

Disseminated Leishmaniasis by *Leishmania viannia* Subgenus: A Series of 18 Cases in Southeastern Brazil

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Background. Disseminated leishmaniasis (DL) is an emerging clinical form of American tegumentary leishmaniasis (ATL) that occurs mainly in Northeastern Brazil. This study describes 18 cases where DL has not yet been reported.

Methods. Disseminated leishmaniasis cases were extracted from ATL recorded cases between 1987 and March 2015. Disseminated leishmaniasis identification was based on ≥ 10 mixed-type lesions, located in ≥ 2 body parts.

Results. Eighteen (5.4%) patients were identified as DL. Polymerase chain reaction followed by enzymatic digestion confirmed *Leishmania viannia* subgenus in 17 patients; amastigotes forms were identified in another one.

Conclusions. Considering that DL diagnosis and management is challenging, clinicians must be aware of this emerging clinical form of the disease.

Keywords. cutaneous leishmaniasis; leishmaniasis.

Leishmaniasis encompasses a spectrum of diseases caused by an obligate intramacrophage protozoa belonging to the genus *Leishmania*. They represent a major public health problem with an annual estimated worldwide incidence of 700 000; a prevalence of 12 million cases; and an estimated mortality of 50 000 patients in 2010 [1].

Three main forms of American tegumentary leishmaniasis (ATL) occur in Brazil: localized cutaneous, mucocutaneous, and disseminated leishmaniasis (DL). In the state of Sao Paulo, Southeastern Brazil, the localized cutaneous and mucocutaneous forms of ATL are associated with *Leishmania (Viannia) braziliensis* and *Leishmania amazonensis* [2].

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Disseminated leishmaniasis was first described in 1986, and it has been mainly reported in Northeastern Brazil [3–7]. Disseminated leishmaniasis is characterized by the presence of a great number of pleomorphic cutaneous lesions spanning 2 or more noncontiguous areas of the patient's body [3–5]. The patients typically report a single initial lesion, usually on the extremities, followed by dissemination that may involve the entire body, sometimes associated with fever and chills [4]. Although DL accounted for 0.2% of the total number of cases of cutaneous leishmaniasis (CL) in the early 1970s, in 2000 this figure rose to 1.9% [5], and between 1988 and 2008 its prevalence increased 3-fold and culminated in 2.4% of the total number of cases [8].

The pathogenesis of DL remains unclear; parasite species, host, and environmental factors may favor parasite dissemination. Disseminated leishmaniasis patients have higher frequency of negative skin test to *Leishmania* antigen [4] as well as poorer interferon-gamma and tumor necrosis factor-alpha responses than patients with CL [3–5, 9]. Therefore, decreased T-cell response has been hypothesized to play a major role in parasite dissemination, and abnormal chemokine production may be associated with this phenomenon. In the same way, different subpopulations of *L (V) braziliensis* have been associated with distinct clinical outcomes and caused distinct in vitro reactivity in peripheral blood mononuclear cells of ATL patients. These findings have suggested that certain strains in this complex *L braziliensis* subpopulations may constitute a major risk of DL [10].

This study describes a series of 18 cases of DL. The first case was diagnosed in 1987 during ongoing outpatient clinic-based surveillance conducted at the University Hospital, Ribeirao Preto Medical School, University of Sao Paulo, Brazil, where DL cases had not been reported before.

SUBJECTS AND METHODS

Data Collection

Patients were attended at the University Hospital of the Ribeirao Preto Medical School, University of Sao Paulo, which is the main reference for the diagnosis and treatment of ATL in the Northeastern region of the state of Sao Paulo, Brazil.

Disseminated Leishmaniasis Diagnosis

Disseminated leishmaniasis was identified according to a case definition of 10 or more pleomorphic lesions located in 2 or more body parts [3–5]. Laboratorial analyses confirmed case patients by identifying amastigotes forms in skin biopsies or *Leishmania* species in biopsy specimens with molecular methods. Patients with DL were identified between June 1987 and March 2015, by review of the medical charts of all the patients

who received a diagnosis of ATL. A standardized entry form was used to extract the demographic, clinical, diagnostic, laboratorial, and treatment information from the medical records.

Leishmania Skin Test

Leishmania skin test (LST) was performed as described previously [11], and induration equal to or higher than 5 mm, between 48 and 72 hours after the injection, was considered a positive result.

Skin Biopsies

The diagnosis was defined as leishmaniasis if the parasites were identified on the hematoxylin-eosin or Giemsa-stained sections. *Leishmania* species was determined in smears and/or skin biopsy samples. DNA was extracted from 5 paraffin-embedded skin samples and from 12 cryopreserved skin samples. There was no sample for one of the patients, who was the oldest case, diagnosed in 1987.

