


Brazilian Journal of Infectious Diseases

 This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License, which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. Fonte: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1413-86702015000300302&lng=en&nrm=iso. Acesso em: 5 abr. 2018.

REFERÊNCIA

SOARES, Killarney Ataíde et al. Field validation of a Leishmania (Leishmania) mexicana exo-antigens ELISA for diagnosing tegumentary leishmaniasis in regions of Leishmania (Viannia) predominance. **Brazilian Journal of Infectious Diseases**, Salvador, v. 19, n. 3, p. 302-307, maio/jun. 2015. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1413-86702015000300302&lng=en&nrm=iso>. Acesso em: 5 abr. 2018. doi: <http://dx.doi.org/10.1016/j.bjid.2015.03.010>.



The Brazilian Journal of INFECTIOUS DISEASES

www.elsevier.com/locate/bjid



Original article

Field validation of a *Leishmania (Leishmania) mexicana* exo-antigens ELISA for diagnosing tegumentary leishmaniasis in regions of *Leishmania (Viannia)* predominance



Killarney Ataíde Soares^a, Ada Amália Ayala Urdapilleta^a, Gilcilene Maria dos Santos^b,
Andréa Lisboa Carneiro^a, Ciro Martins Gomes^{c,d,*}, Ana Maria Roselino^e, Raimunda
Nonata Ribeiro Sampaio^{a,d,f}

^a Pós-Graduação em Ciências Médicas, Universidade de Brasília (UnB), Brasília, DF, Brazil

^b Escola Superior de Ciências da Saúde – ESCS, Faculdade de Medicina, Brasília, DF, Brazil

^c Pós-Graduação em Clínica Médica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (FMRP-USP), Ribeirão Preto, SP, Brazil

^d Dermatology, Brasília, Universidade de Brasília (UnB), Brasília, DF, Brazil

^e Laboratório de Dermatologia – Hospital das Clínicas de Ribeirão Preto, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (FMRP-USP), Ribeirão Preto, SP, Brazil

^f Faculdade de Medicina, Universidade de Brasília (UnB), Brasília, DF, Brazil

ARTICLE INFO

Article history:

Received 3 February 2015

Accepted 17 March 2015

Available online 15 May 2015

Keywords:

Diagnostics

Disease control

Infectious disease

Leishmaniasis

ABSTRACT

Background: Several tests are performed to obtain better accuracy when diagnosing American tegumentary leishmaniasis (ATL). It is believed that antigens released via secretion, excretion and metabolism are more specific than are antigens released by the lysis of *Leishmania* parasites. Such antigens are known as exo-antigens (exo-Ag) and are formed from products released by cultured parasites in a way that is similar to that in which they cause infections in hosts.

Objective: We attempted to validate a *Leishmania mexicana* ELISA exo-Ag for ATL diagnosis in Midwestern Brazil.

Methods: A total of 281 patients were included in the study. We analysed pre-treatment blood from 98 ATL patients; out of those, 85.7% and 14.3% had cutaneous and mucosal forms, respectively.

Results: The exo-Ag accuracy was 83.99% (95% CI=79.24–87.81) with a sensitivity value of 90.82% (95% CI=83.46–95.09) and an overall specificity value of 80.33% (95% CI=73.97–85.44). The positive predictive value and negative predictive value were 71.20% (95% CI=62.72–78.41) and 94.23% (95% CI=89.40–96.94), respectively. Among healthy controls, exo-Ag had a specificity of 91.25% (95% CI=83.02–95.70); additionally, the test had

* Corresponding author at: Laboratory of Dermatomycology, College of Medicine, University of Brasília (UnB), Campus Darcy Ribeiro, Asa Norte, Brasília, Distrito Federal 70910-900, Brazil.

E-mail address: ciromgomes@gmail.com (C.M. Gomes).

<http://dx.doi.org/10.1016/j.bjid.2015.03.010>

1413-8670/© 2015 Elsevier Editora Ltda. All rights reserved.

specificity rates of 66.67% (95% CI=46.71–82.03) in Chagas disease patients, 60.61% (95% CI=43.68–75.32) in patients with rheumatic diseases, 76.92% (95% CI=49.74–91.82) in pemphigus foliaceus patients, 87.50% (95% CI=52.91–97.76) in leprosy patients, 87.50% (95% CI=63.98–96.50) in VRDL-positive patients, and 77.78 (95% CI=45.26–93.68) in deep mycosis patients.

