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Evaluation of HIV-*Leishmania* co-infection in patients from the northwestern Paraná State, Brazil

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ABSTRACT. Leishmaniasis occurs throughout the world and is one of the opportunistic infections that attack HIV-infected individuals. Few data are available on American cutaneous leishmaniasis (ACL) in HIV-infected patients. Current research investigates the occurrence of HIV-*Leishmania* co-infection in HIV-infected individuals in an endemic region in Southern of Brazil. A non-randomized transversal investigation, molecular and serum epidemiologic type, on the occurrence of ACL in 169 HIV-infected patients was undertaken. The patients were followed up at the Integrated Nucleus of Health of the city Maringá, Southern of Brazil. Results showed that 13 (7.7%) of the HIV-infected patients also presented *Leishmania* (*Viannia*) DNA, detectable in blood by PCR. Serology, direct research, culture and PCR in skin material produced negative results. PCR positiveness for *Leishmania* was not associated with CD4 T lymphocytes count, opportunistic disease, treatment, use of proteases inhibitors, tattooing/piercing or use of injectable drugs, residential environment or previous ACL history. Results show that HIV-infected patients who live in endemic areas may reveal *Leishmania* DNA in the blood without any ACL symptoms. Above findings may be attributed to anti-retrovirus medicine that controls viral replication and maintains the functionality of the immune system and to a possible anti-*Leishmania* activity of these drugs.

Keywords: american *cutaneous leishmaniasis*, HIV, co-infection, polymerase chain reaction.

RESUMO. Avaliação da co-infecção HIV-*Leishmania* em pacientes da região noroeste do Estado do Paraná, Brasil. As leishmanioses ocorrem em todo o mundo e são infecções oportunistas que afetam indivíduos portadores do vírus HIV. Este estudo investigou a ocorrência da co-infecção HIV-*Leishmania* em portadores do HIV numa região endêmica para LTA do Sul do Brasil. Foi realizado estudo transversal, não randomizado, utilizando metodologia molecular e sorológica, sobre a ocorrência de LTA em 169 portadores do HIV. Foram estudados pacientes atendidos no Núcleo Integrado de Saúde de Maringá, Paraná, Sul do Brasil. Observou-se que 13 (7,7%) dos pacientes infectados pelo HIV também apresentavam o DNA de *Leishmania* (*Viannia*), detectável no sangue por PCR. A sorologia, pesquisa direta de *Leishmania*, cultura e PCR de lesões de pele foram negativas. A positividade da PCR não estava associada à contagem de linfócitos T $CD4^+$, doença oportunista, tratamento, uso de inibidores de protease, tatuagem, uso de drogas injetáveis, ambiente da residência ou história prévia de LTA. Os resultados mostraram que indivíduos portadores do vírus HIV que residem em área endêmica podem apresentar o DNA de *Leishmania* sem manifestar sintomas de LTA. Estes resultados podem ser atribuídos a ação dos medicamentos anti retrovirais que controlam a replicação viral mantendo a integridade do sistema imunológico ou a uma possível atividade anti-*Leishmania* destas drogas.

Palavras-chave: leishmaniose tegumentar americana, HIV, co-infecção, reação em cadeia da polimerase.

Introduction

American cutaneous leishmaniasis (ACL) is an zoonotic disease caused by protozoa of the genus *Leishmania*. Infections in humans may either be non-apparent or may display a clinical spectrum ranging from localized, sometimes self-healing cutaneous lesions, to severe mutilating mucocutaneous lesions, or diffuse cutaneous leishmaniasis (AKILOV et al., 2007). ACL is widely distributed in Brazil, from the Amazon region to southern Brazil, with most cases caused by *Leishmania* (*Viannia*) *braziliensis* or *Leishmania* (*Leishmania*) *amazonensis* (ALVAR et al., 2008).

Leishmania protozoa are obligate intracellular parasites, and reside within the mammalian host as amastigotes in phagocyte cells, such as macrophages, dendritic cells and neutrophils. Although the *Leishmania* species that cause infection in humans induces strong humoral responses, with production of

significant levels of antibodies, they appear to play no protective role; in fact, they are rather associated with non-healing forms of leishmaniasis (TRIPATHI et al., 2007). The control of *Leishmania* infection is mediated by a Th1-type immune response, and experimental studies in murine models of cutaneous leishmaniasis have established a clear dichotomy between Th1-mediated protection and Th2-mediated disease susceptibility (SACK; NOBEN-TRAUTH, 2002). The CD4 subset of Th1 cells secrete activators of cell-mediated immunity such as IFN-γ, which induces the production of nitric oxide in phagocytic cells which, in its turn, leads to the parasite's destruction.

