VISCERAL LEISHMANIASIS CAUSED BY *Leishmania* (*Viannia*) *braziliensis* IN A PATIENT INFECTED WITH HUMAN IMMUNODEFICIENCY VIRUS

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SUMMARY

The current article reports the case of a 19-month-old-girl, from the state of Minas Gerais, Brazil, with visceral leishmaniasis, by *Leishmania* (*Viannia*) *braziliensis*, and Human Immunodeficiency Virus (HIV) co-infection. The child´s mother and father, aged 22 and 27 years old, respectively, were both HIV positive. The child was admitted to the General Pediatric Center, in Belo Horizonte, presenting high fever, fatigue, weight loss and enlargement of liver and spleen. Indirect immunofluorescent test revealed a titer of 1:320 for *Leishmania*. Such result was confirmed by the presence of amastigotes in bone marrow aspirate samples and culture of promastigote forms. Parasites were identified as being *Leishmania* (*Viannia*) *braziliensis* through PCR, using a *L. braziliensis* complex primer and a generic primer, followed by hibridization. Specific leishmaniasis therapy (Glucantime® antimonial) was intravenously administered.

KEYWORDS: Visceral leishmaniasis; *Leishmania (Viannia) braziliensis;* Human immunodeficiency virus; PCR.

INTRODUCTION

The genus *Leishmania* causes a variety of clinical syndromes ranging from self-healing cutaneous lesions through metastasing mucocutaneous forms to often lethal visceral manifestations. The severity of clinical manifestations in immunocompetent people depends, mainly, on the *Leishmania* species involved. Patients with leishmaniasis, associated with acquired immunodeficiency syndrome (AIDS), have revealed unusual immunopathological aspects. Most patients, suffering from *Leishmania*-HIV co-infections, have shown atypical clinical profiles such as disseminated cutaneous leishmaniasis[5,14], unusual cutaneous lesions[7,20], mucosal lesions containing abundant amastigote forms[4,10,23], atypical mucocutaneous leishmaniasis caused by *L. braziliensis*[6], cutaneous involvement due to viscerotropic parasites[21] and visceral involvement due to dermotropic parasites[12].

Leishmania has been reported to be an opportunistic parasite, in immunosuppressed patients[2] and in immunocompromised individuals such as patients with HIV infection[29], who live in endemic areas. The overlap between VL and AIDS has increased as a consequence of the spread of the pandemic AIDS in rural areas and that of VL to urban areas[9]. Consequently, cases of *Leishmania*/HIV co-infection are becoming more frequent with important clinical, diagnostic, chemotherapeutic, epidemiological and economic implications. The major features of VL clinical picture are: intermittent fever, spleen enlargement, pallor, fatigue, severe weight loss, loss of appetite and pancytopenia[11]. The disease is fatal if not treated. Although the incubation period of VL may vary from only 10 days to a year, the usual incubation period ranges from 2 to 4 months[9,16].

Unfortunately, VL diagnosis is difficult in such patients, since serologic diagnosis is not sensitive enough and culture of *Leishmania* parasites is both time-consuming and not always positive. Molecular techniques for typing and diagnosing the etiological agent are very useful for specific therapy. PACHECO *et al.*[17] described the isolation, genotypic and phenotypic characterization of a monoxenous trypanosomatid, found in the bone marrow of an HIV-positive patient, presenting a visceral leishmaniasis-like syndrome. Several authors have described PCR assays to be used as a diagnosis method[8,19,20,25,26,27]. The present article describes the case report of a VL patient, by *L. braziliensis,* and HIV co-infection.

METHODS

Indirect immunofluorescent assay (IFA): IFA, to detect antibodies in serum diluted from 1:40 to 1:640, was performed according to the manufacturer's instructions, using a commercial kit for the diagnosis of human leishmaniasis (FIOCRUZ/BIO-MANGUINHOS). The serological tests, on different *Leishmania* species, used weakly positive titers ≤ 1:40 and strongly positive titers ≥ 1:80 as IFA cutoff values.