Polymerase Chain Reaction

The used primers were based on a sequence of the minicircle kDNA of *Leishmania* sp 5'-(G/C)(G/C)(C/G)CC(A/C)CTAT (A/T)TTACACCCAACCCC-3' and 5'-GGGGAGGGGCGTTCTGCGAA-3' (Eurofins, MWG Operon, Huntsville, AL) as described previously [2]. The reactions were performed in a Mastercycler Pro Thermocycler (Eppendorf, Hamburg, Germany) at the final volume of 23 μ L, containing 12.9 μ L distilled water, 2 μ L 10 \times buffer (10 μ M Tris-HCl [pH 8.6], 50 μ M KCl, and 1.5 μ M MgCl₂), 2.5 μ L dNTPs (2 mM), 0.2 μ L of each primer (40 μ M), 0.2 μ L Taq DNA polymerase (5 U/ μ L) (Invitrogen, Sao Paulo, Brazil), and 5 μ L DNA extract. The amplification cycles included an initial denaturation step of 3 minutes and 30 seconds at 94°C, followed by 35 cycles at 94°C (30 seconds), 60°C (1 minute), 72°C (1 minute), a final extension at 72°C (10 minutes), and incubation at 4°C.

Polymerase Chain Reaction Product Digestion and *Leishmania* Species Identification

After amplification, *Hae*III and *Bsr*I enzymes (New England Biolabs Inc., Hitchin, England) were used to identify the *Leishmania* species by using the following reaction conditions: 37°C and 1 μ L *Hae*III enzyme, 2 μ L buffer solution, and 12 μ L distilled water, and 5 μ L polymerase chain reaction (PCR) product; 65°C and 1 μ L *Bsr*I enzyme, 2 μ L buffer solution, and 12 μ L distilled water, and 5 μ L PCR product.

Ethical Approval

Informed consent was obtained from all the patients. This report was approved by the Local Human Ethics Committee (number 3605/2006) of the University Hospital of the Ribeirao Preto Medical School, University of Sao Paulo, Brazil, in accordance with the ethical standards of the Helsinki Declaration (1964, amended most recently in 2008) of the World Medical Association.

RESULTS

A series of 18 patients—5.4% of ATL patients—with clinically and laboratory-confirmed DL were identified between June 1987 and March 2015 (Table 1).

Demographic, Epidemiological, and Comorbidities Data

The median age of the patients was 45 years old; the youngest patient was 24 years old, whereas the oldest patient was 75 years old. Seventeen patients were male. Most of the patients were agriculturists (33.3%). No municipalities or farm villages were identified more frequently; however, Pardo River (Sao Paulo state) and Paracatu River (Minas Gerais state) were common places amongst the patients with fishing habit. Twelve patients were smokers; 8 patients were alcoholics. Five patients had diabetes mellitus type 2; just 1 patient had human immunodeficiency virus (HIV) positive serology (no acquired immune deficiency syndrome condition).

Clinical Characteristics

The median evolution time was 6 months (minimum, 1 month; maximum, 48 months). An isolated ulcer was the initial clinical manifestation in most cases (55.5%); it emerged mainly in the face and lower extremities (Figure 1). Seven (38.8%) patients had concomitant mucosal involvement; nasal mucosa and/or septum were affected in 4 of these 7 patients, and the same proportion of patients (4 of 7) had oral lesions covering the hard palate.

Laboratorial Results

Ten (58.8%) of the 17 patients tested positive for LST. The median induration size was 8 mm (minimum, 5 mm; maximum, 14 mm). *Leishmania* skin test had not been registered in the medical chart of 1 patient. The biopsy sections showed infiltration with plasma cells and granulomatous reaction. Amastigotes were identified in 11 (61.1%) skin histological sections. DNA from 17 skin biopsies samples was analyzed by PCR. Amplified *Leishmania* sp was determined for 17 of these samples, which was followed by enzyme digestion to recognize the subgenus *Viannia*.

Treatment Results

Fifteen patients were treated initially with pentavalent antimony (Sb^V; 20 mg/kg per day) for 30 days (1 cycle), and 3 patients were treated with amphotericin B. Patients were evaluated 30 to 60 days after they finished treatment. When they were considered not cured, retreatment was done. Two cycles were necessary to obtain cure for 8 patients. For another one, 3 cycles were necessary. Among patients treated with pentavalent antimony: 1 of them presented secondary effects, and therapy was switched to amphotericin B; and 2 patients died, 1 due a renal acute tubular necrosis and the other due hospital pneumonia. Among the patients treated with amphotericin B (with final doses of 2.5–3.0 g): 1 patient presented impaired liver function, and amphotericin B was switched to pentavalent antimonial;

Table 1. Demographic, Clinical, Laboratorial, and Treatment Data of the Patients With Disseminated Leishmaniasis Seen in Southeastern Brazil

