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Characterization of *Leishmania (L.) infantum chagasi* in visceral leishmaniasis associated with hiv co-infection in Northeastern Brazil

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ABSTRACT

Visceral leishmaniasis, associated with HIV/AIDS coinfection, is becoming a more aggressive disease, complicating an accurate prognosis. A 21-year-old HIV-positive female presenting with clinical features of visceral leishmaniasis was enrolled in this study. Bone marrow cytology, Novy-MacNeal-Nicolle culture and kDNA PCR of peripheral blood were all positive. Typing methods, multilocus enzyme electrophoresis and ITS1-RFLP PCR of peripheral blood confirmed infection by *Leishmania (L.) infantum chagasi*. PCR has proved to be safer and more affordable than other characterization methods; ITS1-RFLP PCR can diagnose and type *Leishmania* spp. in both endemic and non-endemic areas, favoring the prognosis and allowing the appropriate treatment of patients.

KEYWORDS: Characterization. *Leishmania (L.) infantum chagasi.* Visceral leishmaniasis. HIV/AIDS. Coinfection.

Visceral leishmaniasis (VL) caused by *Leishmania (L.) infantum chagasi* is a severe disease that takes the lives of thousands of people every year. Since the 1980s, the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) has increased the number of VL-HIV/AIDS coinfection cases reported in Europe, Africa, Asia and Latin America[1,2].

Clinical diagnosis of VL-HIV/AIDS coinfection is difficult, as *Leishmania* species may initially cause non-specific clinical features in some patients, even when associated with epidemiological data[3].

Cytological examination of spleen, bone marrow and/or lymph node stained-smears are considered the gold standards for VL-HIV/AIDS diagnosis. Nonetheless, cytology may increase the risk of adverse events in patients, as invasive procedures are required to obtain these samples[4]. The culture in NNN (Novy-MacNeal-Nicolle) medium to isolate parasites is the classical procedure recommended for the identification of *Leishmania* spp., and is essential for characterization based on multilocus enzyme electrophoresis (MLEE)[5,6], the gold standard method for *Leishmania* spp. identification[7]. Nonetheless, this may require samples obtained by invasive procedures (e.g., spleen, bone marrow or lymph node). Moreover, culture may be time-consuming and difficult to perform at primary health care facilities[3].

Disease control requires efficient monitoring, rapid identification of *Leishmania* strains and management of molecular tools[8]. Polymerase chain reaction (PCR) is a powerful tool for detecting *Leishmania* DNA in bone marrow aspirates, blood samples and lymph nodes and has shown to be more sensitive than parasitological

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methods. Nowadays, there are too many targets for visceral leishmaniasis detection and the choice of primers is important because each target has its own sensitivity and specificity[9,10]. Le Fichoux *et al.*[11] have used RV1 and RV2 primers to amplify kinetoplast minicircle sequences (kDNA), which are the most commonly used target for VL diagnosis. Schönian *et al.*[12] have used LITSR and L5.8S primers to identify *Leishmania* species and the lineage of strains by ITS1-RFLP PCR.

The present study describes the clinical, epidemiological and molecular characterization of *Leishmania (L.) infantum chagasi* isolated from a visceral leishmaniasis/HIV coinfected patient, outlining the usefulness of the methods adopted here and showing which is the most affordable.

A 21-year-old HIV-positive female lived in the Agreste region of Northeastern Brazil, an area endemic of visceral leishmaniasis. She had presented with clinical features of visceral leishmaniasis and been referred to *Hospital Universitário Oswaldo Cruz*, Recife, in the State of Pernambuco. Anemia associated with anisocytosis and microcytosis, leukopenia with lymphopenia and neutropenia were the most frequent laboratory alterations found. She had presented with episodes of fever, splenomegaly, pale mucous membranes and weight loss of over 5 kg in four months. All these data were available in the patient's clinical records. Bone marrow and peripheral blood samples were collected.

The aim of the study was to carry out a pioneering evaluation that applied a set of characterization methods for the detection of *Leishmania* spp. in an HIV-positive patient. Bone marrow cytology was carried out to search for amastigote forms by optical microscopy. In parallel, 200 µL of bone marrow were inoculated under sterile conditions in NNN culture tubes; 500 µL of NNN/Schneider two-phase medium were added to increase and accelerate the positivity of cultures. Promastigote forms were visualized by optical microscopy using 10 µL of culture[13]. The isolate was submitted to multilocus enzyme electrophoresis (MLEE).

MLEE was performed with the following enzyme extracts: glucose-phosphate isomerase (GPI, E.C.5.3.1.9), glucose-6-phosphate dehydrogenase (G6PDH, E.C.1.1.1.49), isocitrate dehydrogenase with NAD and NADP (IDHNAD IDHNADP, E.C.1.1.1.42) and 6-phosphogluconate dehydrogenase (6PGDH, E.C.1.1.1.43)[7]. This isolate was compared to reference strains of different *Leishmania* species at the Oswaldo Cruz Institute – FIOCRUZ (CLIOC/IOC-FIOCRUZ), e.g. *Leishmania (L.) infantum chagasi*, *L. braziliensis*, *L. guyanensis*, *L. shawi* and *L. naiff* to determine the species.

