Short Report: Identification of a Western Blot Pattern for the Specific Diagnosis of *Trypanosoma cruzi* Infection in Human Sera

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Abstract. A Western blot (WB) method using a lysate from *Trypanosoma cruzi* (Maracay strain) epimastigotes was evaluated. Serum samples from 37 patients with confirmed Chagas disease (cohort I), 27 Spanish patients with visceral leishmaniasis caused by *Leishmania infantum* (cohort II), and 28 Colombian patients with cutaneous leishmaniasis caused by *L. panamensis* and negative serology for Chagas disease (cohort III) were tested. The negative controls were 55 healthy seronegative subjects for *T. cruzi* and *Leishmania*; 28 of the negative controls were from a region endemic for Chagas disease and *Leishmania* (cohort IV), and 27 of the negative controls were from a non-endemic area for *Leishmania* and *T. cruzi* (cohort V). A homogeneous standard band pattern consisting of six antigenic bands corresponding to 28, 32, 38, 39, 40, and 48 kDa was recognized simultaneously for all Chagasic patients' sera. Sera from *Leishmania*-infected patients showed a heterogeneous band pattern that was easily differentiated from the pattern of patients with Chagas disease. WB with *T. cruzi* epimastigote antigen is an efficient method for diagnosis and may be used as an alternative to confirm *T. cruzi* and detect cross-reactivity with *Leishmania*.

INTRODUCTION

Chagas disease is a parasitic infection caused by *Trypanosoma cruzi*. It is mainly found in Central and South America, where the World Health Organization (WHO) estimates that the population at risk is approximately 60 million people and that 9–12 million people are infected.¹ However, the infection is no longer limited to Latin America, because immigration to North America and Europe has meant that Chagas disease is now a major burden to health services in these areas.² In Europe, Spain is the main host country for South American immigrants, many from endemic areas for Chagas disease. In 2008, Spain received over 1,700,000 immigrants from Latin American countries endemic for Chagas disease. Of these immigrants, 5.2% were potentially infected with *T. cruzi*, and 17,390 may develop Chagas disease.³ *T. cruzi* can be non-vector transmitted by vertical transmission, organ transplant, or transfusion of infected blood and its components.^{4–8}

During the acute phase of Chagas disease, parasitological or molecular methods are the main diagnostic tools for detecting *T. cruzi* in blood samples. However, these approaches are not effective in the chronic and undetermined phases of infection, which are characterized by lower parasitemia and the presence of specific antibodies in blood. Therefore, immunological methods are much more reliable for diagnostic purposes.⁹

Although numerous tests are available for diagnosing Chagas disease, the WHO has recommended carrying out at least two assays in parallel. Consequently, a subject is considered infected when the results of the two serological tests are positive.¹⁰ Several serological assays and antigens have been proposed and evaluated for use as confirmatory or supplementary tests of *T. cruzi* infection.¹¹ However, consensus has not been reached to date as regards establishing a reference technique, and no single test is considered the gold standard for unequivocal diagnosis of infection by this parasite.

Although most manufacturers of serological tests for Chagas disease claim sensitivity and specificity levels close to 100%, the occurrence of inconclusive and false-positive results

is a recurrent problem, particularly when serological titers are near the cutoff point. This variability between different methods and laboratories is probably caused by the use of different strains of *T. cruzi*, different antigenic fractions, and different procedures, the immunogenetic capacity of the parasite strain, and the variation in humoral response that each individual develops during infection.¹²

Furthermore, in patients who are coinfecting with other pathogens, especially *Leishmania*, the possibility of cross-reactivity frequently limits the specificity of serological results. This is considered a major drawback for the serological diagnosis of Chagas disease in areas endemic for both *T. cruzi* and *Leishmania* spp. The use of recombinant antigens only partially resolves the problem of cross-reactions.^{13,14}

The use of a qualitative method, such as Western blot (WB) analysis, has an advantage over other serological techniques in that it can be used to identify antibodies that recognize different polypeptide fractions in the complex antigenic mixtures of parasite antigens. Hence, it can perform better than other immunological techniques.

Given that no commercial WB test is currently available, we evaluated the performance of a WB method that uses a crude antigen of *T. cruzi* epimastigotes as an alternative approach to confirm *T. cruzi* infection and detect cross-reactivity with *Leishmania*.