Conclusion: Based on the indicators of validity, we conclude that the results obtained in this study enable the recommendation of the exo-Ag ELISA for ATL diagnosis once it presented a reasonable accuracy compared to classical methods. Cost evaluations are necessary to completely define the role of this technique in large scale.

© 2015 Elsevier Editora Ltda. All rights reserved.

Introduction

American tegumentary leishmaniasis (ATL) is an infectious and difficult-to-control disease that is caused by parasites of the genus *Leishmania*. ATL causes considerable socioeconomic losses, as it affects subjects at their most productive ages.¹

Several complimentary tests are used to diagnose ATL, all of which have limitations. The diagnostic tests include parasite detection techniques (e.g., direct examination, cultures, histopathological examinations and PCR) and immunodiagnostic techniques (e.g., cellular immune response detection, including the Montenegro skin test (MST), antibody detection, including the fluorescent antibody test (IFAT), ELISA, and immunocomplex antigen detection).^{2,3} Serological tests have been given considerable importance in epidemiological investigations. However, for diagnostic purposes, those methods can be associated with a relatively high proportion of false-positive results.

ELISAs have a great advantage among other serological tests because of their higher specificity. In addition to quickly generated results, ELISAs are also relatively low-cost tests.^{4,5} The antigens most commonly used in leishmaniasis diagnostic immunoenzymatic assays are obtained from intact parasites. The sensitivities of these assays fluctuate between 85% and 100% for crude antigens.⁶ Crude antigens from *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis*, when separated by electrophoresis and subjected to Western blotting, can be recognized by sera from patients with Chagas disease.⁷ This phenomenon is believed to occur because of interactions with *Trypanosoma* and *Leishmania* common antigens.⁸

On the other hand, it is believed that antigens released via secretion, excretion and metabolism are more specific than are antigens released by the lysis of *Leishmania* parasites.^{9,10} Such antigens are known as exo-antigens (exo-Ag) and are formed from products released by cultured parasites in a way that is similar to that in which they cause infections in hosts.¹¹ An ELISA with *Leishmania (Leishmania) mexicana* (*L. mexicana*) promastigote antigens, used to detect IgM and IgG antibodies in sera from Brazilian *Leishmania*-infected patients, had a sensitivity of 92.3% and was reactive when tested in 10 patients with ATL via Western blotting.¹²

ELISA with an exo-Ag from *Leishmania* parasites cultured in protein-free medium is thought to be an improved method

with increased accuracy; therefore, we intended to validate this technique for ATL diagnosis in Midwestern Brazil.

Materials and methods

This study was conducted from August 2007 to July 2010 at the Hospital Universitário de Brasília, Brasília, Brazil, a reference centre for ATL in Midwestern Brazil. Ninety-eight consecutive patients with cutaneous (CL) and mucocutaneous (MCL) disease forms, who were specific treatment naive during the previous six months, were included in a cross-sectional study for diagnostic test accuracy.

ATL diagnosis was performed according to a previously validated composite reference standard formed by clinical and laboratory criteria (direct examination, culture, histopathological examination, PCR from lesion fragments, MST and IFAT).¹³ *Leishmania* subgenus identification was performed at the Laboratory of Dermatology, Hospital das Clínicas, University of São Paulo, Ribeirão Preto, Brazil, using restriction fragment length polymorphism (RFLP) as described elsewhere.¹³

Additionally, the study included a convenience sample of 80 healthy patients with no epidemiological history of leishmaniasis, 24 patients with Chagas disease, 13 patients with American pemphigus foliaceus, 8 patients with lepromatous leprosy, 9 patients with deep mycosis, 16 VRDL-positive patients at a minimum dilution of 1:8, and 33 patients with rheumatic disease who were positive for at least one rheumatic marker test, including anti-streptolysin O (ASO), C-reactive protein (CRP), and rheumatoid factor (RF).

Sampling

Two millilitres of patient serum, obtained through venipuncture, was used. After clot retraction, the samples were centrifuged and stored in labelled tubes at -20° C until the ELISA exo-Ag analyses were performed.