Peripheral blood DNA extraction was carried out using the commercial extraction kit-illustra™ tissue & cells genomic Prep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), for the PCR methods. NanoDrop 2000 (Thermo Scientific Nano Drop Products) was used to determine the DNA concentration.

In kDNA PCR, the reaction mixture (25 µL of total volume) was prepared containing a buffer of 1 X Invitrogen, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, 2.5 units of Taq polymerase (Platinum®, Invitrogen, Carlsbad, CA), 2 µL of DNA (14 ng/µL), 25 pmol of each primer, RV1 (5'- CTTTTCTGGTCCCGCGGGTAGG) and RV2 (5'- CACCTGGCCTATTTTACACCA)[11], amplifying a conserved region of the minicircle kDNA of *Leishmania*. After an initial denaturation (5 min at 94 °C), 35 cycles (denaturation, 30 sec at 94 °C; annealing, 1 min at 67 °C; extension 30 sec at 72 °C) were performed and PCR was concluded by a final extension at 70 °C for 10 min. This PCR assay achieved a maximum analytical sensitivity of 100 fg. DNA amplification was performed in a LifePro thermal cycler (Hangzhou Bioer Technology, Binjiang, China). All reactions included a positive and a negative control. The expected final product was 145 bp. The amplification products were visualized by horizontal electrophoresis in 2% ethidium bromide-stained agarose gels.

The ITS1 PCR reaction mixture (50 µL of total volume) was prepared containing 25 µL 2 x GoTaq® Green Master Mix (Promega Corp., Madison, WI), 50 pmol of primers: LITSR (5'CTGGATCATTTTCCGATG) and L5.8S (5' TGATACCACTTATCGCACTT)[12], amplifying a portion of the internal transcribed spacer 1 (ITS1) non-coding region found in the SSU rRNA delimited by the 5.8S and 18S genes. After an initial denaturation (3 min at 95 °C), 35 cycles (denaturation, 40 sec at 95 °C; annealing, 45 sec at 53 °C; extension 1 min at 72 °C) were carried out and PCR was concluded by a final extension at 70 °C for 6 min. This PCR assay achieved a maximum analytical sensitivity of 100 fg. Reactions were performed with 2 µL (14 ng/µL), 5 µL and 10 µL of DNA and included a positive and a negative control. The expected amplification product was 300-350 bp and they were visualized by horizontal electrophoresis in 2% ethidium bromide-stained agarose gels.

Restriction enzyme analyses (RFLP) were performed in a total volume of 15 µL containing the ITS1 PCR product digested by the restriction enzyme *Hae*III without prior purification, under the conditions recommended by the supplier (Hybaid GmbH Heidelberg, GE). ITS1- RFLP PCR was also tested on *Leishmania* strains from both, an endemic and a non-endemic area in Northeastern Brazil. The product underwent electrophoresis on 4.0% ethidium bromide-stained agarose gels. The band patterns from the prototypes were compared to the RFLP patterns of the clinical sample.

The increasing number of visceral leishmaniasis/HIV coinfection cases in Brazil is worrying, in view of the inherent risk of developing VL when HIV is present[14-16]. Additionally, the cumulative immunosuppression accelerates the clinical progression of both infections[17]. In the present study, the female patient presented classical clinical and epidemiological features of VL infection.

In endemic regions, where multiple species of *Leishmania* may coexist, geographical distribution may be an inadequate criterion. Clinical symptoms can be problematic for identification of the infecting species, since some cause both cutaneous and mucocutaneous disease (*L. braziliensis* complex, *L. aethiopica*), while others cause both visceral and cutaneous disease (e.g. *L. donovani* complex)[12].

Bone marrow cytology has variable sensitivities in both immunocompetent and immunosuppressed patients[4,18]. However, higher sensitivities of bone marrow cytology found in HIV-patients have been attributed to more intense *Leishmania* parasitemias in this group. Furthermore, the low number of *Leishmania*-infected cells in patients with pancytopenia, as well as those who have received previous treatments, may lead to false-negative results[3]. Bone marrow cytology should be requested when low sensitivity serological tests were performed alone, in order to avoid false-negative results. The patient in the present study had a positive bone marrow cytology.

It is recommended to perform culture isolation of *Leishmania* in parallel with bone marrow-stained smears in HIV-positive patients with symptoms (e.g. fever, visceromegaly, or hematological abnormalities), as VL/HIV coinfection diagnosis is difficult[3]. The patient had a *Leihmania* spp. positive culture.

