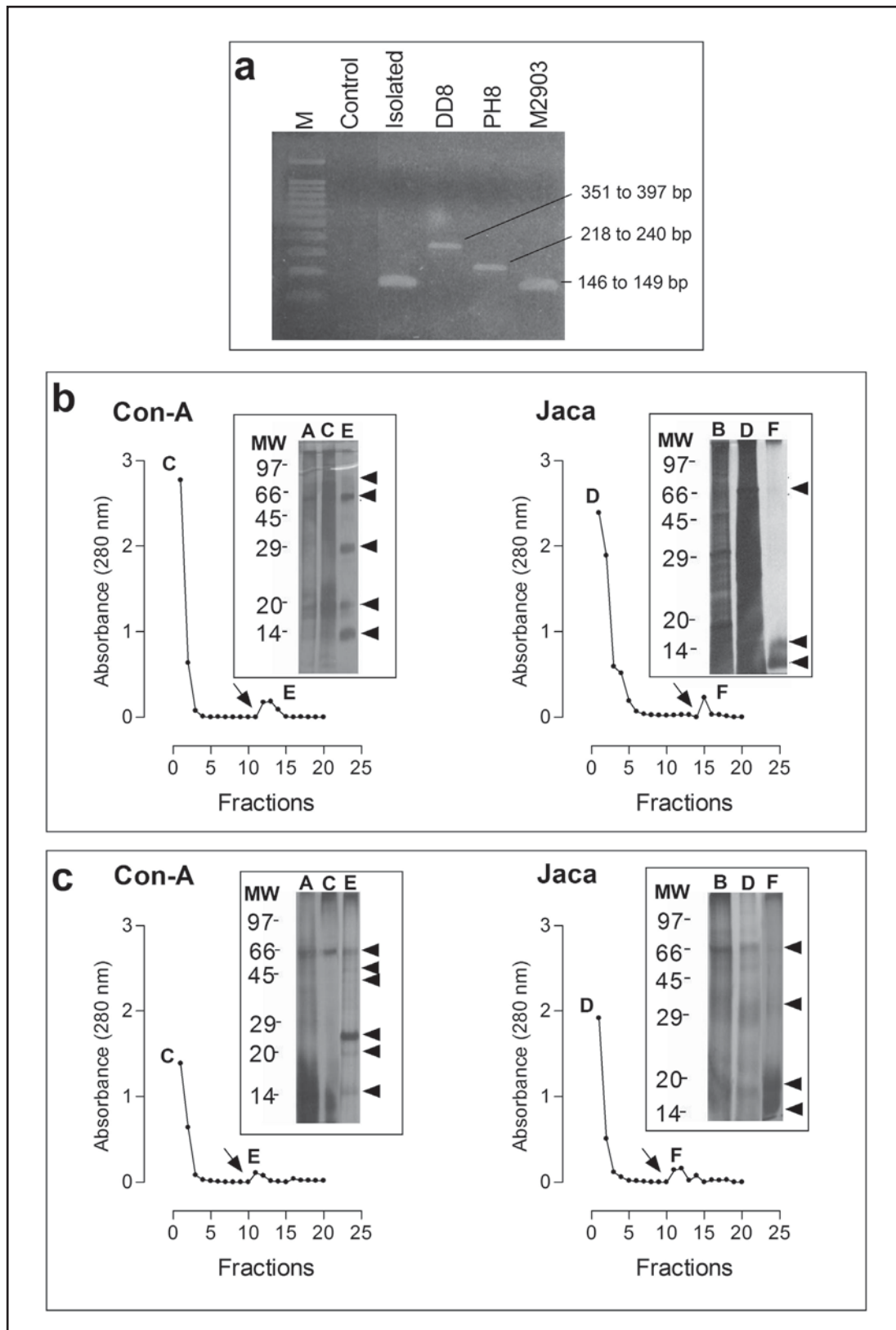
Figure 1 - *a. Multiplex PCR analysis in 1.5% agarose gel. "M" – 100 bp molecular size marker; Control – negative control; Isolated – strain isolated from patient (HC-UFU). "DD8" – Leishmania (Leishmania) donovani (WHO); "PH8" – Leishmania (Leishmania) amazonensis (WHO); "M2903" – Leishmania (Viannia) braziliensis (WHO); b. Leishmania amazonensis; and c. Leishmania braziliensis antigens (chromatography and electrophoresis analysis): A and B, total antigen of both Leishmania species obtained in Con-A buffer or Jaca buffer respectively; C and D, lectin-unbound fractions obtained by respective chromatography (Con-A and Jaca columns); E and F, Con-A-bound or Jaca-bound fractions, respectively. The arrows indicate the addition of 0.2M