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Case Report: Atypical Cutaneous Leishmaniasis in a Patient with Mixed *Leishmania guyanensis* and *Leishmania amazonensis* Infection

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Abstract. The disseminated form of leishmaniasis is a serious and rare disease, being diagnosed in 2% of the cutaneous cases registered per year in Brazil. The main characteristic is the appearance of multiple pleomorphic lesions on the cutaneous surface. A 68-year-old male from the rural area of Tocantins, Brazil, presented atypical disseminated cutaneous leishmaniasis (ACL). The clinical course and histopathological and immunological findings presented a mixed pattern that hindered diagnosis and therapeutic management. Molecular typing revealed a mixed infection with *Leishmania (V.) guyanensis* and *Leishmania (L.) amazonensis*. Molecular identification of the agents responsible for ACL is important for adequate therapeutic planning, minimizing the possibility of sequellae that impact the quality of life of the patient.

INTRODUCTION

Disseminated leishmaniasis is considered a severe and rare form, occurring in approximately 2% of the cutaneous cases diagnosed per year in Brazil. This form of presentation is characterized by the appearance of multiple papular lesions and acneiform appearance that affect several body segments, frequently involving the face and the trunk.[1] There has been an increase in the number of atypical clinical manifestations of atypical disseminated cutaneous leishmaniasis (ACL) reported in several scientific studies,[2,3] justifying the publication of this clinical report that presents an atypical form of disseminated leishmaniasis in individuals with mixed infection by different species of *Leishmania*.

CASE REPORT

Patient L.D.O., male, 68 years old, caucasian, retired by exercise of rural activity, residing in a farm near the municipality of Ponte Alta—Tocantins, Brazil, sought medical assistance because of the acneiform lesion in the lower left limb that progressed with the appearance of multiple pleomorphic lesions (approximately 30) ranging from ulcerated, nodular, and vegetative lesions. Direct parasitological examination of lesion scale was positive for *Leishmania* sp. He was treated with pentavalent antimonial (15 mg Sb^{+5}/kg/day) for 30 days, with no response. The clinical picture deteriorated with lesions increasing in number and size and spreading to the upper limb (two lesions), abdomen (one lesion), and face (one lesion), without mucosal involvement. The case was then classified as disseminated leishmaniasis. Treatment with 100 mg/day of liposomal amphotericin B (AmBisome; Gilead-United, San Dimas, CA) was started and given for 7 days. Three months after treatment, some lesions on the lower limb healed and others showed signs of secondary infection (Figure 1A).

Based on clinical observations and treatment outcome, the following examinations were performed: excisional biopsy of a lesion present in the lower limb for histopathological analysis, immunocytochemistry, and molecular typing of the *Leishmania* species; blood collection for the evaluation of the humoral immune response by indirect immunofluorescence (IFI) and serologies for human immunodeficiency virus and viral hepatitis; Montenegro skin test for the evaluation of the cellular immune response.

Histopathological examination revealed acanthous epidermis exhibiting pseudoepitheliomatous hyperplasia with severe non-granulomatous chronic lymphoplasmacytic inflammatory reaction, compromising superficial and deep dermis. Macrophages with a clear cytoplasm pattern containing phagocytic vacuoles that have spherical structures morphologically compatible with amastigote forms of *Leishmania* sp. were observed (Figure 2A).

Immunocytochemical analysis with specific anti-*Leishmania* antibodies was positive, confirming the presence and abundance of *Leishmania* sp. in the lesion (Figure 2B). The IFI presented 1/320 titer and the Montenegro skin test was negative after 72 hours. Serologies for HIV and viral hepatitis were negative.

The DNA of excisional biopsy was extracted from an injury and the polymerase chain reaction (PCR) was performed with specific fragment digestion using restriction enzymes *Hae III* and *Ava I* (PCR-restriction fragment length polymorphism, Volpini et al.[4]), which revealed *Leishmania (Viannia)* infection.

