International Journal of General Medicine
and Pharmacy (IJGMP)
ISSN(P): 2319-3999; ISSN(E): 2319-4006
Vol. 3, Issue 5, Sep 2014, 99-110

International Academy of Science,
Engineering and Technology
Connecting Researchers; Nurturing Innovations

VALIDATION OF THE DIRECT IMMUNOFLUORESCENCE TEST FOR THE DIAGNOSIS OF AMERICAN CUTANEOUS LEISHMANIASIS IN BRAZIL

KILLARNEY ATAIDE SOARES[1], ADA AMÁLIA AYALA URDAPILLETA[2], GILCILENE MARIA DOS SANTOS[3], ANDREA LISBOA CARNEIRO[4] & RAIMUNDA NONATA RIBEIRO SAMPAIO[5]

[1,2,4,5]Dermatomycology Laboratory, Medical Clinic, Faculty of Medicine, University of Brasilia-UnB, Brasilia-DF, Brazil

[5]Dermatology Service, University Hospital of Brasilia-HUB, University of Brasilia-UnB, Brasília-DF, Brazil

[3]Foundation to Education and Research in Health Sciences (Fepecs), Brasília-DF, Brazil

ABSTRACT

Background: Cutaneous leishmaniasis is a public health problem whose diagnosis depends on the various methods used together to get better sensitivity and specificity. The Direct immunofluorescence (DIF) is a method for the diagnosis of American cutaneous leishmaniasis (ACL) has not been largely studied.

Objetive: to validate DIF for the diagnosis of ACL.

Patients/Methods: this study included 72 patients with confirmed diagnosis of ACL to determine sensitivity and 55 patients with skin lesion, but carriers other diseases to determine specificity. For each patient, were obtained skin biopsy imprints on glass slides. In the next step, was added fluorescein-labeled polyclonal antibody diluted 1:20. Positivity was considered based on the finding of intra or extracellular fluorescent oval-shaped amastigotes.

Results: the clinical results showed a predominance of cutaneous form (84,9%) and only 15,1% of mucosal form. The direct immunofluorescence showed sensitivity of 72,2 ± 10,4% (n = 72, CI 95%) and specificity was 96,3 ± 5,0% (n = 55, CI 95%). The positive predictive value was 96,3 ± 4,3% (n = 74; CI 95%), negative predictive value was 72,6 ± 10,1 (n = 75; CI 95%) and accuracy was 82,7%. Thirty five (89,7%) of 39 samples were identified as *Leishmania Viannia* subgenus by PCR-RFLP.

Conclusions: the indicators of validity were satisfactory and another advantage was quick diagnosis. Therefore, we believed that DIF was validated for the diagnosis of ACL in Brazil.

KEYWORDS: American Cutaneous Leishmaniasis, Diagnosys, Direct Immunofluorescence, Leishmania Viannia Braziliensis

INTRODUCTION

American cutaneous leishmaniasis (ACL) is a difficult-to-control parasitic infectious disease caused by parasites of the *Leishmania* genus. It causes physical and psychological problems, leading to socioeconomic losses for affecting individuals in the most productive phase of their lives[9,30].

The diagnosis of ACL includes clinical, epidemiological and laboratory findings. The existence of a broad spectrum

of clinical aspects that may be confused with those of other diseases and an overlap of endemic regions for ACL and some of these diseases make laboratory tests increasingly relevant in confirming the diagnosis. An investigation of the database of the National Information System for Notifiable Diseases (SINAN), Brazilian Ministry of Health, showed that laboratory tests were decisive for the diagnosis of various diseases in 77.6% of cases of ACL in a historical series from 2001 to 2005[8].

Laboratory tests can be classified into techniques for detecting the parasite (direct demonstration of parasites, culture, hamster inoculation, histopathological examination and Polymerase Chain Reaction) and immunodiagnostic techniques (Montenegro skin test – MST, indirect fluorescent antibody test, and ELISA)[4,19,36].

Parasitological techniques, such as demonstration of amastigotes and culture, are laborious and time-consuming and cannot be automated. Direct demonstration of the parasite has low sensitivity indirectly related to duration of the lesions.

Histopathological examination is recommended by the Brazilian Ministry of Health in the routine diagnosis of ACL. However, it has low sensitivity and may vary, especially when *Leishmania (V.) braziliensis* is the species involved. In many clinical cases, in the absence of the parasite, the diagnosis is based on the description of inflammatory cell manifestations[17]. Another of its disadvantages is sample collection via skin biopsy, which is an invasive technique that requires trained professionals. For these reasons, histopathological examination is not routinely used in the Brazilian health services[21].