methyl a-D-mannopyranoside in Con-A buffer or 0.4M D (+)-galactose in Jaca buffer to the respective columns. The arrowheads indicate the lectin-bound fraction bands.*
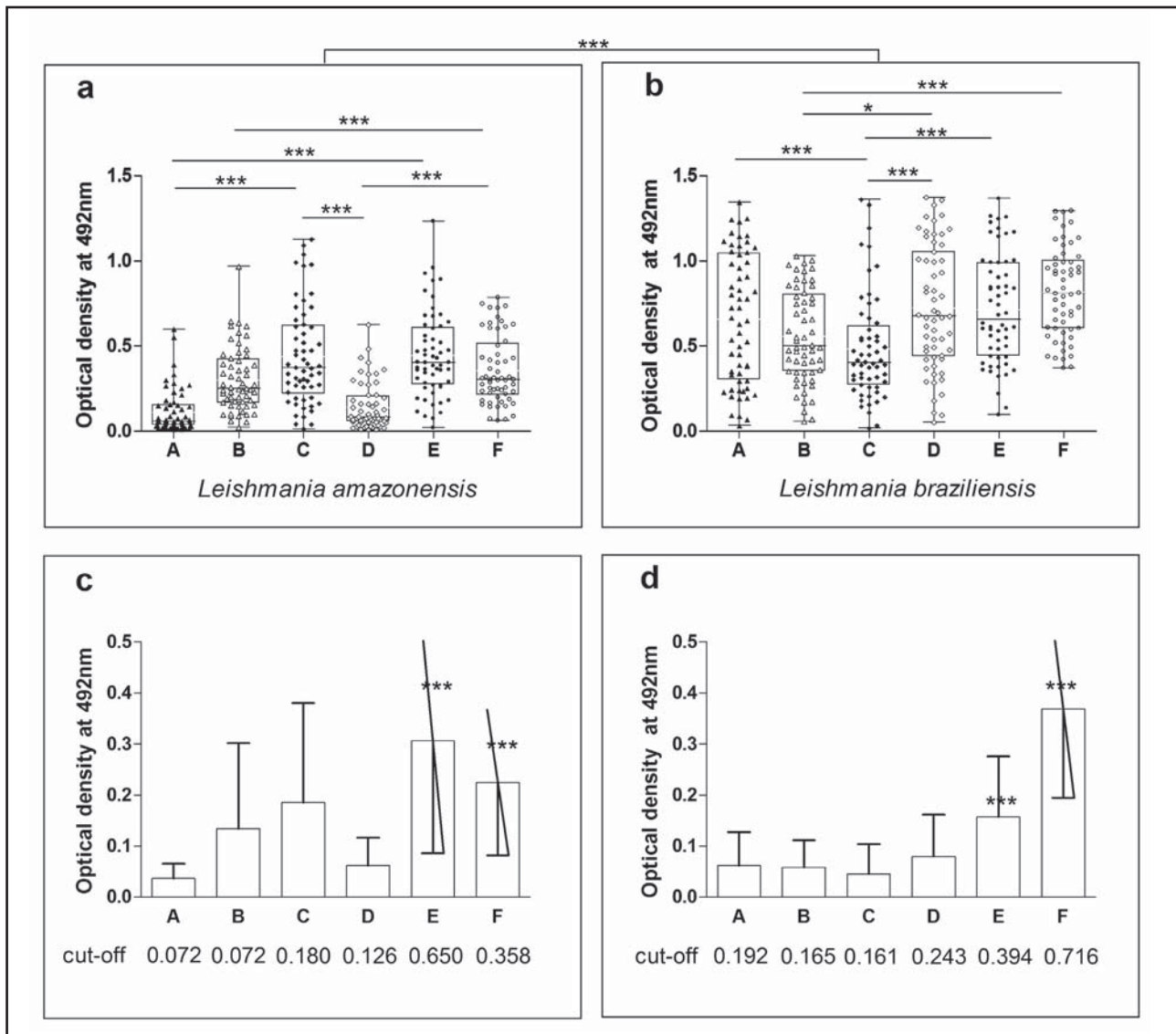
Figure 2 - Reactivity of Leishmania *antigens (a.* Leishmania amazonensis*; b.* Leishmania braziliensis*) with American tegumentary leishmaniasis serum samples measured by means of ELISA; c.* Leishmania amazonensis*; and d.* Leishmania braziliensis *nonspecific reactivity determined by antibody reactivity detection among the healthy subjects. A. Total antigen prepared in Con-A buffer, B. Total antigen prepared in Jaca buffer, C. Con-A-unbound fraction, D. Jaca-unbound fraction, E. Con-A-bound fraction, F. Jaca-bound fraction.* $*$ $p<0.05$, $**$ $p<0.01$, $***$ $p<0.001$.