L. mexicana exo-antigen ELISA

A manufactured kit was used for the ELISA reactions. The kit, developed by the Cellabs laboratory (Brookvale, Australia), contained the following components: one ELISA plate with 12 detachable strips, each of which contained eight wells pre-coated with *Leishmania* exo-antigens; one vial of washing buffer (PBS-Tween, 20 \times concentrate); one vial of

peroxidase-conjugated anti-human IgG (100× concentrate); one vial of substrate (20× concentrate); one vial of substrate buffer; one vial of stop solution; one vial of positive control serum; and one vial of negative control serum.

The patients' sera and the positive and negative controls were diluted 1:200 in the washing solution and pipetted into an ELISA plate that had been pre-coated with *L. mexicana* secretory, excretory, and metabolic antigens. The plate was subsequently incubated for 60 min in a humid chamber. After washing the plate four times with PBS plus Tween 20 (0.05%), the peroxidase-conjugated anti-human IgG (diluted 1:100 in washing solution) was added to the plate. Next, the plate was incubated again for 30 min at room temperature, and the plate was washed four more times with PBS-Tween. One millilitre of chromogenic substrate (TMB) pre-diluted in substrate buffer was added to the wells, and the plate was immediately covered with aluminium foil. After a 30-min incubation at room temperature, 100 µL of stop solution (H₂SO₄) was added to each well. The reactions were observed with an ELISA reader (Spectramax Plus, Molecular Devices LLC, Sunnyvale, CA, USA) that was fitted with a 450/620-nm absorbance filter.

Samples with absorbance values above the cut-off point (0.3; above the grey zone) were considered positive. The ELISA reactions were performed and results were evaluated by a specialized biomedicist who was blind to the real condition of patients included.

Sample size

The sample size was calculated based on the hypothesis test for a proportion, available in Epi Info version 3.3.2 (CDC, Georgia, USA) software. According to the sensitivity of ELISA tests for ATL in previously published studies,^{12,14} considering a confidence level of 95% and an absolute precision of 5%, the number of patients to be included for serological tests was defined as 73 patients and 73 controls.

Statistical analysis

The test properties (sensitivity and specificity values, the positive predicted value, and the negative predicted value, and accuracy) were evaluated using OpenEpi[®] version 3.01 (Emory University, Rollins School of Public Health, Atlanta, Georgia, USA). Confidence intervals were set at 95% (95% CI).

This study was approved by the Ethics in Research Committee of the College of Medicine – University of Brasília – UnB (Process number 046/2007). All patients included signed the informed consent form and the study was in accordance to the Declaration of Helsinki, as revised in 2013.

Results

Epidemiological and clinical aspects

Ninety-eight ATL patients were included, of whom 67.4% were men and 32.6% were women; 70.4% patients had only one skin lesion, while 22.4% had two-four lesions and only three (3.1%) had five or more skin lesions. Regarding the clinical disease forms, 85.7% had CL, while 14.3% had MCL. Additionally, 81.6%

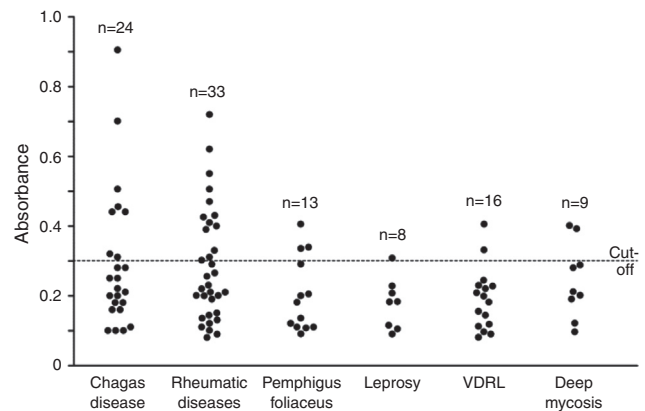


Fig. 1 – Exo-Ag ELISA results for the control subgroups (minimum cut-off value of 0.3).

of the patients had initial infections, while 12.2% had recurrent disease, and no information was available for six patients.

When assessing the sensitivity of the composite reference standard tests, MST was the most sensitive (88.5%), followed by PCR (75.9%) and IFAT (73.1%) (Table 1). RFLP-PCR was performed for samples obtained from 58 patients (Table 1). The subgenera *Viannia* was identified in 95.5% of the 44 positive samples, and the subgenera *Leishmania* was identified in two patients (4.5%).