Indirect immunofluorescence (IIF) is a serological technique widely employed and which shows satisfactory results; however, it fails in terms of specificity in relation to other diseases. MST is antigen-dependent and may be negative early in the disease, in the diffuse form of ACL and in immunocompromised patients. In general, immunological methods have other limitations such as positivity in patients clinically cured[22]. In addition, there is proven cross-reactivity observed against *Leishmania chagasi* and *Trypanosoma cruzi*[44] and other diseases such as lepromatous leprosy and South American pemphigus foliaceus.

Direct immunofluorescence (DIF) is a technique which has not been largely studied for the diagnosis of ACL. There are positive reports of its use in tissue samples of lymph nodes obtained from dogs with Visceral leishmaniasis (VL)[23,24].

Currently, a combination of laboratory techniques is used for confirmation of ACL so that they will complement each other, since all of them present limitations. Considering the above-described picture of the diagnosis of ACL, this study aims to validate the DIF technique for the diagnosis of ACL.

MATERIALS AND METHODS

This study included patients seen at the Dermatology Service of the University Hospital of Brasilia, which is a reference center for the diagnosis and treatment of ACL in Brazil, from August 2007 to July 2010. We included 72 patients according to the following inclusion criteria: patients with a diagnosis of ACL in the cutaneous and mucosal forms confirmed by clinical and epidemiological history, in addition to positivity in at least two routine laboratory tests; treatment-naïve patients or patients with recurrence of the disease who did not undergo specific treatment for ACL 6 months prior to sample collection; and patients who agreed to participate in the study by signing a consent form. For analysis of specificity, we included samples obtained from patients with skin lesions that had received a different diagnosis.

Laboratory Tests for the Diagnosis of ACL: MST was performed via intradermal inoculation of promastigote forms of *Leishmania (L.) amazonensis* (WHO reference strain MHOM/BR/73/PH8 - Center for Immunobiological Production and Research/ Paraná, Brazil). Reading was done through wheal measurement after 48 hours[27]. For IIF, positivity was considered in dilutions starting at 1:40[41]. On histopathological examination, presence of amastigotes in cellular inflammatory infiltrate was investigated[42]. For direct demonstration of amastigotes, *imprints* of skin lesions on slides stained with Giemsa were examined[15]. Culture was performed with a sample obtained from aspirates of the edge of the lesion[18], followed by seeding in NNN medium[32].

Direct Immunofluorescence: we used two kits for DIF (Leishmania Cell, Cellabs, Australia). Each kit was composed of a vial containing 1.25 ml of fluorescein isothiocyanate (FITC)-labeled anti-IgG conjugate and a vial containing 2.5 mL of glycerol and Evans blue in alkaline medium (mounting reagent). Skin tissue fragments were obtained through a 4 mm punch skin biopsy. The fragments were pressed against glass slides (6 compressions per slide) using a tweezer. The slides were identified and stored in a freezer at -30°C until fixation. Then, each slide was dipped in ice acetone bath, and 25 mL of the anti-IgG conjugate diluted 1:20 were added to each *imprint*. The slides were incubated for 30 minutes at 37°C in a moist chamber and washed with saline solution. After drying, a drop of the mounting fluid was added (glycerol and Evans blue in alkaline medium) and covered with a coverslip. Reading of the reaction was done using an immunofluorescence microscopy with a 40x objective. Positivity was considered when fluorescent intra or extracellular amastigotes were found.

Polimerase Chain Reaction – Randon Fragment Length Polimorphysm (PCR-RFLP): each patient had a sample of their damaged skin tissue biopsied and then pressed at three different points of a piece of filter paper. After drying, it was put in an envelope and stored at 4°C. The PCR-RFLP technique followed the methodology previously described[45], but with some modifications concerning DNA extraction, as indicated below. The samples on filter paper were cut with sterile scalpel and placed in a sterile vial previously identified. Next, the nucleic material was extracted using 30 microliters of sterile water, followed by stirring in vortex-like apparatus and subsequent heating at 96°C for 10 minutes in a dry block heater.

