Rev. Inst. Med. Trop. Sao Paulo
55(6):429-431, November-December, 2013
doi: 10.1590/S0036-46652013000600011

CASE REPORT

USEFULNESS OF kDNA PCR IN THE DIAGNOSIS OF VISCERAL LEISHMANIASIS REACTIVATION IN CO-INFECTED PATIENTS

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SUMMARY

It is important to develop new methods for diagnosing relapses in the co-infection of visceral leishmaniasis (VL) and HIV to enable earlier detection using less invasive methods. We report a case of a co-infected patient who had relapses after VL treatment, where the qualitative kDNA PCR showed a good performance. The kDNA PCR seems to be a useful tool for diagnosing VL and may be a good marker for predicting VL relapses after treatment of co-infected patients with clinical symptoms of the disease.

KEYWORDS: Visceral Leishmaniasis; Polymerase Chain Reaction; Diagnosis; Co-infection Visceral Leishmaniasis-HIV.

INTRODUCTION

Visceral leishmaniasis(VL) is a vector-borne disease caused by the *L. donovani* complex (*L. donovani and L. infantum* or its syn. *L. chagasi*). It currently affects around 12 million individuals in 88 countries, although more than 90% of the global VL cases occur in only six countries, namely India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil[1,9]. In Brazil the disease has expanded following a process of rural-to-urban-transmission, and this urbanization has paralleled ruralization of the HIV infection. The VL/HIV co-infection has emerged as a serious disease pattern with a reduction in therapeutic response and a high relapse rate[2]. Both pathogens have the same target cell (macrophage), thus worsening the outcome of both diseases. Therefore, it is essential to improve and make diagnostic techniques available, including molecular tools, which could replace invasive techniques.

Laboratory diagnosis can be reached by serological techniques, such as indirect immunofluorescence [IFI], enzyme-linked immunosorbent assay [ELISA], or immunochromatography [IC]. The diagnosis can also be performed by demonstration of amastigotes in stained bone marrow aspirate which has a sensitivity of 58%-85% and even by culture in Novy-MacNeal - Nicolle [NNN]/BHI medium; the latter can additionally improve sensitivity[10] and molecular tests such as kDNA PCR. Serological tests have limited sensitivity and, as a general rule, should not be used to exclude the diagnosis of VL in HIV-infected patients[3]. The microscopic examination requires an invasive procedure and is extremely subjective and time consuming. Culture has low sensitivity and takes from one to four weeks to give results. Molecular techniques, such as kDNA PCR, in which preserved sequences in *Leishmania kinetoplast* mini-circle DNA are amplified, have shown promise[6,11]. Even when they only allow for diagnosis of the genus, it is possible to diagnose recurrence early since the molecular method can produce a positive result for VL/HIV several weeks before the clinical onset of disease[2]. Consequently, the tests based on DNA detection in peripheral blood (PB) or in bone marrow (BM) aspirates can provide an important approach to the diagnosis of VL reactivation in co-infected VL/HIV patients.

METHODS

PCR kDNA Methodology

DNA extraction: The DNA from PB and BM samples collected with EDTA anticoagulant was extracted according to the manufacturer's protocol of the RBC (Real Biotech Corporation) Genomic DNA Mini Kit and eluded in 25 μL of elution buffer (TE). The quantity and the purity of the DNA in the samples were determined using the Nanodrop Thermo Scientific 1000 spectrophotometer. The samples were subsequently stored in a freezer at 20 °C for later use.

