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Evaluation of different diagnostic methods of American Cutaneous Leishmaniasis in the Brazilian Amazon

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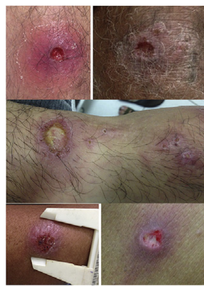
HIGHLIGHTS

- Diagnostic methods were evaluated.
- Clinical forms make difficult diagnosis.
- Parasitological exams is the most indicate and cheaper method.

GRAPHICAL ABSTRACT



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ABSTRACT

Epidemiological studies have been conducted to better understand the dynamics of American Cutaneous Leishmaniasis (ACL) in the Amazon region where distinct species of *Leishmania* circulate. In endemic areas, the optimal diagnosis must be made in the earlier clinical presentation to avoid the complications of chronic disease. The scarcity of financial support, laboratory infrastructure and trained persons are the major obstacles in this reality. This paper describes the result of performing different diagnostic methods for ACL in Amazonas State between the years 2010 and 2011. The tests used were the intradermal skin test (Montenegro's skin test), ELISA (Enzyme-Linked Immunosorbent Assay), direct examination, culture isolation and identification of *Leishmania* species. A total of 38 suspected human cases of ACL were diagnosed by different methods, of which 71.0% (n = 27) were positive by direct examination, 75.6% (n = 28) had positivity in the culture isolates and, of these, 54.0% (n = 19) had infection with *Leishmania* (*Viannia*) *guyanensis*. The positivity of the intradermal skin test with the leishmanin solution was observed in 77.0% of cases analyzed and the serology with detection of IgG and IgM showed the presence of antibodies in 100% of exams realized results, showing variation in the titles of antibodies. The success of Leishmaniasis treatment depends on an effective and early diagnosis. Parasitological diagnosis is highly specific, but sensitivity is subject to variation because the tissue distribution of parasites generally is not homogeneous and depends on the specie of parasite. Moreover, parasitological tests require invasive procedures and depend on restrictive conditions for the collection of biological sample, which limit their use in large-scale for epidemiological studies. ELISA has been the most widely used serological

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method for the diagnosis of Visceral Leishmaniasis (VL) as it is easy to perform and has a low cost. However, flaws in specificity are observed in the diagnosis of cutaneous leishmaniasis. Actually the diagnosis needs to be done as an associated methods depending on the question to be solved.

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1. Introduction

Neglected Diseases consist in a group of diseases that affect mainly the under developed countries, and may be endemic in developing countries and may have a serious threat to industrialized countries (Alvar et al., 2012). The World Health Organization (WHO) estimates that a billion of people in 149 countries suffer from one or more neglected tropical diseases (NTD). Leishmaniasis is among the seventeen diseases considered as an NTD by WHO, occupies the category of emerging and uncontrolled diseases (Lindoso and Lindoso, 2009; Alvar et al., 2012) and is present in 98 countries and territories (WHO, 2010; Alvar et al., 2012). Over 350 million people live in risk areas and, each year, 500 000 develop Visceral Leishmaniasis (VL) and 1.5 million with Cutaneous form of the illness (Desjeux, 2004; Alvar et al., 2012). Factors such as environmental changes, uncontrolled migration, ecotourism, working in dangerous areas, difficulties in treatment, immunocompromised hosts and migration of non-immune people to endemic areas, contribute to the increase in the number of cases (Desjeux, 2001; Shaw, 2007; Gomes et al., 2014).