Statistical Analysis: the indicators of validity provided for diagnostic study were calculated. It was followed the matrix for calculation of indicators for serological tests[13] to obtain the values of sensitivity (SE) and specificity (SP), the positive predictive value (PPV), negative predictive value (NPV), and the accuracy (A) of the DIF test. SE = [TP/(TP + FN)×100], SP = [TN/(TN + FP)×100], PPV = [TP/TVP + FP)×100] and NPV = [TN/(TN + FN)×100] and A = [(TP + TN) /(TP + TN + FP + FN) x100] where TP represents true positive, TN represents true negative, FP represents false positive and FN represents false-negative. To analyze the specificity of the DIF test, we performed tests on glass slides with skin lesions of patients who had received a different diagnosis. For these patients, the diagnosis of ACL had been excluded. A comparison between DIF test and direct demonstration of the parasite was done using the Chi Square test, Epi Info software, version 7 (Centers for Disease Control and Prevention, Georgia, USA). Intervals of prevalence (P) were also calculated to determine the limits for true frequencies of the sample contained. For this purpose, it was used the interval prevalence equation: $P \pm 1.96 \text{ ÖP}(1-P)/n$.

Ethical Issues: this study was approved by the Ethics Committee of the School of Medicine, University of Brasilia (Number 046/2007). All patients included in this study were asked to participate voluntarily and signed an informed consent in accordance with Resolution 196/96 of the National Health Council.

RESULTS

Epidemiological and Clinical Data of Patients with ACL: we included 72 patients, of whom 49 (68.1%) were men and 23 (31.9%) were women. Fifty-five (76.4%) patients had a single skin lesion, while 14 (19.4%) had from 2 to 4 lesions, and only 3 patients (4.2%) had five or more lesions. As for clinical form, 64 (88.8%) of the patients were diagnosed with Cutaneous leishmaniasis (CL) and 8 patients (11.2%) presented the mucosal form (MCL). The disease was diagnosed in 64 (88.8%) cases for the first time, while it relapsed in 5 (7.0%) patients who had already been diagnosed and treated. This datum was not found in the case of three patients.

Routine Tests: the results and sensitivity of each routine method for diagnosis of ACL are shown in Table 1 Regarding parasitological techniques, direct demonstration of amastigotes showed positivity in 36 (50%), and culture showed positivity in 34 (47.2%) samples. MST had a sensitivity of 91.9%, while IIF had a sensitivity of 73.1%.

PCR-RFLP: was performed in 56 samples of 72 patients. The reaction was positive in 39 out of 56 samples (SE = 69.6%). The controls amplified from strains of *Leishmania (L.) amazonensis* and *Leishmania (L.) donovani* showed no digestion, validating the reaction.

PCR-RFLP showed that 35 (89.7%) out of 39 samples had a digestion pattern consistent with the subgenus *Viannia*, while 1 as classified as belonging to the subgenus *Leishmania* and 1 presented a digestion pattern not compatible with the species that cause ACL. Other 2 (5.0%) samples showed different bands, which did not allow classification of the subgenus.

DIF: was positive in 53 (SE = 73.6% ± 10.4%) samples. In most cases (46 out of 53), the finding of a slide was in agreement with the finding of a second slide. Among the samples from patients with MCL, positivity was found in 5 of 8 (62.5%), whereas positivity was found in 48 of 64 (75%) samples from patients with ACL. DIF results were negative in 15 out of 19 patients with a single lesion.

Table 2 relates DIF results with others clinical data and PCR-RFLP results. The DIF test showed more negativity in patients with lesions longer, especially 12 or more months. However, patients with MCL form showed more positivity comparing relative values of patients carriers of CL form.

Regarding specificity, when the DIF technique was performed on slides with imprint from skin lesions of patients with other diseases (n = 55), rounded fluorescent forms were visualized in only two of them. These two cases were diagnosed as Sporotrichosis and Chromomycosis. DIF showed specificity of 96.3% ± 5.3. Considering the results of both sensitivity and specificity, we calculated the positive and negative predictive values and the accuracy of the DIF test. PPV was 96.3%, while NPV was 72.6%, and accuracy was 82.7% (Table 3).

When comparing DIF and direct demonstration of the parasite, we found $x^2 = 7.29$ and $p = 0.0069$ (95% CI, $p < 0.05$), which demonstrates that there was a statistically significant difference between the two techniques (Chi Square).

Discussion: the methods for demonstrating the parasite to achieve the right diagnosis is recommended[22] such as direct demonstration, culture, and histopathological examination. Given the low sensitivity of these methods, immunological techniques such as ELISA, IIF, and MST are usually employed as complementary techniques[22]. In this complex diagnostic scenario, in which sensitivity and specificity are not always satisfactory, DIF is a promising option that can be adopted.