Parasitological test: Cell culture from bone marrow aspirate material was performed in NNN/LIT medium, supplemented with 10% fetal calf serum and incubated at 24-25 ºC for 30 days. For direct examination of bone marrow, the slides were stained with Giemsa and examined under light microscopy.

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DNA extraction : A volume of 20-30 µl of blood and bone marrow aspirates was transferred onto filter paper "FTA Cards" (Gibco BRL) and air-dried. A small portion of the sample, on FTA paper, was removed using a 2 mm punch and placed in a microcentrifuge tube with 200 µl of lysis buffer (FTA processing reagent-Gibco BRL). The sample was washed for 5 min at room temperature and centrifuged three times at 13000 g for 5 min, after which the supernatant was removed and discarded. A volume of 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, [pH 7.5]) was added to, incubated for 5 min at room temperature and the supernatant was discarded after centrifugation, and the pellet ressuspended in 50 µl TE. The paper-bound DNA was then ready for analysis; the paper punch containing purified DNA was directly used for PCR.

PCR 1: In order to amplify the conserved region of the minicircle molecule (Kinetoplastid mitochondrial DNA; kDNA), oligonucleotides (primer A: 5'- (G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACCAACCCC; and primer B: 5'- GGGGAGGGGCGTTCTGCGAA) were used in a hot-start PCR procedure. Briefly, each reaction contained 100 ng of the reverse and forward primers, 200 µM of each deoxynucleoside triphosphate (Pharmacia, Uppsalla, Sweden), 2.5 U of Taq polymerase (Perkin-Elmer, Norwalk, USA) in the buffer, as recommended by the manufacturer, and a small part of the DNA paper sample. PCR amplification was carried out in a DNA thermocycler (Perkin-Elmer) using 33 cycles at 94 °C for 30 s; at 50 °C for 30 s, and at 72 °C for 30 s, with a extension cycle for 10 min at 72 °C.

PCR 2: Specific PCR was carried out using the B1 and B2 primers (B1 – GGGGTTGGTGTAATATAGTGG and B2 – CTAATTGTGCACGGGGAGG) specific for the *L. braziliensis* complex[8]. Cycles consisted of annealing at 63 °C for 1 min, extension at 72 °C for 1 min and desnaturation at 93 °C for 30s. The programmes were run for 35 cycles on a Perkin Elmer thermocycler (Perkin-Elmer, USA), a final extension at 72 °C for 10 min was run.

Agarose gel electrophoresis: Each experiment included a positive control (reference strains of *Leishmania*) and a negative control (rabbit blood). Aliquots of 10 µl of the PCR reaction were analyzed by agarose gel electrophoresis, at 100V, in the presence of ethidium bromide (0.5 µg/ml). The expected amplification products of 120 bp (PCR 1) and 750 bp (PCR 2) were visualized under UV light.

Positive and negative controls: The reference strains *L. (L.) chagasi* MHOM/BR/74/PP75 (code PP75); *L. (V.) braziliensis* MHOM/BR/75/M2903 (code M2903) and *L. (L.) amazonensis* IFLA/BR/67/PH8 (code PH8) were used as positive controls. The promastigotes of such strains were transferred to the filter paper "FTA Cards" (Gibco BRL), to which rabbit blood was added after air dried. Rabbit blood, alone on filter paper, was used as a negative control. DNA from *Leishmania* reference strains and rabbit blood were prepared according to the protocols previously described.

Hybridization: PCR 1 products were transferred to nylon membranes with a dot-blot apparatus and hybridized with cloned *L. braziliensis* minicircles as molecular probe, radio-labeled with [α-^{32}P] dATP, by random hexamer priming. The transferred product was hybridized in Blotto at 65 °C, washed in 0.1X sodium saline citrate at the same temperature, and exposed to X-ray films[22].