Alternatively, the DNA obtained from the biopsy was also used for the amplification of the ribosomal RNA coding region (ITS1, 5.8S, and ITS2).[5] The PCR product was cloned in pGEM-T easy vector and seven independent positive clones were screened by digestion with *Eco* RI restriction enzyme. Two different patterns of digestion were obtained that were sequenced as described.[6] Sequence analysis of one clone representative of each one of the two patterns indicated the presence of one sequence 99% identical with the reference

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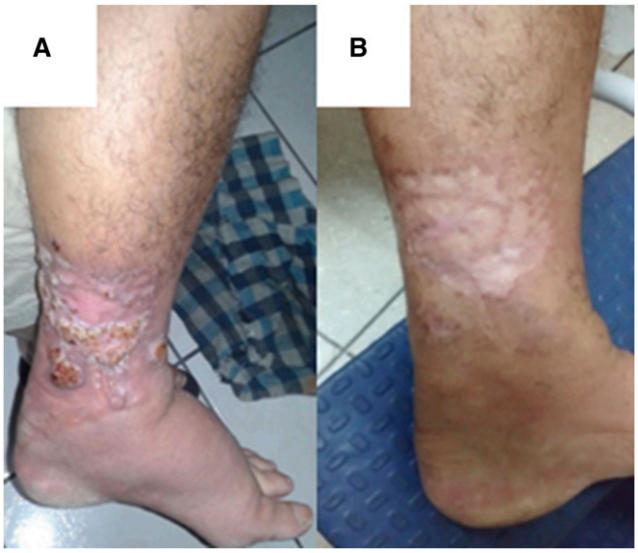

FIGURE 1. Patient with disseminated cutaneous leishmaniasis. (**A**) Skin lesion appearance during treatment. (**B**) Healing of skin lesions after treatment. This figure appears in color at www.ajtmh.org.

strain *Leishmania (Leishmania) amazonensis* (MHOM/BR/73M2269), whereas the second profile corresponded to a sequence presenting 98% identity with the reference strain *Leishmania (Viannia) guyanensis* (MHOM/BR/75/M4147). The comparison between sequences of these two profiles revealed 62% identity (Figure 3). The results obtained, therefore, indicate the presence of a mixed infection with *L. (L.) amazonensis* and *L. (V.) guyanensis*.

A new course of treatment with amphotericin B (50 mg/day) was performed for 12 days, being interrupted because of adverse effects such as acute renal dysfunction (serum creatinine 2.3 mg/dL) and pulmonary thromboembolism. The medication was changed to liposomal amphotericin B (AmBisome; Giléad-United) at a dose of 150 mg/day for 25 days. Lesions healed completely (Figure 1B); however, residual lymphadenopathy in the left ankle remained, disabling the routine activities of the patient.

Eighteen months after the first evaluation of humoral and anti-*Leishmania* cellular immune responses, the examinations were repeated and serology by the IFI technique showed a decrease in circulating antibody titers to 1/80 and the Montenegro skin test remained negative.

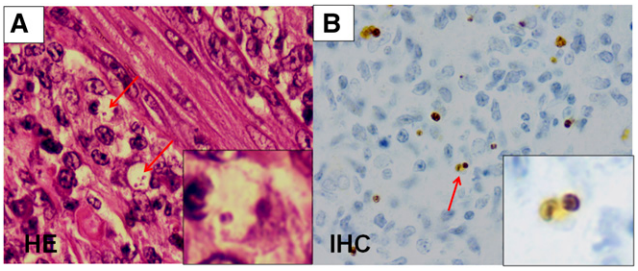

FIGURE 2. Photomicrography of cutaneous lesion. Biopsy fragments were prepared with hematoxylin and eosin (HE) (**A**) and immunocytochemistry (IHC) (**B**). (**A**) Vacuolated macrophages containing amastigotes (arrow). (**B**) Amastigotes labeled IHC. *Leishmania* stained with brown. This figure appears in color at www.ajtmh.org.