MATERIAL AND METHODS

Panel of sera. We studied a panel of 137 cryopreserved sera from the Laboratory of Parasitology Collection at the Universitat de Barcelona, Barcelona, Spain, and 10 validated Chagasic sera provided by the Instituto Carlos III (Madrid, Spain). These samples were as follows: patients with chronic Chagas disease ($N = 37$; cohort I); Spanish patients with visceral leishmaniasis (VL) caused by *L. infantum* ($N = 27$; cohort II); Colombian patients with cutaneous leishmaniasis (CL) caused by *L. panamensis* ($N = 28$; cohort III); healthy subjects who were seronegative for *T. cruzi* and *Leishmania* ($N = 55$), 28 from a region with endemic Chagas disease and leishmaniasis (Colombia; EA; cohort IV) and 27 from a non-endemic area for Chagas disease and leishmaniasis (NEA; Minorca, the Balearic Islands, Spain; cohort V). The study

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was approved by the Ethical Committee of Research of the University of Barcelona (Barcelona, Spain).

The diagnosis of chronic Chagas disease was performed using two enzyme-linked immunosorbent assays (ELISAs): one with commercial recombinant antigens (cutoff according to the manufacturer's instructions [ratio > 1]; BioELISA Chagas; Biokit S.A., Lliçà d'Amunt, Catalonia, Spain) and the other consisting of an in-house ELISA with crude antigen from *T. cruzi* epimastigotes. The results were quantified in units (U), and the cutoff was established as previously described¹⁴ at 20 U. Samples were considered positive if the results of both assays were positive.

Diagnosis of VL and CL were confirmed by the detection of parasites in bone marrow or skin tissue by direct smear observation and/or culture. The serology of *Leishmania* was performed by ELISA and WB.^{15,16} Healthy subjects (cohorts IV and V) were seronegative for Chagas disease and leishmaniasis.

***T. cruzi* antigen.** The antigen used was a total extract of epimastigotes from *T. cruzi* (Maracay strain) cultured in liver infusion tryptose (LIT) medium with 10% heat-inactivated fetal calf serum at 28°C and collected during the exponential growth phase. Cells were washed three times in phosphate-buffered saline (pH 7.4). They were then counted and adjusted to a concentration of 3×10^8 epimastigotes/mL in sample buffer (0.5 M Tris-HCl, pH 6.8, 0.01 M ethylenediaminetetraacetic acid [EDTA], 5% sodium dodecyl sulphate [SDS], 5% 2-mercaptoethanol, 0.0125% bromophenol blue) and boiled for 5 minutes. The antigen was stored at -40°C until use.

Antigen electrophoresis was carried out on 0.1% SDS/12% polyacrylamide gels using molecular mass protein standards (standard low range; Bio-Rad) in a Mini-Protean II (Bio-Rad). Gels were run at 100 V for 2 hours at room temperature. Polypeptides were transblotted onto nitrocellulose sheets (0.45-µm pore size, HAWP 304 FO; Millipore, Bedford, MA) at 100 V for 1.5 hours, and the sheets were blocked with 20 mM Tris and 0.13 mM NaCl, pH 7.6 (TS) with 5% skimmed milk overnight at 4°C. They were then washed in TS. The nitrocellulose sheet was cut, and the strips were introduced into the mini incubation trays. Sera were diluted 1:50 in TS with 1% skimmed milk and 0.2% Tween 20. They were then incubated on the strips for 1.5 hours at 37°C. Bound immunoglobulins were developed by incubation with a 1:1,000 dilution of protein A peroxidase conjugate (Pierce Protein Research Products, US) for 1 hour at room temperature. After three additional washes with TS-0.05% Tween 20 and one wash with TS, the substrate (0.05% H₂O₂ and 0.3% 4-chloro-1-naphthol in methanol dissolved in TS 500) was added. The color was allowed to develop at room temperature, and the reaction was stopped with tap water after 30 minutes. The presence of bound antibodies was analyzed using the Gel Doc System (Bio-Rad).

Statistical analysis. The Student *t* test and Pearson correlation coefficient analysis were performed using the PASW Statistics program. Differences were considered significant at *P* < 0.05.

RESULTS

In our immunoblot analysis, the 37 sera from Chagasic patients revealed a homogeneous polypeptide pattern in the *T. cruzi* epimastigote antigen in which at least 43 reactive bands were recognized, the molecular masses of which ranged from 16 to 100 kDa. The mean number ($x \pm SD$) of polypeptide

fractions detected by sera from patients with Chagas disease was 22.68 ± 3.95 . A direct correlation was observed between the number of bands identified in the WB and the ELISA values ($r = 0.85$ for the in-house ELISA with whole epimastigote antigens and $r = 0.82$ for the commercial ELISA with recombinant antigens).