In Brazil, the American Cutaneous Leishmaniasis (ACL) is distinguished for its wide distribution, occurring in all states of the country. Amazonas State notified 2230 new cases of the disease only in the year 2011 and the transmission occurred mainly in the cities of Manaus (752 cases), Presidente Figueiredo (213) and Rio Preto da Eva (203) (Sinan, 2012). This importance is not only in their high incidence and wide geographical distribution, but also the possibility to assume chronic clinical forms that might determine disfiguring and disabling injuries, with major repercussions in the psychosocial field of the individual (Gontijo and Carvalho, 2003). A total of seven species of *Leishmania* (ATL) are found in Brazil (and all in the north region): *Leishmania* (*Viannia*) *braziliensis*, responsible for localized mucocutaneous and disseminated cutaneous leishmaniasis; *L.* (*V.*) *guyanensis*, *L.* (*V.*) *lainsoni*, *L.* (*V.*) *shawi*, *L.* (*V.*) *lindenbergi* and *L.* (*V.*) *naiffi* by localized cutaneous leishmaniasis; *L.* (*Leishmania*) *amazonensis*, by localized cutaneous and diffuse cutaneous leishmaniasis (Lainson et al., 1987; Silveira et al., 2002; Gramiccia and Gradoni, 2005). The Amazon region is responsible for 37% of new cases and the most prevalent species are *L.* (*V.*) *guyanensis* and *L.* (*V.*) *braziliensis* (Arias and Naiff, 1981). A comparison of clinical and etiologic agents revealed that 83% of reported cases in the region are caused by *L.* (*V.*) *guyanensis* (Naiff et al., 1999; Figueira et al., 2008; Espir et al., 2014). The endemic disease associated with this pathogen affects on rural and urban outbreaks (75.9% and 24.1% of caoty to ulcerated lesions of other diseases such as Virchowian leprosy, paracoccidioidomycosis, tropical ulcer, syphilis, cutaneous tuberculosis, cancer, among others (Gontijo and Carvalho, 2003). Furthermore, there are limitations of conventional diagnostic methods therefore the diagnosis must be made by the association of clinical, epidemiological and laboratory testing aspects. These include the identification of amastigotes in the tissue by immunocytochemical techniques and in imprints (printing affixing biopsy) of aspirate lesion and histopathological evaluation; Isolation of promastigotes *in vitro*; Serological methods such as indirect immunofluorescence assay (IFA), direct agglutination test (DAT), ELISA (Enzyme-linked immunosorbent assay) and Western blot analysis, based on the presence of specific antibodies against parasite antigens; cell mediated immunity as the Montenegro skin test (MST) detection of kDNA by PCR and others (Kar, 1995). One of the most common diagnostic tests, direct observation of amastigotes in stained slides of imprints by Giemsa method under an optical microscope, shows a sensitivity of 50–70%. This technique depends on the number of parasites in the stained smear. The test positivity is inversely proportional to the time of the skin lesion evolution, and is rare after one year (Vega-Lopez, 2003; Bensoussan et al., 2006). Immunological tests employed in clinical practice are indirect indicators of infection by *Leishmania*. The results of MST indicates previous infection and of a cellular immune reaction against the parasite, but does not distinguish whether infection is present or past, so its diagnostic importance is hampered in patients from endemic areas, because asymptomatic individuals may have a positive test (Goto and Lindoso, 2010). In treated patients, the MST still remains positive indefinitely and in cases of infection with *L.* (*L.*) *major* was observed positivity for more than 19 months after treatment (Sassi et al., 1999). MST is positive in more than 80% of patients with cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (ML), while the diffuse cutaneous leishmaniasis (DCL) is persistently negative (Costa et al., 1986; Reis et al., 2009). In this study were evaluated different diagnostic methods of CL in the Amazonas State, Brazil.

2. Materials and methods

2.1. Patients

The study was designed as cross-sectional descriptive and a convenience sample. The results of laboratory tests of 38 patients with cutaneous lesions clinically suspected of ACL, treated between 2010 and 2011 in the Basic Health Unit Manoel Rumão Km 135, from Manaus-Itacoatiara highway and in the Hospital Thomé de Medeiros Raposo located in the municipality of Rio Preto da Eva – Amazonas State, were evaluated. All of the patients came from endemic areas in the north of the Amazon River, Brazil, where predominance of human cases of *L.* (*V.*) *guyanensis* has been reported. Thirty-eight CL patients (23 males and 15 females; mean age 31.22 ± 2.518 (SD) and 37.93 ± 4.113 (SD) years, respectively) with active skin lesions coming from endemic areas located at the Amazonas State-Brazil, were studied. The diagnostic criteria were based on parasitological and immunological parameters as described below. A total of 19 strains isolated from 37 patients were characterized as *L.* (*V.*) *guyanensis* and one as *L.* (*L.*) *amazonensis* by multilocus enzyme electrophoresis (isoenzyme analysis) (Cupolillo et al., 1994).