DISCUSSION

Different species of *Leishmania* of the *Viannia* subgenus *Leishmania (Viannia) braziliensis*, *L. (V.) guyanensis*, and *Leishmania (Viannia) panamensis* and the species *L. (L.) amazonensis* were already recognized as etiological agent of disseminated leishmaniasis.[9,10]

However, there are differences in the clinical, therapeutic, histopathological, and immunological characteristics of the disseminated leishmaniasis caused by the species belonging to the subgenus *Viannia* when compared with those caused by the species *L. (L.) amazonensis*.[11,12]

Disseminated leishmaniasis caused by species belonging to the subgenus *Viannia*, in general, are characterized by the presence of multiple (10–700) pleomorphic skin lesions (acneiform, papular, and ulcerated) in different anatomical regions, appearing simultaneously or secondary to one or more several primary lesions, after a short time. There may be involvement of mucous membranes. There is presence of cellular immune response to *Leishmania* antigen (positive Montenegro skin test) and low titers of circulating antibodies. Histologically, the exudative reaction, lymphoplasmocytic and with few parasitized macrophages, predominates in areas of necrosis associated or not with epithelioid granuloma. Good therapeutic response to pentavalent antimonial and other drugs of choice for the treatment of ACL was observed.[13–16]

When disseminated leishmaniasis is caused by *L. (L.) amazonensis*, a reduced number of primary lesions (1–3) with an erythematous infiltrated plaque appearance are observed. The spread to other anatomical sites is late, taking on average 6–12 months, producing few metastatic lesions. There is no mucosal involvement. The cellular immune response is deficient or absent (negative Montenegro skin test) and may or may not have serum conversion at the end of treatment. Elevated circulating antibody titers are observed. Histologically, there is a predominance of vacuolated, richly parasitized macrophages and rare epithelioid cell groups. Therapeutic resistance, requiring a prolonged period of treatment, but healing is obtained.[11,17,18]

The present study reports a case of atypical disseminated leishmaniasis in a patient with mixed infection with *L. (V.) guyanensis* and *L. (L.) amazonensis* resulting in an ambiguous clinical, therapeutic, histopathological, and immunological picture that made diagnosis and therapy difficult.

The patient's clinical characteristics, pleomorphic lesions in the form of ulcerative and ulcer-vegetative nodules, rapid spread in adjacent areas, and resistance to treatment with pentavalent antimonial are compatible with the disseminated form caused by *Leishmania (Viannia)*.[11,19–21]

Guimarães et al.[22] suggested that amphotericin B should be considered the drug of choice for all patients diagnosed with atypical forms of ACL. Our patient made use of liposomal amphotericin B for 7 days, with partial healing of the lesions. A therapeutic regimen with amphotericin B deoxycholate was required for 12 days, followed by liposomal amphotericin B for another 25 days.

The therapeutic failure observed in this case may be because of the fact that infections caused by *L. (V.) guyanensis* induce less-intense cellular and humoral immune response than *L. (V.) braziliensis*, even though the appearance of ulcers is clinically similar, explaining the greater number of parasites in the lesions and the higher therapeutic failure for this