The mean number of bands detected by sera from other cohorts was 12.19 ± 4.51 for the VL group, 6.79 ± 3.29 for the CL group, 1.89 ± 1.73 for healthy subjects from endemic areas, and 1.15 ± 1.19 for healthy subjects from non-endemic areas. As expected, differences in the number of bands detected between sera from patients with Chagas disease and those patients from other cohorts were significant in all cases (Student *t* test, *P* < 0.001).

A standard pattern of reactivity for sera from patients with Chagas disease was constructed with those bands that were recognized by 100% of sera from cohort I and by less than

TABLE 1
Reactivity of the polypeptide fractions of *T. cruzi* epimastigotes with the sera from patients with Chagas disease and leishmaniasis and healthy subjects

Proteins (kDa)	Cohort I Chagas disease (N = 37; %)*	Cohort II VL (N = 26; %)*	Cohort III CL (N = 28; %)*	Cohort IV healthy EA (N = 28; %)*	Cohort V healthy NEA (N = 27; %)*
16	81.1	30.8	0	0	0
18	89.2	11.5	0	0	0
20	64.9	100	3.6	0	0
21	5.4	100	0	0	0
22	83.8	3.8	0	0	0
23	8.1	100	0	0	0
24	35.1	100	0	0	0
25	86.5	23.1	0	0	0
26	24.3	26.9	17.9	7.1	11.1
27	91.9	7.7	0	0	0
28	100	23.1	3.6	7.1	0
30	21.6	11.5	21.4	0	0
31	45.9	3.8	3.6	0	0
32	100	34.6	21.4	0	0
33	21.6	23.1	0	3.6	0
34	48.6	46.2	0	0	0
35	0	34.6	7.1	0	3.7
36	67.6	53.8	10.7	10.7	7.4
38	100	19.2	35.7	21.4	0
39	100	7.7	32.1	0	0
40	100	38.5	7.1	10.7	14.8
42	2.7	7.7	7.1	3.6	7.4
44	73	57.7	3.6	14.3	0
46	5.4	100	7.1	0	7.4
48	100	38.5	14.3	3.6	14.8
52	24.3	65.4	92.9	7.1	0
56	40.5	84.6	78.6	7.1	7.4
58	0	100	78.6	3.6	0
60	94.6	88.5	10.7	10.7	3.7
62	37.8	50	28.6	10.7	0
64	2.7	7.7	0	0	0
66	75.7	73.1	82.1	28.6	3.7
68	5.4	11.5	0	0	0
70	5.4	38.5	0	3.6	0
72	51.4	23.1	10.7	14.3	11.1
76	94.6	76.9	46.4	3.6	3.7
80	5.4	3.8	0	0	0
82	10.8	7.7	7.1	3.6	0
86	89.2	84.6	3.6	3.6	7.4
88	5.4	7.7	3.6	3.6	0
90	56.8	0	0	0	0
92	16.2	3.8	7.1	0	0
96	100	76.9	32.1	0	11.1
98	2.7	15.4	0	3.6	0
100	70.3	0	0	3.6	0

* Percentage of sera that reacts with the antigen fraction.

FIGURE 1. Profiles recognized in WB with *T. cruzi* epimastigote antigen by sera from Chagasic patients. Lines 1–13 = high ELISA titers; * lines 14–19 = intermediate ELISA titers. † Patients with inconclusive or borderline ELISA titers. ‡ Line 20 = Chagas disease was confirmed by PCR; line 21 = patient diagnosed and treated in country of origin 3.5 years earlier; line 22 = a second blood extraction gave positive ELISA results. *High titers: ELISA in house values > 80 U and ELISA BioKit $r > 4$. †Intermediate titers: ELISA in house values between 40 and 80 U and BioKit r between 2 and 4. ‡Low or borderline titers: ELISA in house between 20 and 40 U and BioKit r between 1 and 2. §Inconclusive when only one of the sera was positive for one of the ELISAs.