Leishmaniasis have been characterized in HIV-1 infected individuals, who may present multiple cutaneous lesions, resembling the picture of diffuse leishmaniasis more frequently found in immunosuppressed patients (RABELLO et al., 2003). HIV-1 infection progressively impairs the immune system function and facilitates the establishment of opportunistic pathogens (CRUZ et al., 2006).

Cases of *Leishmania*/HIV co-infections have been reported in various parts of the world and most co-infections involve leishmaniasis visceral type (ALVAR et al., 2008). Moreover, recurrence of cutaneous Leishmaniasis has been observed in HIV-1–infected patients with only moderate immunosuppression (COUPPIE et al., 2004). Skin lesions have been reported in HIV-infected patients since the first descriptions of the disease (CARDOSO et al., 2002). Cutaneous forms of the disease with disseminated lesions, involving the nasal, oral or pharyngeal mucosa, have been observed in patients with advanced stage Aids, sometimes accompanied by extensively destructive lesions (FERREIRA; BORGES, 2002). In fact, the above reveal the importance of studies on HIV-*Leishmania* co-infection. Current research investigates the occurrence of HIV-*Leishmania* co-infection in HIV/Aids-infected individuals in an ACL endemic region in southern Brazil.

Material and methods

Study of population: A non-randomized transversal research, by molecular and serological methods, was undertaken between March and May 2006 among HIV/Aids-infected patients. 169 individuals out of a total of 204 patients attended to the Brazilian Program Control of HIV-infection were invited to participate of this study. These patients are accomplish every 3-4 months by quantification of viral load, CD4 T lymphocytes count and opportunistic diseases at the Integrated Nucleus of Health of the city of Maringá, Southern of Brazil. Participants were informed on the epidemiological aspects and clinical manifestations of ACL and the importance of a diagnosis of the disease in HIV-infected patients. This work followed Resolution 196/96 from the Health National Council from Brazil's Health Ministry, and was approved by the Permanent Ethics Committee in Research involving Human Beings from the Maringá State University (no. 30/2005). Structured forms were used for data collection whereas data on anti-retroviral drugs and the occurrence of opportunistic diseases obtained from the patients' clinical records.

Biological samples: A sample of blood was collected from all patients by venous puncture and a fraction of samples was used to obtain the plasma for serologic tests. Skin samples were obtained of the patients with lesion, scars and skin spots (small mark different in colour, texture from the surface): the material of the border of the lesion was collected by scrapping, using a metal scraper free from DNA, and distributed in two tubes free from RNAses and DNAses with 100 µL STE buffer (10 mM TRIS; 1 mM $Na_2EDTA.H_2O$; 0.1 M NaCl; pH 8.0) and were stored at –20ºC, for posterior DNA extraction.

Serological Reactions: Indirect immunofluorescence assay (IFA) to ACL was carried out with promastigote forms of *L.* (*V.*) *braziliensis* (SILVEIRA et al., 1999) and for Chagas's disease with *Trypanosoma cruzi*, strain Y (Imuno-cruzi - Biolab) Plasma was diluted as from 1/20, at the ratio of 2, and a human anti-immunoglobulin G conjugated with fluorescein was employed. Titers ≥ 40 were considered significant for ACL and ≥ 80 for Chagas' disease.

Search of *Leishmania* sp.: Skin samples were used for: (a) the preparation of smears, stained by Giemsa stain, and then examined for the presence of amastigote; (b) culture in BBA (Blood Base Agar, Difco) medium with antibiotics, incubated at 25ºC and weekly observed during a month for amastigote of *Leishmania* sp.