In this study, DIF showed higher sensitivity than direct demonstration of amastigotes. DIF was 45% more sensitive than direct demonstration, considering that the sample is obtained in the same way in both techniques. Furthermore, there was a statistically significant difference indicating that the use of an antibody labeled with a fluorescent substance allowed greater visualization of amastigotes compared to those present on stained slides used for direct demonstration ($p < 0.05$).

The indices of positivity for direct demonstration of amastigotes in lesions caused by *Leishmania (V.) braziliensis* vary, especially because of the smaller diameter and scarcity of parasites in ACL[6,7,23,47]. There is considerable variation concerning the sensitivities found for direct demonstration, ranging from 14 to 89.7%[5, 16,21,28,29,31,37].

When compared to culture, DIF also showed satisfactory sensitivity, higher than the average found by several authors, which ranged from 28.6 to 89%[5,16,18,31,35,34,37,46]. In addition, culture presents some limitations, such as the possibility of bacterial contamination and the delay in yielding the final result, which can take up to 30 days.

A fact that drew attention was the good performance of DIF, whose sensitivity was statistically the same as that of IIF, a serological method that has high sensitivities, even though they are quite variable, between 34 and 82.9%[11,12,28,29,31,33]. On the other hand, the IIF test has disadvantages, which include impossibility of automation, cross-reactivity in sera from patients with diseases such as Chagas, Paracoccidioidomycosis, Pemphigus foliaceus, and other deep mycoses[9], and the possibility of a false-negative result in patients with the cutaneous form of the disease, especially in cases with few lesions.

We found few validation studies on DIF for the diagnosis of Leishmaniasis. Two of those studies concern samples obtained from lymph nodes of dogs for the diagnosis of VL. The technique showed a positivity of 93.3%[24] and 92.68%[23] in the samples evaluated. Based on the data, the authors concluded that the DIF method should be used to confirm suspected cases of canine VL in endemic regions.

Parasitological techniques have an ideal specificity; however, they depend on the presence of the parasite so that it can be demonstrated, which leads to a highly variable sensitivity. On the other hand, techniques based on the demonstration of antibodies often present a high detection capability, but positivity reaction is not always specific. Therefore, in spite of the many advances in diagnosis, most reference centers for ACL in Brazil use four or even five techniques to increase sensitivity and specificity.

When DIF was performed on samples from patients with other diseases, we also obtained good results, since fluorescence of structures similar to amastigotes led to non-specific reaction in only 2 (3.7%) of the 55 samples analyzed. These structures were slightly oval, with about the size of a *Leishmania species*, but they were extracellular.

The positive predictive value of DIF was high (96.2%). Thus, when positive, DIF indicates *Leishmania* infection with high reliability. It should be noted that the study of the medical records of the two cases in which there was a positive reaction revealed diagnosis of Chromomycosis and Sporotrichosis, demonstrating possible non-specificity, which should be studied in

the future in other infectious diseases, especially those caused by fungi.

ACL may be confused with Sporotrichosis, considering either clinical signs or cross-reactions in immunological tests; thus the importance of carrying out a differential diagnosis between the two infections[40]. One should not dismiss the possibility, however small, of co-infection with *Sporothrix schenkii* and *Leishmania*[1]. There may be cross-reactivity between the antibodies for both diseases among patients with ACL (R. Almeida-Paes, unpublished results) and among patients with sporotrichosis[3]. Presence of several ellipsoid and round structures inside and outside macrophages were reported and highlighting the similarity with *Leishmania* in a patient with ulcerative lesions with positive culture for *Sporothrix schenkii*[20].

The accuracy of the DIF test was 82.7%. Despite its high specificity, the method, as well as histopathology and direct demonstration of amastigotes, depends on the presence of the parasite in the *imprint*, which directly influences the sensitivity of the technique. In addition to the lack of scientific articles evaluating the sensitivity of DIF, no studies to determine the specificity of the same technique were found in a literature review on the subject.

Of the samples subjected to PCR-RFLP, 36 (92.3%) showed two bands (80 and 40 bp), confirming the subgenus *Viannia*. There is prevalence of *Leishmania (V.) braziliensis* in several regions of Brazil[2,3,10,12,16]. In the Central-West region - place of infection most often reported by patients included in the study - the predominance of this parasite was reported[25,38] as well as the occurrence of specific sand fly vectors in the transmission of the parasite[39].

The sensitivity to PCR found in this study was expected to be higher. It is believed that factors such as collection of the *imprint* on filter paper and the time during which the samples were stored may have influenced the result. It was observed that samples collected early in the project produced more negative results.