Case report: In January 1998, a 19 month-old girl, from the small town of Martinho Campos, in the Brazilian state of Minas Gerais, was admitted to the General Pediatric Center, in Belo Horizonte with fever, pneumonia, lymphodenomegalia and hepatosplenomegalia. The girl's mother and father, aged 22 and 27 respectively, were both HIV-positive. The girl was delivered by caesarian section and weighed 3000 g at birth, with a body length of 50 cm. The child was breast-fed by the mother until 3 months old. She suffered bouts of pneumonia at 3, 5, 6, 8, 9 and 13 months of age. At 8 months, she received two blood transfusions due to her recurrent medical profile of malnutrition, anemia, fever, pallor, hepatomegalia and splenomegalia. At 19 months, she was diagnosed as HIV-positive and received treatment with Zidovudine (AZT) (120 mg/m^2 every 8h), Didadosine DDI (100 mg/m^2 every 12h) and intravenous gammaglobulin. On physical examination, she was chronically ill appearing, febrile and pale. The spleen and liver were palpable, extending to 5 and 8 cm below the coastal margin. Laboratory data revealed platelets (35,600/µL), hemoglobin (8.7 g/dL), normal erythrocyte, leukocytopenia. The patient subsequently experienced respiratory failure, due to AIDS-related pneumocytosis, and died 8 weeks later.

RESULTS

Immunological test: A positive titer was observed for *Leishmania* antibodies (IFA 1:320). After 2 weeks of intermittent fever, another positive titer for *Leishmania* antibodies (IFA 1:160) was noted.

Parasitological studies: The results of *Leishmania* IFA were confirmed by visualization of amastigote forms, from bone marrow aspirate, after Giemsa staining (Fig. 1) and isolation of promastigote forms in culture. Specific therapy was initiated with intravenous administration of antimonial Glucantime® (20 mg/Kg body weight).
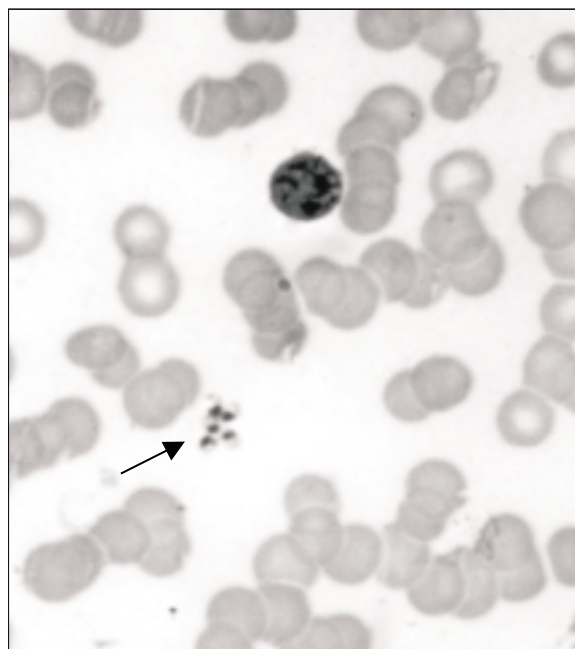

Fig. 1 - Smear of the bone marrow aspirate from a child with visceral leishmaniasis indicating presence of amastigotes (2-3 µm in diameter).