The polymerase chain reaction (PCR): In the PCR reaction, kDNA 20 (5'GGG KAG GGG CGT TCT SCG AA 3') and kDNA 22 (5' SSS WCT ATW TTA CAC CAA CC CC3') were used to target the amplification of the conserved region of kDNA mini circles of *Leishmania* sp, producing a fragment of 120 base pairs[5]. According to the amount of

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DNA from the extracted material, volumes between 2 and 4 µL (100 ng) of sample were added to 20 µL of reaction buffer containing 50 mM of KCl, 10 mM of Tris-HCl (pH 8.0), 0.2 mM of dNTPs, 1.0 mM of $MgCl_2$, 0.4 µM of each primer and one unit of Taq DNA polymerase (Fermentas, Canada). Two negative controls were employed containing all of the components of the reaction together with milli-Q water. The positive control was obtained from the culture material of the *Leishmania infantum* strain (reference M6445). Each reaction was performed at an initial denaturation temperature of 94 °C for five minutes followed by 35 cycles of denaturation at 94 °C for one minute, annealing at 58 °C for one minute, extension at 72 °C for 30 seconds, and a final extension of 72 °C for five minutes. Reactions were performed in a thermocycler (Mini Cycler MJ Research/USA). PCR products were visualized on 2% agarose gel. Immediately after electrophoresis, the gel was stained with 0.5 µg/mL of ethidium bromide. The amplified products were visualized by UV light over a transilluminator (AlphaImager) (Fig. 1).

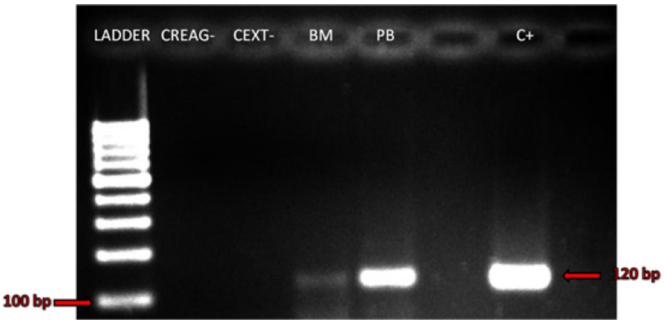

Fig. 1 - Detection of kDNA-120 bp amplification in the sample from bone marrow (BM) and peripheral blood (PB) of the patient with LV.

CASE REPORT AND RESULTS

Herein, we report a case of a co-infected (HIV/VL) 32-year-old female, who was hospitalized six times over a period of one year and a half with clinical suspicion of VL reactivation.

The patient has had the HIV infection since she was 19 years old. The highly active antiretroviral therapy (HAART) has been used irregularly and the count of CD4+ T cell never exceeded 100 cells/mm^3. The first diagnosis of VL was made in 2008.

In the patient's first admission to the hospital, she clinically presented chills, fever, anorexia, and enlargement of liver and spleen. Leishmania was found in samples from smear examination of BM, and kDNA PCR was positive in the PB. She received liposomal amphotericin B, 4 mg/kg/day for five days. At this time, the NNN/BHI culture medium from BM was negative. She was discharged without symptoms.

In a second hospitalization, six months later, the patient had fever and asthenia. Laboratory diagnosis of leishmaniasis was established through PB samples only; evaluation of smear and culture yielded negative results, but PCR turned out positive, allowing the patient to receive the same treatment as on the first admission.

The patient was hospitalized a third time five months later due to fever, prostration, and dyspnea. Once more, using only PB samples, smear microscopy, culture examination, and kDNA PCR were carried out. Again, only the kDNA PCR yielded positive results. This led to a diagnosis of reactivation and the patient underwent an additional treatment.

Yet another time, five months later, the patient was hospitalized with symptoms of headache, fever, liver and spleen enlargement, and pancytopenia. Samples of BM and PB were obtained to perform microscopic examination of smear, culture, and kDNA PCR. The smear from PB only suggested the presence of amastigotes; the culture was negative. Once again, because of the positivity of kDNA, the patient was treated. One month later, the patient was admitted to the hospital once more, this time due to anemia and again to pancytopenia. In the culture of BM aspirate, promastigotes were observed by microscopy. The microscopic examination of smear after panoptic staining revealed the presence of amastigotes. Only at this time PCR was not performed. Finally, in the patient's last hospitalization two months later, positive results were obtained in samples of both BM and PB using kDNA PCR, and in BM alone using smear. The PB smear only suggested the presence of amastigotes; cultures were negative. The patient received the same treatment as before and was well when discharged.