2.2. Inclusion criteria and diagnostic methods

All patients included in this study were selected from a population of individuals attending at the ambulatory of the Basic Health Unit Manoel Rumão, and in the Hospital Thomé de Medeiros Raposo, Rio Preto da Eva, Amazonas State, that presented a positive clinical picture (skin lesion) and suggestive epidemiology for ATL.

Also the inclusion criteria were: individuals of both genders, resident in Rio Preto da Eva town, Amazonas State, with ages between 18 and 70 years old, presenting characteristic disease symptoms, with 15 to 90 days of lesion evolution, accept to participate in the study by signing the Informed Consent Term (ICT) and who filled a standard form consisting of information regarding age, gender, professional activity, size and number of lesions, time of infection, use of topical or oral medication and/or alternative medicine. As to ATL infection is no “gold standard method” was defined alternatively for this study that the positivity in culture or smear microscopy is a composite reference standard, but false negative was considered. All patients included in this study presented primary infection of *Leishmania* spp. (Table 1). The exclusion criteria used were: absence of skin lesions, pregnancy, previous infection or in current treatment for ACL and individuals that no accept to participate in this investigation and with below 18 years of age. A control group of healthy individuals at the same study area were included in this study and were used to perform the ELISA's tests. All patients were submitted to direct examination by scarification of cutaneous lesions edges and search for amastigote forms in smear, stained using Panoptycal kit (LB Laborclin®) and analyzed by optical microscopy (1000X of magnification). The smear was the initial test performed. Smears were obtained by both slit skin method. Material from the scarification of lesion edges was also inoculated in biphasic blood agar NNN growth media (Novy and McNeal, 1904; Nicolle, 1908) and when positive, the parasites were subsequently expanded in complete liquid media *Schneider Drosophila Medium* (pH 7,2) supplemented with 10% Inactivated Bovine Fetal Serum (iBFS) to obtain mass of parasites to use in biochemical characterization and cryopreservation of flagellate forms in liquid nitrogen. The *L.* (*L.*) *amazonensis* leishmanin, produced and standardized by Ministry of Health, Brazil was used in the study. This leishmanin contains killed *L.* (*L.*) *amazonensis* (MHOM/BR/73/PH8) promastigotes at a concentration of 40 μg/mL proteic nitrogen (10^7promastigotes per mL in sodium chloride 0,0098 g/mL and phenol 0.005 g/mL). Leishmanin skin test was carried out on the first reporting day of clinically suspected cases of CL. The definitive parasitological diagnosis was made by smear microscopy or parasite culture, fulfilling the above-mentioned criteria. For the MST, the skin over the volar surface of the forearm was cleaned with alcohol 70% and 0.1 mL of the leishmanin antigen was injected intradermally. The induration area of the

Table 1
Result of different methods of diagnosis performed in cutaneous leishmaniasis patients suspected in the state of Amazonas, BR*.