L. mexicana exo-antigen ELISA

The exo-Ag ELISA overall accuracy was 83.99% (95% CI = 79.24–87.81) with a sensitivity value of 90.82% (95% CI = 83.46–95.09) and an overall specificity value of 80.33% (95% CI = 73.97–85.44). The positive predictive value and the negative predictive value were 71.20% (95% CI = 62.72–78.41) and 94.23% (95% CI = 89.40–96.94), respectively.

The exo-Ag ELISA specificity varied according to the group studied: healthy patients, patients with other diseases, and also in VDRL-positive sera (Table 2). Fig. 1 shows the exo-Ag ELISA reaction results for the groups of patients with other diseases and in VDRL-positive sera (minimum cut-off value, 0.3) (Fig. 1). The exo-Ag ELISA showed false positive results in 13 of the 33 patients with rheumatic diseases (Table 3).

Discussion

Parasite detection methods are recommended as a way to achieve an accurate diagnosis. Given the low sensitivity of these tests, immunological techniques such as ELISA, IFAT, and MST have also been employed. The exo-Ag ELISA test has been studied as a potentially better option for ATL diagnosis.

Direct examination sensitivity ranges from 14 to 89.7%, depending on the parasite species and the disease evolution period.¹³ Parasite cultures from ATL patients also yield low and variable positivity rates (28.6–89%).^{13,15,16} Additionally, the limitations of this technique, including bacterial and fungal contamination and delays to provide the final result (up to 30 days), should be taken into account.

Table 1 – Sensitivity of assays used to diagnose samples from 98 patients with American cutaneous leishmaniasis who were treated at the University Hospital of Brasília from August 2007 to July 2010.

Result	Culture	DE	IFAT	MST	PCR	HE	Exo-Ag ELISA
Positive	47	45	68	62	44	19	89
Negative	43	46	24	8	14	73	9
NP	8	7	6	28	40	6	0
Sensitivity	(52.2)	(49.5)	(73.9)	(88.5)	(75.9)	(20.7)	(90.8)

NP, exam not performed; DE, direct examination; IFAT, immunofluorescence antibody test; MST, Montenegro skin test; *HE, histopathological examination with the presence of amastigote forms.

Table 2 – *Leishmania mexicana* exo-Ag ELISA specificity according to the group of control subjects evaluated.

Group	Samples (n)	False positive (n)	Specificity: (95% CI) ^a
Healthy subjects	80	7	91.25 (83.02–95.70)
Chagas disease	24	8	66.67 (46.71–82.03)
Pemphigus foliaceus	13	3	76.92 (49.74–91.82)
Leprosy	8	1	87.50 (52.91–97.76)
Deep mycosis	9	2	77.78 (45.26–93.68)
VRDL-positive patients	16	2	87.50 (63.98–96.50)
Rheumatic diseases	33	13	60.61 (43.68–75.32)
Total	183	36	80.33 (73.97–85.44)

^a 95% CI, 95% confidence interval.

PCR is considered as the most accurate method for ATL.³ However, a variable sensitivity is expected depending on the specific technique used. Our results are aligned with previous data and confirm that *Leishmania (Viannia) braziliensis* is the most frequent species in the region studied.^{16–19} Similar to other techniques for immunologic tests in leishmaniasis, such as the MST,¹³ the decision of using *L. mexicana* antigens for the ELISA tests is based on the fact that parasites of the *Leishmania* subgenus are more easily managed in laboratory experiments and grow faster in culture media. This action can have an important impact on cost reduction when considering its reproduction on larger scale.

The exo-Ag ELISA performed well by exhibiting a higher sensitivity than did the IFAT, another highly sensitive serological method that may present highly variable results, with rates ranging from 34 to 82.9% (Table 1).^{14,20} Additionally, the IFAT has other disadvantages such as not being suitable for automation, high cross-reactivity in patients with Chagas disease, paracoccidioidomycosis, pemphigus foliaceus, and some deep mycoses.²¹ All those diseases are considered endemic

in some parts of South America, especially in Midwestern Brazil.^{3,22}

The exo-Ag ELISA showed similar results when compared to the index of positive tests obtained by other authors with different antigens. The exo-Ag ELISA sensitivity values were close to those of tests that satisfactorily used chromatography-purified antigenic fractions.^{23,24} As an antigen, the intact parasite contains membrane glycoproteins that act as powerful epitopes due to constant exposure to the host immunological system. Despite comprising glycoproteins that are not present on the parasite membrane, the exo-Ag ELISA sensitivity was similar to that of immunoenzymatic assays that used intact promastigotes.²⁵