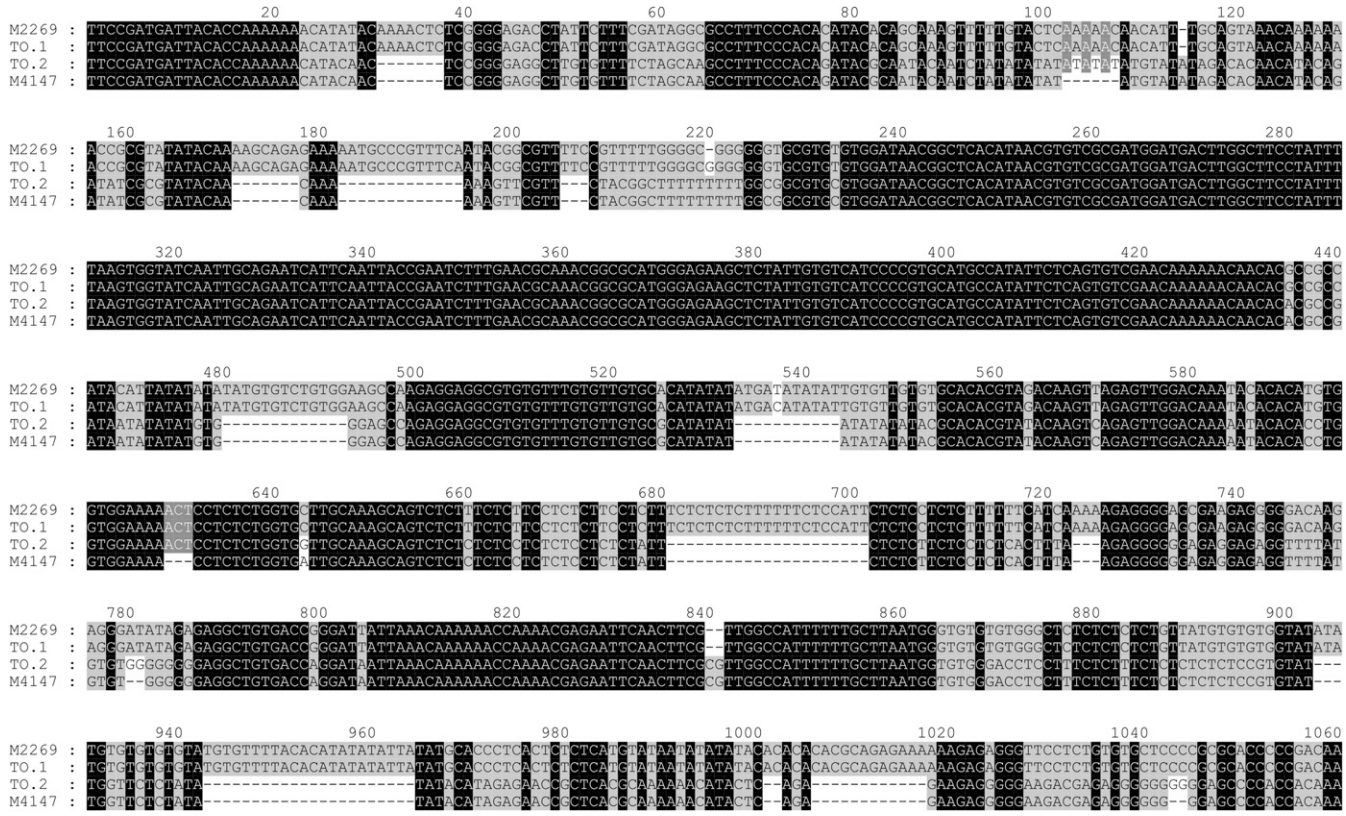

FIGURE 3. Nucleotide alignment of cloned fragments of the ribosomal RNA coding region (ITS1, 5.8S and ITS2) of approximately 1 kb. Two clones, corresponding to *Leishmania (L.) amazonensis* and *Leishmania (L.) guyanensis*, representative of each restriction pattern, were sequenced (TO.1 and TO.2, respectively). For polymerase chain reaction amplification, the primers used were IR1 (5′-GTCGTAGGTGAACCTGCAGCAGCTGGATCATT-3′) and IR2 (5′-GCGGGTAGTCCTGCCAAACACTCAGGTCTG-3′).[5] The amplified products were analyzed in a 1% agarose gel electrophoresis stained with ethidium bromide and then photographed. The amplified products were purified from agarose gels using the GenElute Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO) and cloned in pGEM-T easy (Promega Corporation, Madison, WI). The nucleotide sequence of two positive clones containing ITS fragment were performed in an automated DNA sequencer (ABI PRISM Big Dye Terminator Cycle Sequencing) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Waltham, MA). Primers used for sequencing were pUC/M13, IR1 and IR2, and 5.8SF (5′-GCAGTAAAGTGCGATAAGTGG-3′) and 5.8SR (5′-GGAAGCCAAGTCATCCATC-3′) primers. Consensus sequences were generated from at least four forward and four reverse sequences. Nucleotide sequence analyses were performed using Lasergene Software (DNASTAR, Madison, WI) and Clone Manager 9.0 Software (Denver, CO). The sequences obtained were aligned using ClustalW[7] (Kyoto, Japan) and edited to improve the alignment with the program GeneDoc 2.6 (Bloomington, IN).[8] Nucleotide sequences of *Leishmania (V.) guyanensis* M4147 and *Leishmania (L.) amazonensis* M2269 were obtained from GenBank (accession numbers AJ000299.1 and DQ300194.1, respectively). Identical nucleotides among the four sequences are in a black background, whereas those that are similar among the nucleotide sequences are in two different gray backgrounds (corresponding to 75% and 50% of identity among the four sequences). The remaining residues are in a white background.

species.[23] In addition, *L. (L.) amazonensis* presents a high capacity to block specific cellular immune responses to *Leishmania* antigens, making it difficult to control infection.[24]

Histopathological and immunocytochemical analysis revealed a mixed profile of inflammatory response. A large number of macrophages with amastigote forms were observed, with one to three parasites per infected cell, sheltered in large parasitophorous vacuoles, and compatible with *L. (L.) amazonensis* infection.[25,26] In addition, a large non-granulomatous inflammatory response with lymphoplasmacytic infiltrate and epithelial cells in the superficial and deep dermis was observed, similar to that reported by Couppié et al.[9] in a patient with a disseminated form caused by *L. (V.) guyanensis*.