PCR for the genus *Leishmania*: Purified DNA from the positive controls and the DNA, isolated from blood and bone marrow aspirate of the patient, also yielded an amplification product (Fig. 2a). The DNA purified from rabbit blood was negative for *Leishmania,* based on PCR analysis. No contamination or inhibition was detected.
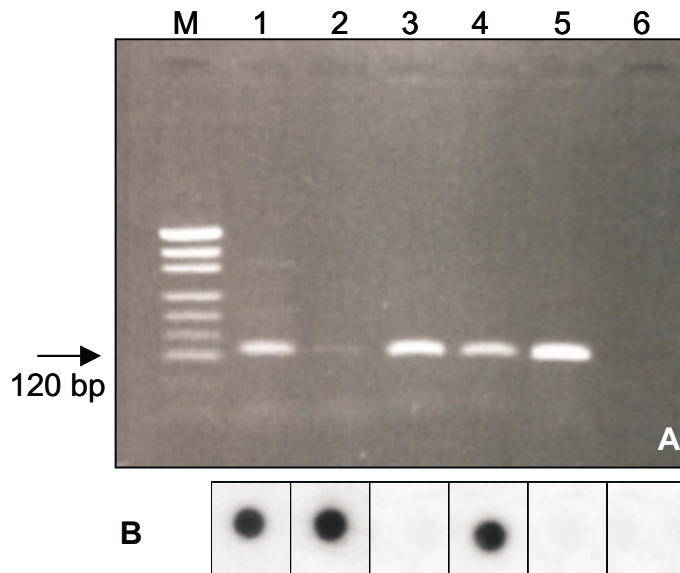

Fig. 2 - A. PCR products obtained with primers of the genus *Leishmania* visualized *after* 2% agarose gel electrophoresis stained with ethidium bromide. MW 50base-pair DNA ladder; lanes 1 and 2: bone marrow and blood from patient, lane 03: DNA of *L. chagasi*, lane 4: DNA of *L. braziliensis*, lane 5: DNA of *L. amazonensis* and lane 6: negative control. B. Hybridization with a cloned minicircle from *L. braziliensis*.

Specific PCR: The specific amplification of isolated DNA, from blood and bone marrow, provided an amplification band similar to the 750 bp kDNA amplification band of the *L. (V.) braziliensis* reference strain M2903. PCR did not amplify DNA of *L. chagasi* and *L. amazonensis* (Fig. 3).
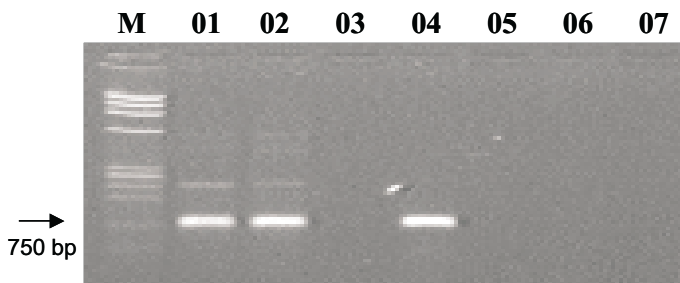

Fig. 3 - Specificity of PCR, with primers specific for *L. braziliensis* complex. Lane M: molecular weight marker (MWM) φX174 *Hae* III, lanes 1 and 2: blood and bone marrow from patient, lane 3: *L. (L.) chagasi*, lane 4: *L. (V.) braziliensis*, lane 5: *L. amazonensis*, lane 6: negative control, lane 7: No DNA.

Hybridization: A hybridization signal was observed in the PCR product when hybridized against a cloned minicircle of *Leishmania (Viannia) braziliensis* used as probe (Fig. 2 b) thus, confirming the specificity of the primers B1/B2 for the *L. braziliensis* complex.

The parasite was identified as *L. (V.) braziliensis* using blood and bone marrow aspirate, the specific primer B1/B2[8] and PCR-hybridization analysis.

DISCUSSION

Leishmania/HIV co-infection is considered to be an emerging disease and a threat in several countries in accordance with WHO data. The global experience, mainly in European countries, is marked by an increasing number of co-infection cases in this decade, leading to modifications concerning the epidemiological profile, presentation and clinical outcome of visceral leishmaniasis in various countries.

Recent changes in the epidemiological profiles, identified by the AIDS and leishmaniasis control programs, in Brazil, such as the ruralization and pauperization of HIV infection and the simultaneous urbanization of leishmaniasis, point to a possible expansion of people at risk of acquiring both infections.