50% of sera from any other cohort (Table 1). This resulted in a homogeneous standard band pattern consisting of six antigenic bands corresponding to 28, 32, 38, 39, 40, and 48 kDa that were recognized simultaneously for all Chagasic patients, regardless of their ELISA titer values (high, intermediate, or low) (Table 1 and Figure 1). In contrast, sera from patients with VL and CL showed a heterogeneous pattern of reactivity in which distinct antigenic fractions of *T. cruzi* epimastigote antigen were recognized, mainly the polypeptides of 20, 21, 23, 24, 34, 36, 44, 46, 48, 52, 56, 58, 60, 62, 66, 76, 86, and 96 kDa for VL cases and 52, 56, 58, 66, and 76 kDa for CL cases (Figure 2). The number of bands in the established standard pattern of reactiv-

TABLE 2

Number of bands in the standard pattern of Chagas disease (polypeptides of 28, 32, 38, 39, 40, and 48 kDa of *T. cruzi* epimastigote antigen) recognized by sera from cohorts of non-Chagasic patients

Number of bands	Cohort II VL		Cohort III CL		Cohort IV healthy EA		Cohort V healthy NEA	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
6	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0
4	2	7.4	1	3.5	0	0	0	0
3	3	11.1	1	3.5	0	0	0	0
2	9	33.3	11	39.3	3	10.7	0	0
1	7	25.9	3	10.7	6	21.4	8	29.6
0	6	22.2	12	7.1	19	67.8	19	70.3
Total	27		28		28		27	

ity for Chagas disease that were simultaneously recognized by sera from cohorts II to V was never higher than four (Table 2). Accordingly, a serum may be considered positive for Chagas disease when it reacts simultaneously against at least five antigenic bands of the standard pattern (28, 32, 38, 39, 40, and 48 kDa).

DISCUSSION

WB analysis using a range of strains and antigenic preparations has been used to diagnose Chagas disease in Central and South America.^{12,17–23} The main limitation of this technique is that it is difficult to perform interlaboratory comparisons, mainly due to the heterogeneity of the strains and antigenic preparations used. In addition, the size of the slab gels and the concentration of polyacrylamide used as well as the different molecular weight markers all introduce additional variations in band separation and the establishment of respective molecular weights.

Although some slight differences in antigen molecular weights were found when our method was compared with other studies that shared the same source of antigens (crude epimastigote antigens) and similar electrophoretic conditions,^{12,24,25} our results confirm a similar pattern of reactivity in Chagasic sera.

Various criteria of positivity have been used to detect Chagas disease in WB analysis—for example, serum recognition

FIGURE 2. Profiles recognized in WB with *T. cruzi* epimastigote antigen by sera from (A) patients with visceral leishmaniasis, (B) patients with cutaneous leishmaniasis, and (C) healthy subjects from endemic and non-endemic areas.

of at least 3 bands from a group of 7 bands (14, 19, 27, 30, 34, 37, and 75 kDa)²⁴ or at least 5 bands from a set of 10 (25, 30, 33, 37, 39, 45, 52, 70, 75, and 92 kDa).¹⁸ Using an excreted-secreted antigen (TESA), it was considered that serum was positive when the 150- to 160-kDa antigen fractions were recognized.¹⁸ Unlike the other criteria mentioned, we found that the homogeneous and repetitive patterns of at least five of six bands of 28, 32, 38, 39, 40, and 48 kDa correlated with Chagas disease.

Antigenic cross-reactivity with *Leishmania* is a major drawback of conventional serological techniques for the diagnosis of *T. cruzi* infection.^{17,26–28} The bands recognized by the sera of patients with Chagas disease and American CL, mucocutaneous leishmaniasis, and VL using homologous and heterologous antigens have been addressed previously, with different results.^{17,20,29–32} In our study, individual bands from the standard pattern were recognized by sera from patients with VL or CL, but the general pattern of reactivity of these sera was very different from the pattern of sera from Chagasic patients; in no case did it include more than four bands of those bands included in the standard pattern for Chagas disease.

In summary, our results show that WB is an efficient alternative method for the diagnosis and confirmation of *T. cruzi* infection when the criteria defined in the present study are applied. This finding is particularly relevant when other serological tests are discordant or borderline. Given that the homogeneous pattern observed in Chagasic sera is easily distinguishable from the heterogeneous pattern shown by leishmanial sera, cases of cross-reactivity with *Leishmania* can be differentiated using WB.