State	Gender	Age at Onset (Years)	Year of Diagnosis	Occupation	Evolution Time (Months)	Origin at Diagnosis	LST	Mucosal Involvement	Amastigotes in Skin Samples	PCR	<i>Leishmania</i> Subgenus	Initial Treatment/Follow Up
SP	M	24	1987	Hiker	48	Ribeirao Preto City	Positive	No	Present	No sample	No sample	Glucantime ² /Cure
SP	M	49	1988	Agriculturist	3	Pardo River	Negative	No	Present	+ (P)	<i>Leishmania viannia</i>	Glucantime ² /Cure
SP	M ^a	56	1988	Agriculturist	3	Santa Cruz da Esperança City	Positive	No	Present	+ (P)	<i>Leishmania viannia</i>	Glucantime ¹ /Cure
MG	F	33	1991	Agriculturist	6	Santa Maria de Itabira City	Positive	Oral	Present	+ (P)	<i>Leishmania viannia</i>	Amphotericin-B ^b /Cure
MG	M	50	1995	Agriculturist	18	Paracatu River	Positive	No	Present	+	<i>Leishmania viannia</i>	Glucantime ¹ /Death
MG	M ^a	49	1998	Bricklayer	1	Paracatu River	Negative	No	Present	+	<i>Leishmania viannia</i>	Glucantime ² /Cure
SP	M ^c	37	1999	Driver	1	Ribeirao Preto City	Not registered	Nasal	Present	+ (P)	<i>Leishmania viannia</i>	Amphotericin-B/Death
SP	M ^{a,d}	75	2000	Agriculturist	8	Guatapara City	Negative	Nasal and oral	Present	+ (P)	<i>Leishmania viannia</i>	Glucantime ² /Death
MG	M	47	2001	Welder	2	Paracatu River	Positive	No	Absent	+	<i>Leishmania viannia</i>	Glucantime ¹ /Cure
SP	M ^a	40	2002	Manager Fruit Market	3	Ribeirao Preto City	Negative	No	Absent	+	<i>Leishmania viannia</i>	Glucantime ² /Cure
SP	M ^a	39	2008	Bricklayer	6	Espírito Santo do Pinhal City	Positive	Oral	Absent	+	<i>Leishmania viannia</i>	Glucantime ² /Cure
SP	M	43	2009	Bricklayer	8	Pardo River	Positive	Nasal	Present	+	<i>Leishmania viannia</i>	Glucantime ^e /Cure
SP	M	67	2010	Traveling Salesman	12	Jurucê City	Positive	Nasal	Present	+	<i>Leishmania viannia</i>	Glucantime ¹ /Cure
SP	M	38	2010	Agriculturist	8	Santa Ernestina City	Positive	No	Absent	+	<i>Leishmania viannia</i>	Glucantime ³ /Cure
SP	M	37	2013	Driver	6	Pardo River	Negative	No	Absent	+	<i>Leishmania viannia</i>	Glucantime ² /Cure
SP	M	58	2014	Bar Owner	6	Pardo River	Negative	No	Absent	+	<i>Leishmania viannia</i>	Glucantime ² /Cure
SP	M	43	2014	Machine Operator	2	Pardo River	Positive	Oral	Absent	+	<i>Leishmania viannia</i>	Glucantime ² /Cure
SP	M	58	2015	Electrician	7	Pardo River	Negative	No	Present	+	<i>Leishmania viannia</i>	Amphotericin-B/Control

Abbreviations: HIV, human immunodeficiency virus; LST, *Leishmania* skin test; MG, Minas Gerais; (P), paraffin-embedded tissue; PCR, polymerase chain reaction; SP, Sao Paulo.

^a Patient with diabetes mellitus type 2.

^b Switched to glucantime² due impairment liver function.

^c Patient with HIV positive serology.

^d Patient with chronic pulmonary obstructive disease.

^e Switched to amphotericin B due phlebitis and tremors.

^{1,2,3} Number of cycles of 30 consecutive days with interval of 30 to 60 days prescribed to be cured.



Figure 1. Disseminated leishmaniasis caused by *Leishmania viannia* subgenus. Concomitant mucosal involvement (A) was found in 7 patients. In most cases, an isolated or coalescent ulcers (B), characteristic leishmaniotic, or uncharacteristic ulcer (C) antecede the spreading lesions to cephalic segment, mainly the face, trunk, and limbs, represented by a myriad of acneiform, nodular, or papular lesions, some of them superposed by crusts.

and another one suffered hospital pneumonia (HIV-positive patient), followed by septic shock, and subsequently died.

DISCUSSION

The expansion of urban boundaries is accompanied by appearance of diseases that had been previously restricted to rural areas. Southeastern Brazil is not the exception; population growth associated with the constant increase in the agricultural market in these regions have resulted in urban invasion of forest regions [12]. This scenario presents a new stage of cohabitation with local fauna that was previously confined outside the cities. Nonetheless, outdoor work (such as agriculturist and bricklayer) and outdoor activities (such as fishing) seem to place people at higher risk of acquiring DL due to greater exposure to the *Leishmania*-transmitting mosquito [5].