The exo-Ag ELISA specificity was not perfect in a control group of apparently healthy patients with no epidemiological history of ATL (Table 2). Although they do not fulfil the composite reference standard for the definition of ATL, it is not possible to completely rule out subclinical *Leishmania* infections once the disease is presenting a growing incidence in otherwise free areas, such as periurban regions. Other subclin-

Table 3 – Results of *Leishmania mexicana* exo-Ag ELISA among 33 rheumatic disease patients, testing positive for at least one rheumatic marker.

Rheumatic marker	ELISA with <i>Leishmania mexicana</i> exo-antigens results	(n)	Specificity (95% CI)
ASO	Positive	3	
	Negative	9	
CRP	Positive	6	60.61
	Negative	7	
RF	Positive	4	(43.68–75.32)
	Negative	4	
	Total	33	

ASO, anti-streptolysin O; CRP, C-reactive protein; RF, rheumatoid factor.

ical diseases that could produce nonspecific antibody binding should also be considered.^{7,26}

The specificity was moderate when Chagas patients' sera were tested (Table 2). Studies have shown that the specificity values for Chagas disease range from a total lack of specificity²⁷ or low values such as 5.5%²⁸ to values as high as 83.33%.²⁴ The latter study used purified glycoprotein antigens that were less likely to cause nonspecific reactions. However, the challenge of cross-reactivity with other trypanosomatids remains and is likely due to the presence of antigenic determinants with similar structures.^{8,29} Therefore, it is necessary to purify the exo-Ag ELISA antigens in order to improve the test specificity against other parasitic diseases such as Chagas disease.

When the serum samples of patients with rheumatic diseases were studied, substantial numbers of nonspecific reactions were observed in all sera in which rheumatic markers were present (Table 3). We highlight the C-reactive protein that presented a considerable number of false positive results. This protein is present in patients with inflammatory conditions as a result of classical pathway activation, which relies on the presence of immunoglobulins. These immunoglobulins seem to exhibit nonspecific behaviours in these patients, which would explain the high exo-Ag ELISA cross-reactivity.

In samples from patients with deep mycoses, two reactions with absorbances above the cut-off value were observed in sera from patients with sporotrichosis. ATL can be mistaken for sporotrichosis either by clinical manifestations or by immunological test cross-reactions, and therefore the differential diagnosis between these two infections is relevant.³⁰ It is also important to remember the possibility of coinfection by *Sporothrix schenckii* and *Leishmania*, as described previously.³¹

It should be pointed out that the use of *L. mexicana* antigens, a species not endemic in Midwestern Brazil, can result in higher frequency of false positive and false negative results. However, as previously stated, the relative difficulty in managing *Leishmania (Viannia) braziliensis* can be an obstacle for species-specific antigens production. By observing the occurrence of nonspecific reactions, we can note the need to study immunoenzymatic reactions with *L. mexicana* exo-antigen antigenic fractions. Additionally, we might suggest the separation of the proteins that comprise *L. mexicana* exo-antigens for using in immunoblot tests with sera from patients with other diseases, in order to identify the less reactive bands that provide higher specificities.

Based on the indicators of validity, we conclude that the results obtained in this study allow for the recommendation of the exo-AG ELISA for ATL diagnosis once it presented a reasonable accuracy compared to classical methods. Cost evaluations are necessary to completely define the role of this technique on large scale.

Funding

Fundação de Amparo à Pesquisa do Distrito Federal; FAP/DF (Process 193.000.332/2007) and Conselho Nacional de Pesquisa; CNPq) (Process 478575/2008-4).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

We thank the preceptors and residents of the Dermatology Service of University Hospital of Brasília, the technicians of the Dermatomyology Laboratory of UnB, and Tércio Rodrigues Pereira, Viviane Medeiros da Costa, and Professor Pedro Luis Taulil.