It should be noted that it is easy to perform the DIF test, since there is only one incubation step, which takes 30 minutes. Thus, it is possible to prepare 30 slides (15 patients) for reading in one hour. However, it is important to consider that there are limitations to this technique, since collection of the material is invasive and requires professional expertise and adequate sanitation. We suggest studies with other forms of sample collection, such as direct apposition of the slide at the edge of the scarified lesion or preparation of slides with aspirate specimens.

The World Health Organization classifies Leishmaniasis as a neglected disease that predominantly affects people with low purchasing power in a development countries[48]. Investment in diagnosis and treatment has been relegated because it does not show to be profitable. The choice of the most affordable and effective method should be made by the institutions responsible for public health, since early initiation of treatment depends on the diagnosis of the disease[43].

The validity of a diagnostic test result is measured by its capacity to accurately determine positivity in people who are truly sick and negativity in people who are healthy[14]. This is important for public health measures, since this is necessary in order to apply diagnostic techniques that are appropriate for population studies with the objective of diagnosing individuals affected by the disease in its preclinical and clinical stage.

Based on the good results of the validation tests and on the advantage of fast results, it is believed that DIF can be adopted as a routine technique for ACL in outpatient care, especially in cases of infection with *Leishmania (V.) braziliensis.*

Acknowledgments: the authors thank Cellabs Biotechnology for providing the immunofluorescence kit, the professors. Ana Maria Roselino (School of Medicine of Ribeirão Preto, University of São Paulo - FMRP/USP) and Dr. Pedro Luis Tauil (Center for Tropical Medicine, University of Brasilia - NMT/UnB). We also thank the staffs of the Dermatology Unit of University Hospital of Brasília and Dermatomycology Laboratory's technicians of the School of Medicine of UnB.

Financial Support: This work was supported in part by Conselho Nacional de Desenvolvimento e Pesquisa (CNPq): number 478575/2008-4. Fundação de Amparo à Pesquisa do Distrito Federal–FAP/DF: Number 193.000.332/2007.

Conflicts of Interest none

REFERENCES

1. Agudelo SP, Restrepo S, Velez ID. Cutaneous New World leishmaniasis-sporotrichosis coinfection: report of 3 cases. *J Am Acad Dermatol* 1999; **40:** 1002-1004.
2. Andrade MS, Brito MEF, Silva ST, Lima BS, Almeida EL, Albuquerque EL, Marinho JF, Ishikawa E, Cupolillo E, Brandão-Filho SP. American cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis*, in a military training área, at Zona da Mata of Pernambuco. *Rev Soc Bras Med Trop* 2005; **38**(3): 229-233.
3. Barros MBL, Schubach AO, Valle ACF, Gutierrez-Galhardo MC, Schubach TMP, Conceição-Silva F, Matos-Salgueiro M, Mouta-Confort E, Reis RS, Madeira M, Cuzzi T, Quintella LP, Passos JPS, Conceição MJ, Marzochi MCA. Positive Montenegro skin test among patients with sporotrichosis in Rio de Janeiro. *Acta Trop* 2005; **93**: 41–47.
4. Castilho TM, Shaw JJ, Floeter-Winter LM. New PCR assay using glucose-6-phosphate dehydrogenase for identification of leishmania species. *J Clin Microbiol* 2003; **4**(2): 540-546.
5. Choulli E, Amri F, Bouslimi N, Siala E, Selmi K, Zallagua N, Abdallah RB, Bouratbine A, Aoun K. Les cultures sur milieu NNN dans le diagnostic biologique des leishmanioses. *Pathol Biol* 2009; **57**: 219-224.
6. Cuba CAC, Marsden PD, Barreto AC, Rocha R, Sampaio RR, Parzlaff L. Parasitologic and Immunologic Diagnosis of American (mucocutaneous) Leishmaniasis. *PAHO Bull* 1981; **15**(3): 249-259.
7. Cuba CAC, Marsden PD, Barreto AC, Rocha R, Sampaio RR, Patzlaff L. Diagnóstico parasitológico e imunológico de leishmaniose tegumentária americana. *Bol Of San Panam* 1980; **89**(3): 195-205.
8. Datasus. *National Information System for Notifiable Diseases, historical series: 2001 to 2005*. 2009. Avaliable at: http://dtr2004.saude.gov.br/sinanweb/tabnet/tabnet?sinan/lta/bases/ltabr.def. (last acessed 13 april 2009).
9. Desjeux P. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 2004; **27**: 305-318.
10. Falqueto A, Sessa PA, Ferreira AL, Vieira VP, Santos CB, Varejão JBM, Cupolillo E, Porrozzi R, Carvalho-Paes LE, Grimaldi Jr G. Epidemiological and Clinical Features of *Leishmania (Viannia) braziliensis* American Cutaneous and Mucocutaneous Leishmaniasis in the State of Espírito Santo, Brazil. *Mem. I. Oswaldo Cruz* 2003; **98**(8): 1003-1010.