Surveillance conducted in the University Hospital of the Ribeirao Preto Medical School, University of Sao Paulo, between 1987 and 2015 showed a total of 335 patients with ATL. The localized cutaneous form corresponded to 70.4% (236 of 335) of the patients (data not published). Disseminated leishmaniasis was diagnosed in 5.4% of ATL cases. The latter percentage represents an important proportion compared with other reported

series in which DL corresponded to 2.4% of CL cases [8]. Although ATL is endemic in Southeastern Brazil, the series of cases reported herein show a considerable number of DL cases. These cases must be monitored and evaluated as a new emerging clinical form of ATL in this region. It is of interest to note that the first DL case in our clinically diagnosed cases was diagnosed in 1987, whereas the first DL case reported in Brazil dates back to 1986 [3].

According to Turetz et al [5], old age is a risk factor for DL development—these authors established an odds ratio of 2.57 (95% confidence interval [CI], 1.25–5.70) for age over 20 years old. This information is consistent with the age range described in our study (24–75 years old); however, no difference was found when age was categorized in <20 years old, and ≥ 20 years old comparing DL to total ATL cases ($P = .334$); in fact, there is no DL patient under 20 years old.

In the present report, DL was more frequently diagnosed among male patients (17 of 18 were males). Our DL cases tend to be more frequently diagnosed in males compared with our total ATL cases, but no significant difference was found (odds ratio [OR] = 6.2; 95% CI, 0.82–47.8; $P = .06$). Nevertheless, in a larger series, male gender has been considered as

a risk factor for DL (OR = 2.2; 95% CI, 1.03–5.31; $P = .045$) [5], and another study has also showed this tendency (12 males in 18 DL cases) [13].

Immune response in DL is able to control parasite growth, and only a few amastigotes remain in the lesion site. As a result of the local inflammatory response, the lesion becomes ulcerated later on [4]. It is of interest to note that 2 male patients who did not present the amastigote form in the skin biopsy and who tested negative for *Leishmania* sp by PCR analysis, but who fulfilled the clinical definition of DL, were excluded from this analysis. In these 2 cases, *Leishmania* sp etiologic involvement was supported by the epidemiological background, clinical manifestation, suggestive skin histopathological description for *Leishmania* infection (lymphoplasmacytic infiltrate with granuloma formation), and positive therapeutic response to *N*-methylglucamine; however, they did not fulfill the inclusion criteria of this report, emphasizing the challenge in DL diagnosis.

In most of our cases, the initial clinical manifestation was the presence of an isolated ulcerated lesion with subsequent development of several cutaneous lesions, including a mixture of acneiform, nodular, and papular lesions as described previously [3, 5].

Seven (38.8%) patients presented simultaneous mucosal commitment and disseminated skin lesions. Mucosal lesions (MLs) are more frequent in DL patients compared with CL patients [5, 14]. Normally, MLs occur in 3% of patients with a history of CL and typically appear after several months or years after the initial cutaneous infection [1].

Pentavalent antimony was the preferred drug used to treat DL. Thirteen patients (81.2%) required 2 or 3 cycles of 30 consecutive days of antimonial treatment to be cured, although a high rate of therapeutic failure is described for DL [5]. In 1 case, amphotericin B was used with success, and the last diagnosed patient is still under medical control (less than 1-year follow up). Three deaths occurred in the casuistic: 1 was of them due to antimonial side effect (renal acute tubular necrosis), and the others 2 cases were due to pneumonia followed by sepsis or respiratory insufficiency.

In our casuistic, *L viannia* was identified as the subgenus in all DL cases. Considering the epidemiological profile in northeastern Sao Paulo state and the experience in our hospital, this subgenus highly suggests the *L (V) braziliensis* involvement; notwithstanding, further techniques should be performed to confirm the species. Influence of the parasite genotype on the clinical manifestation of the disease results from a cause-effect relationship. Single-nucleotide polymorphisms and indels associated with DL occur in 6 polymorphic loci in the parasite's genome [10]. Reports on ATL clusters in Northeastern Brazil support the idea that the clinical manifestation of DL is related with a particular strain of the parasite [7, 10, 15]. The genetic

background of the *Leishmania* parasite reported here should be determined for the detection of strains.

CONCLUSIONS

The present case series has reported on a detailed epidemiological, clinical, and laboratorial profile of an emerging form of ATL. Considering the alarming growing incidence of DL cases, clinicians must be aware of this emerging form of ATL. The challenges in this field include the difficulty to perform differential diagnosis amongst neglected dermatosis and to manage the disease.

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