REFERENCES

- Rodrigues AM, Hueb M, Santos TA, Fontes CJ. Factors associated with treatment failure of cutaneous leishmaniasis with meglumine antimoniate. *Rev Soc Bras Med Trop*. 2006;39:139-45.
- Medeiros AR, Roselino AMF. American cutaneous leishmaniasis: from the beginning until the present time. *An Bras Dermatol*. 1999;74:329-36.
- Gomes CM, de Paula NA, Cesetti MV, Roselino AM, Sampaio RN. Mucocutaneous leishmaniasis: accuracy and molecular validation of noninvasive procedures in a *L. (V) braziliensis*-endemic area. *Diagn Microbiol Infect Dis*. 2014;79:413-8.
- de Arruda MM, Figueiredo FB, Cardoso FA, et al. Validity and reliability of enzyme immunoassays using *Leishmania major* or *L. infantum* antigens for the diagnosis of canine visceral leishmaniasis in Brazil. *PLOS ONE*. 2013;8:e69988.
- Cataldo JI, de Queiroz Mello FC, Mouta-Confort E, et al. Immunoenzymatic assay for the diagnosis of American tegumentary leishmaniasis using soluble and membrane-enriched fractions from infectious *Leishmania (Viannia) braziliensis*. *J Clin Lab Anal*. 2010;24:289-94.
- Szargiki R, Castro EA, Luz E, Kowalthuk W, Machado AM, Thomaz-Soccol V. Comparison of serological and parasitological methods for cutaneous leishmaniasis diagnosis in the state of Paraná, Brazil. *Braz J Infect Dis*. 2009;13:47-52.
- Vega Benedetti AF, Cimino RO, Cajal PS, et al. Performance of different *Trypanosoma cruzi* antigens in the diagnosis of Chagas disease in patients with American cutaneous leishmaniasis from a co-endemic region in Argentina. *Trop Med Int Health*. 2013;18:1103-9.
- Vexenat Ade C, Santana JM, Teixeira AR. Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa *Trypanosoma cruzi*, *Leishmania chagasi* and *Leishmania (viannia) braziliensis*. *Rev Inst Med Trop Sao Paulo*. 1996;38:177-85.
- Martin SK, Thuita-Harun L, Adoyo-Adoyo M, Wasunna KM. A diagnostic ELISA for visceral leishmaniasis, based on antigen from media conditioned by *Leishmania donovani* promastigotes. *Ann Trop Med Parasitol*. 1998;92:571-7.
- Pinedo-Cancino V, Kesper N, Barbiéri CL, Lindoso JA, Umezawa ES. The efficacy of *L. (L.) chagasi* excreted-secreted antigens (ESAs) for visceral leishmaniasis diagnosis is due to low levels of cross-reactivity. *Am J Trop Med Hyg*. 2013;88:559-65.
- Rajasekariah GH, Smithyman AM, Gupta RK, Martin SK. The utility of exoantigens for detection of *Leishmania* infections. *Mil Med*. 2007;172:482-5.
- Ryan JR, Smithyman AM, Rajasekariah GH, Hochberg L, Stiteler JM, Martin SK. Enzyme-linked immunosorbent assay based on soluble promastigote antigen detects