The IFI technique is a useful method for the diagnosis, control of clinical cure, and analysis of the efficacy of the adopted therapeutic regimen.[27] In patients with disseminated leishmaniasis caused by *L. (V.) braziliensis*, low to moderate levels (mean 640) of anti-*Leishmania* immunoglobulin G were observed by Chagas et al.,[17,28] by contrast, cases of disseminated leishmaniasis by *L. (L.) amazonensis* presented high levels of these antibodies (mean titer 5,120), indicating a lower activation of the $CD4^+$/Th1 immune response in these patients. In our findings, the immune response also presented a mixed pattern because low antibody titers were detected during disease (1/320) and decreased after clinical resolution (1/80). However, the cellular immune response to *Leishmania* antigen remained negative, even after different therapeutic regimens and clinical cure, similar to that observed by Carvalho et al.[11] in cases of disseminated leishmaniasis by *L. (L.) amazonensis*.

During the DNA sequencing of the clones, a ratio of 6:1 was observed, corresponding to *L. (L.) amazonensis* and *L. (V.) guyanensis*. This difference in *Leishmania* concentration confirms the increased resistance of *L. (L.) amazonensis* to the therapies used because the sample was collected after treatment with pentavalent antimonial and liposomal amphotericin B for 7 days. Thus, it is explained that all the parasitophore vacuoles observed had large dimensions. Thus, in atypical cases, more detailed molecular investigation may be required.

According to Raja et al.,[29] the appearance of unusual clinical lesions of leishmaniasis is attributed to a depressed host immune response, comorbidities, pregnancy, or involvement of an atypical parasite strain in the lesions. In this case, the coinfection of *L. (L.) amazonensis* and *L. (V.) guyanensis* may have influenced the natural course of disease evolution, resulting in mixed clinical, therapeutic, histopathological, and immunological characteristics of the different patterns of disseminated leishmaniasis.

Molecular identification of the agents responsible for ACL is important for adequate therapeutic planning, minimizing the possibility of sequelae that impact the quality of life of the patient.

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