DISCUSSION AND CONCLUSION

Leishmania/HIV co-infection emerges as a serious disease, demanding a fast diagnostic method for detecting active disease and relapse so as to enable prompt treatment. The patient herein reported had several predictors of recurrence, such as CD4+ cell count below 100 cells/mL at the time of the primary diagnosis of VL, failure to recover the number of CD4+ lymphocytes after the first episode of VL, and previous episodes of failure[4]. Significantly, the patient was always a poor adherent to antiviral therapy. However, even patients who use HAART regularly and have a recovery of the CD4+ cell count and an undetectable viral load ,and who also receive secondary prophylaxis can suffer VL relapses.

Since co-infection favors a poor outcome, amastigotes can be found in PB[1]. Hence, it is possible to avoid invasive and painful techniques, such as bone marrow aspiration, by replacing them with others that use peripheral blood. The kDNA PCR assay can be a useful technique especially when PB is utilized. In a study comparing PCR with parasitological and serological techniques, PIARROUX *et al.* obtained a sensitivity of 82% for PCR[7]. In our report, kDNA PCR investigation produced positive results in all five molecular diagnoses in the patient's total of six hospitalizations, suggesting the hypothesis of VL reactivation. Only once was PCR not performed. In three of her six hospital stays, when both BM and PB were investigated, PCR was always positive. In two of the six hospitalizations, only PB was examined, and only kDNA PCR was positive. Amastigotes were observed by microscopic smear examination four times, and in three of these, the sample was obtained from BM, necessarily using an invasive technique. On the sixth admission, the NNN/BHI culture medium was positive in a sample proceeding from BM. Considering these findings and our aim to apply a non-invasive technique for obtaining samples, in order to avoid unnecessary pain and to prevent time-consuming techniques and subjective test interpretations, it is a priority to improve molecular tools for monitoring patients who are liable to VL reactivation, which is usual in HIV/VL co-infected patients.

It is important to emphasize that co-infected patients do not clear *Leishmania* DNA from peripheral blood nor can they show a rapid

reappearance of *Leishmania* DNA after therapy and these situations can or cannot be associated with clinical disease.

A negative result is strongly indicative of successful control of infection and efficacy of treatment, whereas a positive PCR result could be indicative only of the presence of the parasite, but not necessarily of relapse during an asymptomatic period[8]. Therefore, qualitative PCR testing positive in the absence of clinical symptoms of the disease is not always related to VL reactivation in co-infected patients.

The kDNA PCR seems to be a useful tool for diagnosing VL, and it may be a good marker for predicting VL relapses after treatment in co-infected patients with clinical symptoms of the disease.

RESUMO

Utilidade da kDNA PCR no diagnóstico de reativação de leishmaniose visceral em pacientes co-infectados sintomáticos

É importante a pesquisa de novos métodos laboratoriais para o diagnóstico de recidivas em casos de co-infecção leishmaniose visceral (LV) e vírus da imunodeficiência humana (HIV), que permitam o diagnóstico precoce das recidivas, utilizando métodos menos invasivos. Descrevemos aqui, o caso de paciente co-infectada que apresentou recidivas após o tratamento da LV e onde a PCR qualitativa demonstrou bom desempenho. A kDNA PCR parece ser ferramenta útil para o diagnóstico de recidivas de LV após o tratamento em pacientes co-infectados com sintomas clínicos da doença.

FUNDING

This study was supported by São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, Proc. 2010 50304-8).

CONFLICTS OF INTEREST STATEMENT

The authors have no associations or commercial relationships which might represent conflicts of interest.

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Received: 15 April 2013
Accepted: 26 June 2013