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REFERENCES

- World Health Organization, 2007. Guhl F, Lazdins-Helds JK, eds. *Reporte del grupo de trabajo científico sobre la enfermedad de Chagas*. Geneva, Switzerland: World Health Organization.
- Schmunis GA, 2007. Epidemiology of Chagas disease in non-endemic countries: the role of international migration. *Mem Inst Oswaldo Cruz* 102: 75–85.
- Schmunis GA, Yadon ZE, 2010. Chagas disease: a Latin American health problem becoming a world health problem. *Acta Trop* 115: 14–21.
- Riera C, Guarro A, Kassab HE, Jorba JM, Castro M, Angrill R, Gállego M, Fisa R, Martín C, Lobato A, Portús M, 2006. Congenital transmission of *Trypanosoma cruzi* in Europe (Spain): a case report. *Am J Trop Med Hyg* 75: 1078–1081.
- Centers for Disease Control and Prevention (CDC), 2006. Chagas disease after organ transplantation—Los Angeles, California, 2006. *MMWR Morb Mortal Wkly Rep* 55: 798–800.
- Muñoz J, Portús M, Corachán M, Fumadó V, Gascón J, 2007. Congenital *Trypanosoma cruzi* infection in a non-endemic area. *Trans R Soc Trop Med Hyg* 101: 1161–1162.
- Young C, Losikoff P, Chawla A, Glasser L, Forman E, 2007. Transfusion-acquired *Trypanosoma cruzi* infection. *Transfusion* 47: 540–544.
- Flores-Chávez M, Faez Y, Olalla JM, Cruz I, Gárate T, Rodríguez M, Blanc P, Cañavate C, 2008. Fatal congenital Chagas' disease in a non-endemic area: a case report. *Cases J* 1: 302.
- Chang CD, Cheng KY, Jiang LX, Salbilla VA, Haller AS, Yem AW, Bryant JD, Kirchhoff LV, Leiby DA, Schochetman G, Shah DO, 2006. Evaluation of a prototype *Trypanosoma cruzi* antibody assay with recombinant antigens on a fully automated chemiluminescence analyzer for blood donor screening. *Transfusion* 46: 1737–1744.
- World Health Organization Expert Committee, 2002. *Control of Chagas Disease. World Health Organization Technical Report Series 905*. Geneva, Switzerland: World Health Organization, 1–109.
- Frade AF, Luquetti AO, Prata A, Ferreira AW, 2011. Western blotting method (TESAcruzi) as a supplemental test for confirming the presence of anti-*Trypanosoma cruzi* antibodies in finger prick blood samples from children aged 0–5 years in Brazil. *Acta Trop* 117: 10–13.
- Reiche EM, Cavazzana M Jr, Okamura H, Tagata EC, Jankevicius SI, Jankevicius JV, 1998. Evaluation of the western blot in the confirmatory serologic diagnosis of Chagas' disease. *Am J Trop Med Hyg* 59: 750–756.
- Flores-Chávez M, Fernández B, Puente S, Torres P, Rodríguez M, Monedero C, Cruz I, Gárate T, Cañavate C, 2008. Transfusional Chagas disease: parasitological and serological monitoring of an infected recipient and blood donor. *Clin Infect Dis* 46: 44–47.
- Riera C, Vergés M, López-Chejade P, Piron M, Gascón J, Fisa R, Gállego M, Portús M, 2009. Desarrollo y evaluación de una técnica ELISA con antígeno crudo de *Trypanosoma cruzi* para el diagnóstico de la Enfermedad de Chagas. *Enf Emerg* 11: 22–29.
- Riera C, Valladares JE, Gállego M, Aisa MJ, Castillejo S, Fisa R, Ribas N, Carrió J, Alberola J, Arboix M, 1999. Serological and parasitological follow-up in dogs experimentally infected with *Leishmania infantum* and treated with meglumine antimoniate. *Vet Parasitol* 84: 33–47.
- Aisa MJ, Castillejo S, Gállego M, Fisa R, Riera MC, de Colmenares M, Torres S, Roura X, Sentís J, Portús M, 1998. Diagnostic potential of Western blot analysis of sera from dogs with leishmaniasis in endemic areas and significance of the pattern. *Am J Trop Med Hyg* 58: 154–159.
- Malchiodi EL, Chiaramonte MG, Taranto NJ, Zwirner NW, 1994. Cross-reactivity studies and differential serodiagnosis of human infections caused by *Trypanosoma cruzi* and *Leishmania* spp; use of immunoblotting and ELISA with a purified antigen (Ag163B6). *Clin Exp Immunol* 97: 417–423.
- Umezawa EF, Nascimento NS, Kesper N Jr, Coura JR, Borges-Pereira J, Junqueira AC, Camargo ME, 1996. Immunoblot assay using excreted-secreted antigen of *Trypanosoma cruzi* in serodiagnosis of congenital, acute, and chronic Chagas disease. *J Clin Microbiol* 34: 2143–2147.
- Oelemann WM, Vanderborght BO, Verissimo Da Costa GC, Teixeira MG, Borges-Pereira J, De Castro JA, Coura JR, Stoops E, Hulstaert F, Zrein M, Peralta JM, 1999. A recombinant peptide antigen line immunoassay optimized for the confirmation of Chagas' disease. *Transfusion* 39: 711–717.
- Bucio MI, Cabrera M, Segura EL, Zenteno E, Salazar-Schettino M, 1999. Identification of immunodominant antigens in Mexican strains of *Trypanosoma cruzi*. *Immunol Invest* 28: 257–268.
- Sánchez B, Monteón V, Reyes PA, Espinoza B, 2001. Standardization of micro-enzyme-linked immunosorbent assay (ELISA) and Western blot for detection of *Trypanosoma cruzi* antibodies using extracts from Mexican strains as antigens. *Arch Med Res* 32: 382–388.
- Berrizbeitia M, Ndao M, Bubis J, Gottschalk M, Aché A, Lacouture S, Medina M, Ward BJ, 2006. Purified excreted-secreted antigens from *Trypanosoma cruzi* trypomastigotes as tools for diagnosis of Chagas' disease. *J Clin Microbiol* 44: 291–296.
- Campos Y, Briceño L, Reina K, Figarella K, Pérez JL, Mosca W, 2009. Serological diagnosis of Chagas disease: evaluation and characterisation of a low cost antigen with high sensitivity and specificity. *Mem Inst Oswaldo Cruz* 104: 914–917.
- Teixeira MG, Borges-Pereira J, Netizert E, Souza ML, Peralta JM, 1994. Development and evaluation of an enzyme linked