[a]*Leishmania* isolates	[b]Species	Sex	Occupation	Direct examination	Cultivation	MST[c]	IgG [e]BT	IgM [e]BT	Length (days)
MHOM/BR/10/IM 5637	*L. guyanensis*	F	Biologist	+	+	+	1:80	1:40	60
MHOM/BR/10/IM 5653	*L. guyanensis*	M	Farmer	−	+	+	1:160	1:20	90
MHOM/BR/10/IM 5657	*L. guyanensis*	M	Farmer	+	+	+	1:20	1:80	40
MHOM/BR/10/IM 5679	*L. amazonensis*	F	Farmer	+	+	−	UR[d]	UR[d]	30
MHOM/BR/10/IM 5681	*Leishmania* sp	M	Farmer	−	−	+	1:40	1:80	30
MHOM/BR/10/IM 5684	*Leishmania* sp	M	Farmer	+	+	−	1:40	1:80	30
MHOM/BR/10/IM 5686	*Leishmania* sp	M	Farmer	−	+	UR[d]	1:20	1:40	60
MHOM/BR/10/IM 5690	Leishmania sp	F	Farmer	+	+	+	1:160	1:80	60
MHOM/BR/10/IM 5691	*Leishmania* sp	F	Businessman	−	−	UR[d]	1:160	1:80	60
MHOM/BR/10/IM 5692	*Leishmania* sp	F	Businessman	+	+	+	1:80	1:20	15
MHOM/BR/10/IM 5694	*L. guyanensis*	M	Farmer	+	+	UR[d]	1:160	1:80	60
MHOM/BR/10/IM 5696	*L. guyanensis*	F	Farmer	+	+	UR[d]	1:80	1:80	30
MHOM/BR/10/IM5697	*L. guyanensis*	M	Student	+	+	+	1:40	1:80	15
MHOM/BR/10/IM5698	*Leishmania* sp	M	Student	−	−	+	1:40	1:80	15
MHOM/BR/11/IM 5747	*L. guyanensis*	M	Farmer	−	+	+	1:40	1:80	15
MHOM/BR/11/IM 5748	*Leishmania* sp	M	Student	+	+	UR[d]	1:20	1:40	20
MHOM/BR/11/IM 5749	*L. guyanensis*	M	Farmer	+	+	UR[d]	1:40	1:80	30
MHOM/BR/11/IM 5750	*L. guyanensis*	F	Housewife	−	+	+	1:20	1:40	25
MHOM/BR/11/IM 5751	*Leishmania* sp	M	Farmer	−	−	+	1:40	1:40	15
MHOM/BR/11/IM 5752	*L. guyanensis*	M	Businessman	+	+	+	1:40	1:80	20
MHOM/BR/11/IM 5756	*Leishmania* sp	F	Businessman	−	+	−	1:40	1:20	15
MHOM/BR/11/IM 5757	*Leishmania sp*	M	Farmer	+	C	+	1:40	1:40	30
MHOM/BR/11/IM 5759	*Leishmania* sp	M	Public servant	+	+	UR[d]	1:80	1:80	30
MHOM/BR/11/IM 5760	*Leishmania* sp	M	Farmer	+	−	UR[d]	1:40	1:80	30
MHOM/BR/11/IM 5771	*Leishmania* sp	F	Farmer	+	−	+	1:20	1:40	30
MHOM/BR/11/IM 5772	*L. guyanensis*	M	Farmer	+	+	−	1:20	1:80	30
MHOM/BR/11/IM 5773	*L. guyanensis*	M	Farmer	+	+	+	1:40	1:80	20
MHOM/BR/11/IM 5797	*Leishmania* sp	F	Student	+	+	UR[d]	1:40	1:40	30
MHOM/BR/11/IM 5826	*L. guyanensis*	M	Farmer	+	−	+	1:40	1:80	30
MHOM/BR/11/IM 5827[f]	*Leishmania* sp	F	Farmer	−	−	+	UR[d]	UR[d]	30
MHOM/BR/11/IM 5828	*L. guyanensis*	F	Farmer	+	+	+	1:40	1:80	30
MHOM/BR/11/IM 5829	*L. guyanensis*	F	Farmer	+	+	+	1:40	1:80	30
MHOM/BR/11/IM 5830	*L. guyanensis*	M	Farmer	+	+	−	1:80	1:80	30
MHOM/BR/11/IM 5832	*L. guyanensis*	M	Farmer	+	+	−	1:20	1:80	20
MHOM/BR/11/IM 5833	*L. guyanensis*	F	Farmer	+	−	UR[d]	1:20	1:40	30
MHOM/BR/11/IM 5840	*Leishmania* sp	F	Farmer	−	+	UR[d]	1:40	1:80	60
MHOM/BR/11/IM 5847	*Leishmania* sp	M	Farmer	+	+	+	1:40	1:20	60
MHOM/BR/11/IM 5869	*L. guyanensis*	M	Farmer	+	+	+	1:80	1:40	60

*All biological material was collected by healthcare professionals at the Joint Health Unit Thomé de Medeiros Raposo and the Basic Health Unit Manoel Rumão, Municipality of Rio Preto da Eva.