11. Ferreira MP, Roselino AMF, Nascimento MMP, Aires JM, Figueiredo JFC. Sensitivity of an immunoenzymatic test for detection of ant-*L. brasiliensis* antibodies compared to other tests used for the diagnosis of American cutaneous leishmaniasis. *Rev Inst Med Trop* 2006; **48**: 215-217.

12. Garcia FCB, Santos SSR, Chociay MF, Medeiros ACR, Roselino AMF. Subsidiary methods for the diagnosis of American tegumentar leishmaniasis (ATL): comparison of sequencing of DNA and PCR-RFLP for identification of leishmania species in skin samples. *An Bras Dermatol* 2005; **80**(3): 339-344.

13. Guimarães MC, Coutinho SG, Antunes CMF. Rules for serology of parasitary diseases. *Rev Soc Bras Med Trop* 1987; **20**: 55-58.

14. Hennekens CH, Buring JE *Epidemiology in medicine.* Boston/Toronto: Little, Brown and Company, 1987, p. 383.

15. Ikeda-Garcia FA, Feitosa MM. Diagnostic methods of canine visceral leishmaniasis. *Clin Vet* 2006; **62**: 32-38.

16. Luz ZMP, Silva AR, Silva FO, Caligiorne RB, Oliveira E, Rabello A. Lesion aspirate culture for the diagnosis and isolation of *Leishmania* sp. from patients with cutaneous leishmaniasis. *Mem Inst Oswaldo Cruz* 2009; **104**(1): 62-66.

17. Magalhães AV, Moraes MA, Raick AN, Llanos-Cuentas A, Costa JM, Cuba C, Marsden PD. Histopatology of cutaneous leishmaniasis caused by *Leishmania leishmania braziliensis*. Histopatological classification. *Rev Ins Med Trop* 1986; **28**: 428-430.

18. Marzochi MC, Teixeira PC, Marzochi KF, Conceição NF, Coutinho W, Brito DB. Vaccum aspiratory puncture system for *Leishmania* culturing, isolation and transport preliminary report. *Rev Inst Med Trop* 1993; **35**(3): 301-303.

19. Medeiros ACR, Roselino AMF. Leishmaniose Tegumentar Americana: do histórico aos dias de hoje. *An Bras Dermatol* 1999; **74**: 329-336.

20. Mello CX, Schubach AO, Madeira MF. Can yeast-like form of *Sporothrix schenkii* confuse the direct parasitological diagnosis of American cutaneous leishmaniasis. *Rev Soc Bras Med Trop* 2011; **44**(1): 127.

21. Menezes da Silva JA, Moura NFO, Elkhoury ANSM, Alves WA, Souza M; Oliveira SM, Dantas MCS, Oliveira GM. Laboratorial diagnosis of American cutaneous leishmaniasis in Brazil by analysis of National Information System for Notifiable Diseases. *Rev Soc Bras Med Trop* 2007; **40**(1): 139.

22. Ministry of Health, Brazil, Secretary of Health Surveillance. *Manual to control of American Leishmaniasis Tegumentary*. 2nd edn. Brasília, 2007; 180 p.

23. Moreira MAB, Luvizotto MCR, Garcia .JF, Corbett CEP. Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. *Vet Parasitol* 2007; **145** (3-4): 245-252.

24. Moreira MAB, Luvizotto MCR, Nunes CM, Silva TCC, Laurenti MD, Corbett CEP. Aplicação da técnica de Imunofluorescência direta para o diagnóstico da leismaniose visceral canina em aspirado de linfonodo. *Braz J Vet Res Anim Sci* 2002; **39**(2): 103-106.

25. Mota JOC, Sampaio, RNR. A pilot study comparing low-dose liposomal amphotericin B with N-methyl glucamine for the treatment of American cutaneous leishmaniasis. *J Eur Acad Dermatol Venereol* 2011; **26**(3): 331-335.

26. Navin TR, Arana FE, Mérida AM, Arana BA, Castillo L, Silvers DN. Cutaneous Leishmaniasis in Guatemal: comparison of diagnostic methods. *Am J Trop Med Hyg* 1990; **42**(1): 36-42.