- immunotransfer blot technique for serodiagnosis of Chagas' disease. *Trop Med Parasitol* 45: 308–312.
25. Marcipar IS, Risso MG, Silber AM, Revelli S, Marcipar AJ, 2001. Antibody maturation in *Trypanosoma cruzi*-infected rats. *Clin Diagn Lab Immunol* 8: 802–805.
 26. Camargo ME, Rebonato C, 1969. Cross-reactivity in fluorescence tests for *Trypanosoma* and *Leishmania* antibodies. A simple inhibition procedure to ensure specific results. *Am J Trop Med Hyg* 18: 500–505.
 27. Guimarães MC, Celeste BJ, de Castilho EA, Mineo JR, Diniz JM, 1981. Immunoenzymatic assay (ELISA) in mucocutaneous leishmaniasis, kala-azar, and Chagas' disease: an epimastigote *Trypanosoma cruzi* antigen able to distinguish between anti-*Trypanosoma* and anti-*Leishmania* antibodies. *Am J Trop Med Hyg* 30: 942–947.
 28. Araujo FG, 1986. Analysis of *Trypanosoma cruzi* antigens bound by specific antibodies and by antibodies to related trypanosomatids. *Infect Immun* 53: 179–185.
 29. Dos Santos JI, Morgado MG, Galvão-Castro B, 1987. Human visceral leishmaniasis: analysis of the specificity of humoral immune response to polypeptides of *Leishmania donovani chagasi*. *Am J Trop Med Hyg* 37: 263–270.
 30. Evans TG, Krug EC, Wilson ME, Vasconcelos AW, de Alencar JE, Pearson RD, 1989. Evaluation of antibody responses in American visceral leishmaniasis by ELISA and immunoblot. *Mem Inst Oswaldo Cruz* 84: 157–166.
 31. Vexenat Ade C, Santana JM, Teixeira AR, 1996. Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa *Trypanosoma cruzi*, *Leishmania chagasi* and *Leishmania (Vianina) braziliensis*. *Rev Inst Med Trop Sao Paulo* 38: 177–185.
 32. Gonçalves CC, Reiche EM, De Abreu Filho BA, Silveira TG, Felizardo TC, Maia KR, Costacurta R, Padovesi EJ, Dias Filho BP, Jankevicius SI, Jankevicius JV, 2002. Evaluation of antigens from various *Leishmania* species in a Western blot for diagnosis of American tegumentary leishmaniasis. *Am J Trop Med Hyg* 66: 91–102.