Polymerase Chain Reaction – PCR: Skin samples in STE buffer (NaCl 0.1 M; TRIS 10 mM pH 8.0; $Na_2EDTA.2H_2O$ 1 mM pH 8.0) were incubated at 95ºC during 30 minutes in Biometra PC Thermal Cycler. The samples were centrifuged (1 min. at 13,000 x g) and the supernatant was stored at 4ºC till amplification. With regard to the blood, leukocyte layer was separated and stored at -20ºC till use. DNA was then extracted by guanidine isothiocyanate–phenol method (CHOMCYNSKI; SACCHI, 1987). Samples were thus centrifuged (3.500 x g during 15 min.); sediment was washed with 1 mL PBS (3.500 x g during 15 min.) and 300 µL guanidine isothiocyanate–phenol

(GF) were added, homogenized for 1 min, and 50 μL chloroform were added by stirring. After centrifugation at 9.200 x g during 10 min., the supernatant was transferred to another tube with 300 μL absolute ethanol, stirred during 1 min., centrifuged at 9.200 x g during 15 minutes and the sediment washed with 300 μL absolute ethanol. Centrifuge at 9.200 x g during 10 min. followed. Ethanol was evaporated from the sediment in a dry bath (BIOPLUS IT-2002) at 95ºC. DNA was hydrated by 50 μL buffer TE (TRIS 10 mM pH 8.0; $Na_2EDTA.2H_2O$ 1 mM pH 8.0) and incubated in a rotating stirrer during 6h at room temperature, stored at 4ºC till amplification. For extraction controls were used buffy coat samples of normal human added of *L.* (*V.*) *braziliensis* promastigotes (positive) or not (negative), and the DNA was extracted in the same way. A positive and a negative extraction controls were used for every 22 samples.

DNA amplification was carried out using the MP1L (5´ - TAC TCC CCG ACA TGC CTC TG – 3´) and MP3H (5´ - GAA CGG GGT TTC TGT ATG C – 3´) primers described by Venazzi et al. (2007). Reaction mixture for PCR (25 μL) contained 1 μM of each primer (Invitrogen), 0.2 mM dNTP (Invitrogen), 2U *Taq* DNA Polymerase (Invitrogen), 3 mM $MgCl_2$, enzyme buffer and 5 μL of extracted DNA. DNA amplification was undertaken in a Biometra PC Thermocycler, with initial warming at 95°C, during 5 min. Thirty cycles followed, each divided into three stages: de-naturation (95°C – 1.5 min); annealing (56°C – 1.5 min.); polymerization (72°C – 2 min.). A final extension was carried out at 72ºC during 10 min. The PCR products were analyzed in 3% agarose gel, stained with ethidium bromide (10 μg μL^{-1}) and visualized in a Transilluminator (Macro Vue UV-20 Hoefer). Positive and negative controls for PCR reactions were respectively DNA from *L.* (*V.*) *braziliensis* promastigote and water.

Viral load and Counting of T CD4 Lymphocytes: Patients were analyzed and followed up according to the Brazilian Health Ministry protocol (BRASIL, 2007). The HIV viral load followed RT-PCR methodology (COBAS Amplicor hiv-1 MONITOR™ Test, Roche). T CD4 lymphocytes were counted by flow cytometry with FACSCount Reagents™ (Becton Dickinson).

Statistic Analysis: The association of opportunistic diseases with T CD4 lymphocyte counts was analyzed by chi-square test with Statistica 6.0. A probability value $p < 0.05$ was considered statistically significant.

Results

One hundred and sixty-nine HIV-infected patients, 93 males (55%) and 76 females (45%), age ranging between 20 and 67 years old, predominantly between 40 and 57 years (41.4%) were analyzed. PCR showed *Leishmania* (*Viannia*) DNA in 13 (7.7%) of blood samples (Figure 1) and negative in all the 10 skin samples. The latter had also negative results by direct examination and culture. All HIV-infected patients had IFA for leishmaniasis negative, although one was IFA positive for Chagas's disease, titer 80. Most HIV-infected patients (94.7%) inhabited the urban area, of whom 165 (97.6%) did not have a previous ACL history. Four of them reported that at least 15 years a ago they had ACL which was treated with pentavalent antimony drugs. However, the thirteen HIV infected patients with DNA *Leishmania* were among those without any previous ACL history (Table 1). However, PCR positiveness for *Leishmania* was not associated with CD4 T lymphocytes count ($p = 0.7799$), opportunistic disease ($p = 0.3812$), treatment ($p = 0.9225$), residential environment ($p = 0.8047$) or previous ACL history ($p = 0.7150$), use of proteases inhibitors ($p = 0.2315$), tattoo/piercing ($p = 0.3232$) or use of injectable drugs ($p = 0.3290$).