[a] Designations: Host [M = Mammalia: HOM = *Homo sapiens*]/Country of origin/year of isolation/original code used by INPA.
[b] Stock identification was established by enzyme electrophoresis.
[c] MST: Montenegro skin test.
[d] UR: unrealized.
[e] Imunoglobulin before treatment.
[f] Imprint made one week after first examination with a positive result.

reaction was read after 48 h. MST was measured using the ballpoint technique (Sokal, 1975; Skraba et al., 2015). An induration of ≥5 mm was taken as a positive reaction.

The antigen protein dosage was done by Bradford method (Bradford, 1976) and concentration adjusted (2.5 μg antigen/mL) for serological assays (ELISA/enzyme linked immunosorbent assay) was performed in poliestirene microplates (Nunc Denmark, Maxi Sorp), with 96 well coat with 100 μL per well, diluted in Carbonate Buffer (pH 9.6). The plates was sealed and incubated for minimum of 18 h or overnight at 4 °C in damp chambers and, subsequently washed with Rins age Solution (RS) containing 0.05% Tween20® and 0.9% de NaCl. Afterwards, remaining plate sites were blocked with 150μL/well of Blockage Buffer containing 2% skimmed milk (Molico®/WM/whole milk), incubated for 30 min at 37 °C in damp chamber and submitted for subsequent rinsing procedures with RS. Serum samples were added (100μL/well, in pairs) in serial dilutions (1/10 to 1/160) in incubation buffer containing 0.05% Tween20® and 0.25% WM. After incubation for 30 min at 37 °C, plates were washed and received immunoenzimatic conjugate (polyclonal SIGMA® antibodies) anti-IgG (A-0293) or Human IgM (A-0420), produced in goats, linked to peroxidase, diluted to 1:40.000, 1:50.000 respectively in volume of 50μL/well and after incubated for 30 min at 37 °C. After that, plates were washed again, then added enzimatic substrate containing ortho-Phenylenediamine (OPD/SIGMA® P5412-100TAB] in Sodium Citrate/citric acid buffer, pH 5 with 4 μL H_2O_2 (50μL/well) at room temperature. After colour development for 15 min, reaction was interrupted with H_2SO_4 4 N and readings accomplished in microplate reader (BIO TEK®) at 492 nm.

2.3. Statistical analysis

Comparative analysis between groups was performed by ANOVA followed by the *Kruskal-Wallis* test using *Graph Pad Prism 5.0* (San Diego, CA, USA). Statistical significance was defined in both cases $p < 0.05$.

2.4. Ethical approval

Experimental investigation was done by consent obtained from all human adult participants and according to the requirements of the National Health Council Resolution 196/96, and approved by the ethics committee for research involving human subjects at the National Institute of Amazonian Research (INPA) under number 006/2010.

3. Results

Out of the total 38 patients studied 23 (61%) were males and 15 (39%) were females (Table 1). Age range was 18–72 years old. Sample collection time for diagnostic tests ranged according to the time patients seeked medical care. History of wilderness activity was observed in 75% of cases and all individuals reported having acquired the disease in rural areas in populational settlements situated along Rodovia AM-010 (AM-010 State Express way) where cycles of wild transmission occur. Among parasitological exams performed, 71% (n = 27) showed positive in the direct examination and 75, 6% (n = 28) in the blood agar NNN culture media, isolation of *L. (V.) guyanensis* was observed in 54% (n = 19) of the samples, followed by *L. (L.) amazonensis* which was observed in one sample (n = 1), parasitic growth during one sample showed bacterial contamination and was discarded.

The ELISA test was performed in 36 patients because two samples were unsuitable for testing. Concerning IgM and IgG titers, all patients (100%) showed reactivity to the immunoglobulins at the time of disease diagnosis. Based on cut-off values, cases were considered positive if IgM titers ≥1/20 and IgG titers≥1/20. Control group (no-infected individuals) was composed of eight patients who will accepted to take part in the study by signing an Informed Consent Term. Among them reactivity was 1/10 for both immunoglobulins. For test positivity classification criteria, titers were classified as weakly positive (1:20), positive (1:40 e 1:80) and strongly positive (1:160).