values among the tested antigens (Table 1). However, low specificity and low positive predictive values were also observed with this group of antigens. Total antigen of *Leishmania amazonensis* in Jaca buffer (B) and Con-A-unbound fraction (C) presented moderate sensitivity and negative predictive values (Table 1). Con-A and Jaca-bound fractions from *Leishmania amazonensis* and *Leishmania braziliensis* (E and F) showed

poor to moderate sensitivity with low positive and negative predictive values (Table 1).

Leishmania sp total antigen triggered strong serological cross-reactivity with VL and CD serum samples. Con-A or Jaca-bound fractions from both species of *Leishmania* presented even higher cross-reactivity. Serum samples from M or TB did not behave differently from healthy controls.

Table 1 - ELISA serological parameters for Leishmania amazonensis *and Leishmania braziliensis antigens.*

Serological parameters	Leishmania (Leishmania) amazonensis						Leishmania braziliensis complex					
	A	B	C	D	E	F	A	B	C	D	E	F
Sensitivity (%)	41.0	84.0*	81.0*	38.0	19.0	38.0	91.0*	95.0*	91.0*	91.0*	81.0	60.0
Specificity (%)	54.0	44.0	54.0	57.0	71.0*	65.0	57.0	56.0	53.0	56.0	58.0	66.0*
Positive predictive value (%)	24.0	41.0*	37.0*	23.0	19.0	27.0	42.0	42.0	40.0	41.0	39.0	37.0
Negative predictive value (%)	73.0	92.0*	89.0*	73.0	73.0	76.0	95.0*	97.0*	95.0*	95.0*	54.0	83.0

A: Total antigen prepared in Con-A buffer, **B**: Total antigen prepared in Jaca buffer, **C**: Con-A-unbound fraction, **D**: Jaca-unbound fraction, **E**: Con-A-bound fraction, **F**: Jaca-bound fraction.

* Significant statistical difference, *p<0.05*.

DISCUSSION

In this study, we compared the efficiency of antigenic preparations from two *Leishmania* strains from distinct phylogenetic complexes for detecting anti-*Leishmania* antibodies in ATL serum samples. Antigens obtained by means of lectin affinity chromatography on Con-A or Jaca columns presented differences in the number of bands observed in SDS-PAGE profiles for both *Leishmania* complexes. These data agree with previous reports of distinct lectin agglutination patterns depending on the *Leishmania* species. These differences are due to an array of glycoconjugate structures in the parasite membrane, which varies according to the species and is recognized with differing affinity by the lectin[16]. The presence of bands of 53 and 47kDa in *Leishmania braziliensis* Con-A preparations and RCA (*Ricinus communis* agglutinin) revealed some similarity in the profiles of the bound glycoproteins[22].

The glycoconjugates isolated by affinity chromatography for both *Leishmania* species showed marked nonspecific reactivity for negative serum samples and other infections, in comparison with the respective total antigen or unbound fractions. Glycoconjugates isolated by means of either Con-A or Jaca resulted in moderate to poor specificity and sensitivity scores on ELISA, despite high OD values (Figure 2C and D). The increased nonspecific reactivity with Con-A and Jaca-bound fractions that was observed with negative control samples led us to increased cutoff values, which compromised the serological scores for sensitivity and specificity. This suggests that there is cross-reactivity between *Leishmania* glycoconjugates and carbohydrate epitopes that are naturally present in healthy serum. Our serological assays with Con-A and Jaca-bound fractions suggest that shared carbohydrate epitopes are present among *Trypanosomatidae* parasites, which are in part responsible for the high levels of cross-reactivity between ATL serum samples and samples from CD and VL patients[5][9]. Previous reports have shown that the sensitivity and specificity of serological diagnoses depend on the type, source and purity of the antigen used, since some of the antigens from *Leishmania* parasites have common cross-reactive epitopes shared with other microorganisms like *Trypanosoma*, *Plasmodium* and *Mycobacterium*[17]. Our study failed to identify Con-A or Jaca-binding glycoconjugate antigens presenting optimum characteristics of sensitivity and specificity for diagnostic purposes.

Efficient immunoglobulin detection was observed when *Leishmania braziliensis* antigens were used to test ATL serum samples. This result agrees with previous findings, thus suggesting that the use of species-specific antigens can improve the serological diagnostic assays for leishmaniasis[8][10]. Within this context, it has been shown that homologous *Leishmania* antigens result in higher titers of antibody detection in direct agglutination assays, thereby leading to more efficient serological diagnosis of infection[14].

Epidemiological surveys conducted in both areas from which the samples were collected confirm that there is higher prevalence of *Leishmania (Viannia) braziliensis* than of *Leishmania (Leishmania) amazonensis* in ATL cases[24]. Therefore, the lower reactivity with *Leishmania amazonensis* antigen observed in our study was probably due to the predominance of *Leishmania braziliensis* in the regions where the patients were infected. In addition, we have been systematically analyzing biopsies from ATL patients diagnosed at the UFU Hospital in Uberlândia, Minas Gerais for the last five years. Species-specific PCR performed on material from these biopsies show predominant presence of the *Leishmania braziliensis* complex in our region (Silva AG, Afonso-Cardoso SR, Ferreira MS, Souza MA: unpublished data).

Taken together, our data showed that the use of species-specific antigens leads to better detection of anti-*Leishmania* antibodies. Our findings suggest that glycoconjugates isolated by Con-A or Jaca affinity chromatography from promastigote forms of *Leishmania* sp showed limited diagnostic value. Finally, our data show that the specific aspects of an endemic area, such as the prevalent species of *Leishmania* parasites, can influence the efficiency of diagnostic methods.

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