In Brazil, the number of cases of *Leishmania*/HIV co-infection is growing in several states, including both the cutaneous and visceral clinical presentation. However, the under-notification cases of co-infection remains a problem with important clinical and epidemiological implications[29]. DA-CRUZ *et al.*[6] reported an atypical case of AIDS-associated mucocutaneous leishmaniasis, due to *L. braziliensis*. There are also a few reports of HIV-infected mucocutaneous leishmaniasis patients, associated or not with cutaneous lesions[4,10,15,23]. In a recent case report CHEHTER *et al.*[3] described the involvement of the gastrointestinal tract and dissemination forms of *Leishmania,* in co-infection visceral leishmaniasis and HIV patient. In the present paper, atypical VL was caused by *L. braziliensis* and the patient did not show cutaneous or mucosal lesions. HERNANDEZ *et al.*[13] also demonstrated *L. braziliensis* as causing VL, in a patient with HIV infection. In our laboratory, we have isolated various parasite samples from inhabitants of Martinho Campos and characterized them as *L. braziliensis*; no other cases of VL have yet been reported from the region.

In the last few years, the number of human cases of visceral and cutaneous leishmaniasis, in the metropolitan region of Belo Horizonte (MRBH), has increased, suggesting a rise in the transmission rates of *Leishmania* species[18,24]. The clinical incubation period of VL typically ranges from 6 weeks to 6 months but may vary from 10 days to 10 years[1]. The course of the disease is identical in children and adults. It may suddenly begin with high fever, vomiting, diarrhea, poor appetite, weight loss, lassitude and pallor and later produce splenomegaly, hepatomegaly, pancytopenia, lymphadenopathy and hyperglobulinemia. Untreated, the disease is fatal in 90% of cases within 1-3 years[9].

HIV infection and *Leishmania* infection each induce important similar immunological changes, worsened when there is concomitant infection, such as a Th 1 to Th 2 response switch. However, the consequences of the viral infection predominate[1]. The inhibitory effect of HIV and *Leishmania* on cell proliferation and INF-gamma production is not due to IL-10 alone, but probably operates at the level of regulation of IFN-gamma-inducing factors, such as IL-12 and IL-18[28]. Patients with HIV infection have depressed CD4+ cells, contributing to the visceralization of *Leishmania* infections[6,28]. In the present study, however, the patient had detectable anti-*Leishmania* antibodies (1:320), and parasitological tests showed to be positive for *Leishmania* in bone marrow samples. Positive PCR were obtained from the blood and bone marrow samples. The parasite was identified as *Leishmania* (*V.) braziliensis* using specific PCR and Southern blot analysis. The results reported here

reinforce the hypotesis that *L. (V.) braziliensis* is an opportunistic parasite producing severe manifestations that normally do not occur in immunocompetent individuals.

Specific diagnosis of leishmaniasis and characterization of parasites are, therefore, important prerequisites for treatment of immuno-compromised patients in *Leishmania*-endemic areas, due to their increased risk of morbidity and mortality.

RESUMO

Leishmaniose visceral causada por *Leishmania (Viannia) braziliensis* em paciente infectado com HIV

No presente artigo os autores relatam caso de uma criança de 1 ano e 07 meses proveniente do estado de Minas Gerais com leishmaniose visceral causada por *Leishmania (Viannia) braziliensis* e co-infecção HIV. A mãe e o pai da criança de 22 e 27 anos de idade respectivamente também HIV positivo. A criança foi internada no Centro Geral de Pediatria em Belo Horizonte com febre alta, fadiga, perda de peso e aumento de fígado e baço. Foi realizado teste de imunofluorescência indireta para *Leishmania* e detectado título de 1:320. Este resultado foi confirmado com o encontro de amastigotas em aspirado de medula óssea e o crescimento de promastigotas em meios de cultura. Os parasitos foram identificados como *Leishmania (Viannia) braziliensis* utilizando PCR com primer específico para o complexo *L. braziliensis*, e primer genérico seguido de hibridização. Terapia específica para leishmaniose (antimonial de Glucantime) foi administrado por via intravenosa.

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