Out of 36 ELISA exams performed for IgG, 22% (8) showed weakly positive reactions (Titer 1:20), 67% (24) presented positive reactions, with titer ranges between 1:40 and 1:80, and the majority (18 individuals) showed titers of 1:40. Strongly positive reactions were observed in 11% (4) of analyzed samples, with observed titers of 1:160. On IgM titers analysis, 89% (32) showed positive reactions (1:40 or 1:80) and weakly positive reactivity was found in 10% (4) of the total. There were no strongly positive IgM titers among the samples studied. Reactivity difference of IgG and IgM comparing patients and controls was significant $p < 0.0001$.

Delayed type hypersensitivity (DTH) is an important feature of all forms of CL. The skin test or Montenegro skin test (MST) measures DTH reactions to an intradermal injection of a suspension of killed promastigotes. It is a useful and important tool for epidemiological, immunological, and diagnostic studies. The immunological procedure is similar to others tests like the tuberculin test. MST was performed in 38 patients, of whom 29% (11) did not return to reading the test within 48 h. Of the 27 subjects who returned for reading, positive result was observed in 77% (21) of the patients and in 33% (6) the result was negative. The standard response to MST was quite varied and the reactions showed the following values: Weakly reactive (values close to negative; 5 − ≤ 10 mm) 37% reactive (>10 mm ≤ 20) and 50% strongly reactive (>20 mm) 12.5% (Table 2).

4. Discussion

Cutaneous leishmaniasis is proved to be endemic in Amazonas State (Guerra et al., 2006; Figueira et al., 2014; Espir et al., 2014) considered as a serious public healthcare issue as well as in several Brazilian States (Alvar et al., 2012). This study was realized in a municipality of Amazonas State named as Rio Preto da Eva. This town in Amazonas State has been created in the 1960 serving as a road connected for Manaus and Itacoatiara towns. It is located close to Km 80 of AM-010 Road (3°07′06“ S, 59°W), 57,5 Km away from the state capital, and is part of Manaus metropolitan area. Population consists of 26.948 people and its territory comprises 5.813 Km^2, according to the IBGE (Brazilian Institute of Geography and Statistics). The area consists of firm land damp tropical forest (rain forest) with a great diversity in species. Climate is tropical with an average of year temperature around 26 °C, precipitation over 2.000 mm/year and relative humidity above 80%. In this town the circulation of four species of *Leishmania*, had already detected in human, causing different clinical forms of ATL, however the major incidence is caused by *L.* (*V.*) *guyanensis*, followed by *L.* (*L.*) *amazonensis*, *L.* (*V.*) *naifi* and *L.* (*V.*) *braziliensis* (Figueira et al., 2008; Espir et al., 2014).

Traditional diagnostic tests such as blood smear analysis, culture and skin biopsy histopathology are not always conclusive in patients with clinical diagnosis of cutaneous leishmaniasis (Faber et al., 2003). Actually, the methods used to make the diagnosis show low sensitivity operational disadvantage in endemic areas since that the infrastructure is still rather deficient in these regions (Silveira et al., 1999). In this study MST presented 60% positivity. It is important to notice that many patients (eleven individuals) did not make it back for induration readings, showing the difficulty in applying the test in the diagnosis of ACL in theses areas. It was

Table 2
Result of Montenegro skin test of Cutaneous Leishmaniasis in the Amazonas State, BR.