- immunoglobulin M (IgM) and IgG antibodies in sera from cases of visceral and cutaneous leishmaniasis. *J Clin Microbiol.* 2002;40:1037–43.
13. Gomes CM, de Paula NA, de Moraes OO, Soares KA, Roselino AM, Sampaio RN. Complementary exams in the diagnosis of American tegumentary leishmaniasis. *An Bras Dermatol.* 2014;89:701–9.
 14. Ferreira MP, Roselino AM, Nascimento MM, Aires JM, Figueiredo JF. Sensitivity of an immunoenzymatic test for detection of anti-*L. brasiliensis* antibodies compared to other tests used for the diagnosis of American cutaneous leishmaniasis. *Rev Inst Med Trop Sao Paulo.* 2006;48:215–7.
 15. Chouih E, Amri F, Bouslimi N, et al. Cultures on NNN medium for the diagnosis of leishmaniasis. *Pathol Biol (Paris).* 2009;57:219–24.
 16. Luz ZM, Silva AR, Silva Fde O, Caligorne RB, Oliveira E, Rabello A. Lesion aspirate culture for the diagnosis and isolation of *Leishmania* spp. from patients with cutaneous leishmaniasis. *Mem Inst Oswaldo Cruz.* 2009;104:62–6.
 17. Falqueto A, Sessa PA, Ferreira AL, et al. Epidemiological and clinical features of *Leishmania (Viannia) braziliensis* American cutaneous and mucocutaneous leishmaniasis in the State of Espírito Santo, Brazil. *Mem Inst Oswaldo Cruz.* 2003;98:1003–10.
 18. Garcia FCB, Santos SSR, Chociay MF, Medeiros ACR, Roselino AMF. Subsidiary methods for the diagnosis of American tegumentary leishmaniasis (ACL): comparison of sequencing of DNA and PCR-RFLP for identification of leishmania species in skin samples. *An Bras Dermatol.* 2005;80:S339–44.
 19. Andrade MS, Brito ME, Silva ST, et al. American tegumentary leishmaniasis caused by *Leishmania (Viannia) braziliensis* in military training area of Zona da Mata in Pernambuco. *Rev Soc Bras Med Trop.* 2005;38:229–33.
 20. Reis Lde C, Brito ME, Almeida EL, et al. Clinical, epidemiological and laboratory aspects of patients with American cutaneous leishmaniasis in the State of Pernambuco. *Rev Soc Bras Med Trop.* 2008;41:439–43.
 21. Ministério da Saúde, Brasília, 180 pp. Secretaria de Vigilância em Saúde. Manual de controle da Leishmaniose Tegumentar Americana [Manual of American Tegumentary Leishmaniasis control]. 2nd ed; 2007.
 22. Gomes CM, Cesetti MV, de Moraes OO, Mendes MS, Roselino AM, Sampaio RN. The influence of treatment on the development of leishmaniasis recidiva cutis: a 17-year case-control study in Midwestern Brazil. *J Eur Acad Dermatol Venereol.* 2015;29:109–14.
 23. Gomes-Silva A, Souza MA, Afonso-Cardoso SR, et al. Serological reactivity of different antigenic preparations of *Leishmania (Leishmania) amazonensis* and the *Leishmania braziliensis* complex. *Rev Soc Bras Med Trop.* 2008;41:135–41.
 24. Vidigal Cde P, Marcussi VM, Marcussi LM, et al. Enzyme immunoassay using *Leishmania (Viannia) braziliensis* antigens for laboratorial diagnosis of American cutaneous leishmaniasis. *Acta Trop.* 2008;107:208–12.
 25. Gil JF, Hoyos CL, Cimino RO, et al. Role of three ELISA tests using promastigote homogenates of *Leishmania braziliensis*, *L. amazonensis* and *L. guyanensis* in the diagnosis of tegumentary leishmaniasis. *Medicina (B Aires).* 2011;71:420–8.
 26. Gomes CM, Ávila LR, Pinto SA, et al. *Leishmania braziliensis* amastigotes stimulate production of IL-1 β , IL-6, IL-10 and TGF- β by peripheral blood mononuclear cells from nonendemic area healthy residents. *Parasite Immunol.* 2014;36:225–31.
 27. Mendes DG, Lauria-Pires L, Nitz N, et al. Exposure to mixed asymptomatic infections with *Trypanosoma cruzi*, *Leishmania braziliensis* and *Leishmania chagasi* in the human population of the greater Amazon. *Trop Med Int Health.* 2007;12:629–36.
 28. Yoneyama KA, de Peder LD, Lonardoni MV, Silveira TG. Diagnosis of American cutaneous leishmaniasis by enzyme immunoassay in patients from Northern Paraná State, Brazil. *Braz J Infect Dis.* 2007;11:360–4.
 29. Ferreira LR, Kesper N, Teixeira MM, et al. New insights about cross-reactive epitopes of six trypanosomatid genera revealed that *Crithidia* and *Leptomonas* have antigenic similarity to *L. (L) chagasi*. *Acta Trop.* 2013;131:41–6.
 30. de Lima Barros MB, Schubach A, Francesconi-do-Valle AC, et al. Positive Montenegro skin test among patients with sporotrichosis in Rio De Janeiro. *Acta Trop.* 2005;93:41–7.
 31. Agudelo SP, Restrepo S, Vélez ID. Cutaneous New World leishmaniasis-sporotrichosis coinfection: report of 3 cases. *J Am Acad Dermatol.* 1999;40 Pt 1:1002–4.