27. Oliveira MRF, Macedo VO, Carvalho EA, Barral A, Marotti JG, Bittencourt A, Abreu MVA, Orge MGO, Lessa HA, Marsden PD. Follow-up of 7-17 years of patients with mucosal leishmaniases caused by *Leishmania (Viannia) braziliensis* in Três Braços, Bahia. *Rev Soc Bras Med Trop* 1995; **28**, 325-332.

28. Perles TF, Curti MCM, Barros FMF, Fernandes ACBS, Zanzarini PD, Venazzi EAS, Silveira TGV, Arraes SMAA, Lonardoni MVC. Características laboratoriais da leishmaniose tegumentar americana em pacientes com as formas cutânea ou mucosa, na Região Noroeste do Estado do Paraná. *Rev Soc Bras Med Trop* 2006; **39**(3): 184.

29. Reis LC, De Brito MEF, Almeida EL, Felix SM, Medeiros ACR, Silva CJ, Pereira VRA. Aspectos clínicos, epidemiológicos e laboratoriais de pacientes com leishmaniose cutânea americana no Estado de Pernambuco. *Rev Soc Bras Med Trop* 2008; **41**(5): 439-443.

30. Rodrigues AM, Hueb M, Rodrigues-dos-Santos TAR, Fontes CJF. Fatores associados ao insucesso do tratamento da leishmaniose cutânea com antimoniato de meglumina. *Rev Soc Bras Med Trop* 2006; **39(**2): 139-145.

31. Rodrigues EHG, Brito MEF, Mendonça MG, Werkha RP, Coutinho EM, Souza WV, Albuquerque MFPM, Jardim ML, Abath FGC. Evaluation of PCR for diagnosis of American Cutaneous Leishmaniasis in an area of endemicity in Northeastern Brazil. *J Clin Microbiol* 2002; **40**(10): 3572-3576.

32. Romero GAS, Ishikawa E, Cupollilo E, Toaldo CB, Guerra MV, Paes MG, Macedo VO, Shaw JJ. Identification of antigenically distinct populations of *Leishmania (Viannia) guyanensis* from Manaus, Brazil, using monoclonal antibodies. *Acta Trop* 2002; **82**: 25–29.

33. Romero GAS, Orge MGO, Guerra MVF, Paes MG, Macêdo VO, Carvalho EM. Antibody response in patients with cutaneous leishmaniasis infected by *Leishmania (Viannia) braziliensis* or *Leishmania (Viannia) guyanensis* in Brazil. *Acta Trop* 2005; **93**: 49-56.

34. Romero GAS, Sampaio RNR, Macêdo VO, Marsden PD. Sensitivity of lymph node aspiration in localized cutaneous leishmaniasis due to *Leishmania (Viannia) braziliensis*. *Mem Inst Oswaldo Cruz* 1999; **94**(4): 509-511.

35. Salinas G, Valderrama L, Palma G, Montes G, Saravia NG. Detection of amastigotes in cutaneous and mucocutaneous leishmaniasis using the immunoperoxidase method, using polyclonal antibody: sensibility and specificity compared with conventional methods of diagnosis. *Mem Inst Oswaldo Cruz* 1989; **84**: 53-60.

36. Salman SM, Rubeiz NG, Kibbi AG. Cutaneous Leishmaniasis: Clinical Features and Diagnosis. *Clin Derm* 1999; **17**: 291-296.

37. Sampaio RNR, Andrade GB, Pereira AC, Da Silva EA, Cuba CAC. Estudo comparativo de técnicas de demonstração de amastigotas e isolamento de promastigotas no diagnóstico da leishmaniose tegumentar americana. *An Bras Dermatol* 2002; **77**(5): 557-561.

38. Sampaio RNR, De Paula CDR. American cutaneous leishmaniasis in Federal District, Brazil. Rev Soc Bras Med Trop 1999; **32**(5): 523-528.

39. Sampaio RNR, Gonçalves MC, Leite VA, França BV, Santos GM, Carvalho MSL, Tauil PT. Study on the transmission of American cutaneous leishmaniasis in the Federal District. *Rev Soc Bras Med Trop* 2009; **42**(6): 686-690.

40. Santos IB, Schubach TMP, Leme LRP, Okamoto T, Figueiredo FB, Pereira SA, Quintela LP, Madeira MF, Oelho FS, Reis RS, Schubach AO. Sporotrichosis: the main differential diagnosis with tegumentary leishmaniosis in dogs from Rio de Janeiro, Brazil. *Vet Parasitol* 2007; **143**: 1-6.