Montenegro skin test			
Leishmania isolates	[a]Values close to negative	[b]Reactive	[c] Strongly reactive
MHOM/BR/10/IM5637		18 × 14	
MHOM/BR/10/IM5653	7 × 6		
MHOM/BR/10/IM5657		11 × 11	
MHOM/BR/10/IM5681		15 × 12	
MHOM/BR/10/IM5690			20 × 20
MHOM/BR/10/IM5692	6 × 5		
MHOM/BR/10/IM5697		15 × 14	
MHOM/BR/10/IM5698		10 × 13	
MHOM/BR/11/IM5747	8 × 9		
MHOM/BR/11/IM5750	5 × 6		
MHOM/BR/11/IM5751	9 × 8		
MHOM/BR/11/IM5752	7 × 6		
MHOM/BR/11/IM5757		16 × 14	
MHOM/BR/11/IM5771		14 × 14	
MHOM/BR/11/IM5773	5 × 3		
MHOM/BR/11/IM5826		16 × 15	
MHOM/BR/11/IM5827		15 × 12	
MHOM/BR/11/IM5828			27 × 23
MHOM/BR/11/IM5829		15 × 7	
MHOM/BR/11/IM5847	6 × 6		
MHOM/BR/11/IM5869		11 × 6	

[a] Values close to negative (5 – ≤ 10 mm).
[b] Reactive (>10 a ≤20).
[c] Strongly reactive (>20 mm).

noticed that reactivity MST antigen showed random induration sizes among patients testing positive, varying from weakly positive to strongly positive. Beginning in the first month of lesion evolution, 57% (17) of patients showed MST positive tests and had significant titers of antibody in ELISA assays, attesting the relevance of using these techniques to laboratorial diagnosis even in patients bearing recent lesions.

Direct examination for parasite detection in slides showed 65% positivity in samples analyzed and has shown to be a useful and relatively fast and inexpensive test for disease diagnosis.

Patients were also included if they already presented parasite positivity in culture or smear microcopy, however sometimes patients make use of different substances for topical treatment before diagnosis, that affects the results of parasitological examinations, for this reason was included in this study all suspect patients for ATL, mainly with skin lesions and epidemiological characteristics of disease transmission. It is often necessary for patients to return to the realization of the exams a week later after the lesion care with hygiene just soap and water. This result could be seem in Table 1, with five patients that had the *Leishmania* infection and the parasitological diagnosis were negative and the others exams were positive. For this reason, these patients with negative parasitological tests were considered as positive to CL.

Rodríguez-Cortés et al. (2010) verified that the use of serological tests in experimental infections by *Leishmania* is highly effective for detection of IgG_2 anti-*Leishmania* and the use of this test for disease diagnosis can be useful in patients who host parasites. The same to occur in *L.* (*V.*) *guyanensis* infections observed in our study, where reactivity to serologic tests to homologous *Leishmania* antigen was 100% for IgG and IgM.

Nevertheless, until today there is no consensus to a “gold standard” method for disease diagnosis (Rodríguez-Cortés et al., 2010). Polymerase chain reaction (PCR) exams is highly sensitive for ACL diagnosis (Gontijo and Carvalho, 2003) a could be done. Faber et al., in 2003, in a study comprising 46 patients with proven Cutaneous Leishmaniasis, detected positivity in all samples tested by PCR and traditional testing, culture being the most sensitive, however, there were no statistically significant difference for sensitivity when comparing direct exam, histopathological exam and MST, such data are comparable to the ones obtained in our study. Molecular biology diagnosis methods, such as PCR are still expensive and restricted to experimental studies in spite of undeniable demonstrations of advantages in its sensitivity and specificity (Oliveira et al., 2005; Rhajaoui et al., 2007). Tojal et al., 2006 using PCR mKDNA, found 100% positivity in 50 patients studied from Rio Branco town – Acre/Brazil, and 98% positivity for MST in the same patients, thus demonstrating that the skin test is an excellent auxiliary tool for the disease clinical diagnosis.

The disease occurs in both males and females and at any age range with possible domiciliar and peridomiciliary transmission (Faber et al., 2003). The *L.* (*V.*) *guyanensis* (77, 5%) and *L.* (*L.*) *amazonensis* (2.5%) species were characterized. This observation leads us to emphasize that the association of techniques such as MST, ELISA (detection of specific immunoglobulins) and direct examination for the detection of parasites, adds up to a more effective CL laboratorial diagnosis. This fact points out to the necessity of professional healthcare worker training and adequacy of laboratories both in public and private networks for the diagnosis of CL in the State of Amazonas since it is not performed on a routine basis in most clinical labs.

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