The allelic variation of this strain was tested using a panel of five enzymes by MLEE, confirming the presence of *Leishmania (L.) infantum chagasi* IOC-L 3328 (MHOM/BR/2011/COS). One PCR target was the kinetoplast minicircle, which led to detection of *Leishmania* DNA in this clinical sample (data not shown). Amplification of this region is frequently chosen as the target for human kala-azar molecular diagnosis because of its higher sensitivity due to multi-copy number sequences[9,10]. PCR has consistently shown to be better than direct examination and culture, particularly in samples with low parasitic load[19]. Kinetoplast DNA PCR has been successfully used to identify *Leishmania* in blood samples of symptomatic and asymptomatic, immunocompetent and immunocompromised patients. Nevertheless, the kinetoplast is a heterogeneous target for which a great diversity of primers has been described and this may result in loss of assay accuracy, complicating the construction of RFLP patterns. However, it does distinguish a few selected species, such as *L. infantum*, *L. donovani* and *L. major*[9,10,18].

The last PCR assay, targeting the ITS1 region, detected *Leishmania* DNA in a blood sample. PCR targeting the non-coding region found in the SSU rRNA has provided good results and proved to be substantially more sensitive than microscopic examination, especially in cases of HIV-coinfection[10]. The main advantage of ITS1 PCR over kDNA PCR is that the former can distinguish many clinically important *Leishmania* species through RFLP patterns[9,12]. ITS1-RFLP PCR in *Leishmania* trains from endemic and non-endemic areas in Northeastern Brazil was able to establish specific patterns (Figure 1). In this clinical sample, 2 µL (14 ng/µL) and 5 µL of DNA were insufficient to establish RFLP patterns after ITS1 PCR, but 10 µL did work (Figures 2A, 2B), thus confirming *Leishmania (L.) infantum chagasi* species.

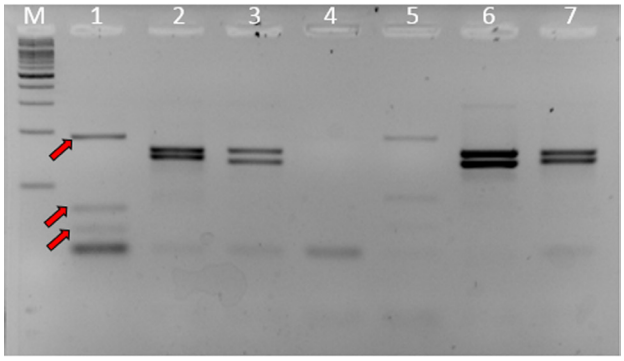

Figure 1 - ITS1-RFLP PCR of different *Leishmania* species with the restriction endonuclease Hae III. M - 100 bp marker (Promega®); 1 - *Leishmania (L.) infantum chagasi*; 2 - *L. braziliensis*; 3 - *L. amazonensis*; 4 - *L. guyanensis*; 5 - *L. mexicana*; 6 - *L. lainsoni*; 7 - *L. shawi*. Specific fragments of *Leishmania (L.) infantum chagasi* are indicated by the arrow

Characterization of *Leishmania* spp. makes it possible to conduct epidemiological studies and assess the *Leishmania* population, besides targeting more accurate disease control measures. PCR-based methods that use blood samples have proved to be safer and more affordable than the other characterization methods mentioned above; especially ITS1-RFLP PCR, which can determine the levels of *Leishmania* spp. in both endemic and non-endemic areas favoring the prognosis and allowing the appropriate treatment of patients.

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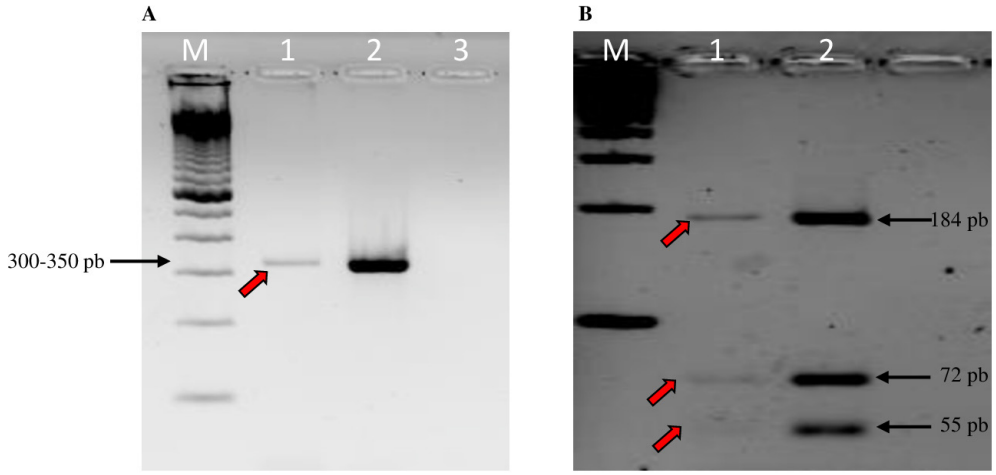

Figure 2 - ITS1 PCR product (A) and RFLP PCR with Hae III (B) for typing of *Leishmania* spp. The positive control and the clinical sample had amplification products of 300-350 bp (MHOM/BR/1974/PP75). The clinical sample (MHOM/BR/2011/COS), indicated by the arrow, showed a banding pattern matching the one of *Leishmania (L.) infantum chagasi*. M –100 bp marker (Promega®); 1- Clinical strain; 2- Positive control; 3-Negative control

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