41. Silveira TGV, Arraes SMAA, Bertolini DA, Teodoro U, Lonardoni MVC, Roberto ACBS, Ramos M, Sobrinho AN, Ishikawa E, Shaw, J. Observations on laboratory diagnosis and cutaneous leishmaniasis epidemiology in the State of Paraná, South of Brazil. *Rev Soc Bras Med Trop* 1999; **32**(4): 413-423.

42. Stolf HO, Marques MES, Yoshida ELA, Dillon NL. Surto de leishmaniose tegumentar americana em Itaporanga, São Paulo (Brasil). *Rev Inst Med Trop* 1993; **35**(5): 437-442.

43. Szargiki R, De 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 Inf Dis* 2009; **13**(1): 47-52.

44. Vexenat AC, Santana JM, Teixeira ARL. Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa *Trypanosoma cruzi*, *Leishmania chagasi* and *Leishmania (Viannia) braziliensis. Rev Inst Med Trop S Paulo* 1996; **38**(3), 177-185.

45. Volpini AC; Passos VMA; Oliveira GC; Alvaro JR. PCR-RFLP to identify *Leishmania (Viannia) braziliensis* and *L. (Leishmania) amazonensis* causing American cutaneous leishmaniasis. *Acta Trop* 2004; **90**: 31-37.

46. Weigle KA, Labrada LA, Lozano C, Santrich C, Barker DC. PCR-based diagnosis of acute and chronic cutaneous leishmaniasis caused by *Leishmania (Viannia). J Clin Microbiol* 2002; **40**: 601-606.

47. Weigle KA, Santrich C, Martinez F, Valderrama L, Saravia NG. Epidemiology of cutaneus leishmaniasis in Colombia: environmental and Behavioral risk factors for infection, clinical manifestations, and pathogenecity. *J Inf Dis* 1993; **168**: 709-714.

48. WHO: World Health Organization. *Distribution of cutaneous leishmaniasis worldwide, 2009. Map production: Control of Neglected Tropical Diseases.* Avaliable at: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_leishmaniasis_cutaneous_2009.png. (last acessed 15 january 2011).

APPENDICES

Table 1: Results Regarding Sensitivity of Diagnostic Tests in Samples of 72 Patients with American Cutaneous Leishmaniasis Seen at the University Hospital of Brasilia from August 2007 to July 2010

Result	Culture	Direct Demonstration	IIF	MST	Histopathological Examination
Positive (sensitivity%)	34 (47.2)	36 (50)	49 (73.1)	57 (91.9)	18 (25.7)
Negative	38	36	18	5	52
NP	-	-	5	10	2

NP: test not performed. **IIF:** Indirect Immunofluorescence Test. **MST:** Montenegro skin test.

Table 2: IFD Results Related with Clinical and Laboratorial Data of 72 Patients with Confirmed Diagnosis of American Cutaneous Leishmaniasis Seen at the University Hospital of Brasilia from August 2007 to July 2010

Clinical Data	IFD	
	Positive	Negative
Number of Patients	52	20
Clinical Leishmaniasis form		
Cutaneous	48	17
Mucosal	04	03
Number of Skin Lesions		
01	37	15
02-04	12	03
05-09	02	01
Not related	01	01
Duration of Lesions		
< 1 month	02	0
1-3 months	23	08
4-6 months	15	04
7-12 months	03	01
> 12 months	04	05
Not related	05	02
Recidive form		
Yes	02	03
No	47	17
Not related	03	0
***Leishmania* Subgenous by PCR-RFLP**	**In 39 Patients**	**In 16 Patients**
L. (Viannia)	26	09
L. (Leishmania)	01	0
Not classified*	01	02
Negative	11	05

Table 3: Indicators of Validity (Sensitivity, Specificity, Predictive Values, and Accuracy) for the Direct Immunofluorescence Test Performed on Tissue *Imprint* Samples Obtained from Skin Lesion Biopsy of 72 Patients with Confirmed Diagnosis of American Cutaneous Leishmaniasis Seen at the University Hospital of Brasilia from August 2007 to July 2010

Imprints on Slides	Sensitivity% (95% CI)	Specificity% (95% CI)	Predictive Value (95% CI)		Accuracy (%)
			Positive	Negative	
Patients diagnosed with ACL	73.6 ± 10.4 (No. = 72)		96.3 ± 4.3 (No. = 74)	72.6 ± 10.1 (No. = 75)	82.7
Patients with other diseases		96.3 ± 5.3 (No. = 55)			

CI: confidence interval.