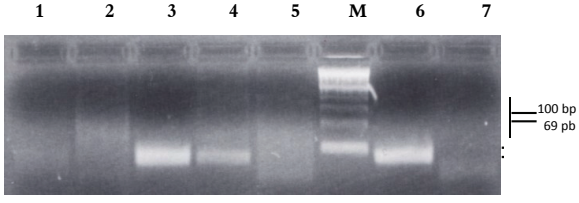

Figure 1. Representative gel showing of 69 base pairs fragment of the *Leishmania* (*Viannia*) k-DNA amplified by PCR with MP1L and MP3H primers. Lanes 1, 2 and 5 show negative blood samples; lanes 3 and 4, positive blood samples; lane 6, positive control (DNA of *L.* (*V.*) *braziliensis*); lane 7, negative control (water); M, Molecular Marker Standard.

It has also been reported that 98 (58.0%) of HIV/Aids infected patients had one or two opportunistic diseases. Cytomegalovirosis was the most frequent with 128 cases, followed by toxoplasmosis with 61 cases, by candidiasis with 28 cases, by herpes zoster with 20 cases, and by tuberculosis with 11 cases. PCR was positive for six toxoplasmosis-infected patients, one with toxoplasmosis and herpes zoster, two with herpes zoster, one with tuberculosis and three with no opportunistic disease (Table 2).

Treatment with anti-retroviral drugs had been prescribed for 119 (70.4%) infected patients, out of which 53 (44.5%) employed protease inhibitors: one of the infected patients was being treated with Tenofovir; two with Indinavir; 14 with Atazanavir, 13

with Nelfinavir and 23 with Ritonavir associated with another protease inhibitors (Lopinavir, Atazanavir, Amprenavir and Indinavir). PCR was positive in six (Table 3) among the latter.

Table 1. Characteristics of 169 HIV/Aids-infected subjects, according to results of Polymerase Chain Reaction for *Leishmania* (*Viannia*). Maringá, Paraná State, Brazil, March to May 2006.

	PCR for *Leishmania Viannia*		p
	Positive	Negative	
CD4 T cells μL			
> 350	8	102	0.7799
≤ 350	5	54	
Opportunistic disease			
Yes	10	88	0.3812
No	3	68	
Anti-retroviral therapy			
Yes	9	110	0.9225
No	4	46	
Residential environment			
Urban	13	147	0.8047
Rural	0	9	
Previous ACL history			
Yes	0	4	0.7150
No	13	152	
Protease Inhibitors			
Yes	6	47	0.2315
No	7	109	
Tattoo/piercing			
Yes	1	29	0.3232
No	12	127	
Injectable drugs			
Yes	0	21	0.3290
No	13	135	

Table 2. Occurrence of opportunistic diseases in the 169 HIV/Aids-infected patients, according to results of Polymerase Chain Reaction for *Leishmania* (*Viannia*). Maringá, Paraná State, Brazil, March to May 2006.

Opportunistic disease	PCR		p
	Positive	Negative	
Toxoplasmosis			
Yes	7	54	0.1654
No	6	102	
Tuberculosis			
Yes	1	10	0.8571
No	12	146	
CMV infection			
Yes	10	118	0.9175
No	3	38	
Candidiasis			
Yes	4	24	0.0847
No	9	156	
Herpes zoster			
Yes	3	17	0.1915
No	10	139	

Table 3. Medicine prescribed to 169 HIV/Aids-infected patients, according to results of Polymerase Chain Reaction for *Leishmania* (*Viannia*). Maringá, Paraná, Brazil, March to May 2006.

Medicine	PCR		Total
	Positive	Negative	
Proteases inhibitors⋆	6	47	53
Others⋆⋆	3	63	66
None	4	46	50
Total	13	156	169

⋆Atazanavir, Ritonavir, Neolfinavir and Indinavir; ⋆⋆Lamivudina, Estavudina, Efavirens, Tenofovir, Neviropina and Didanosina.

Discussion

Results show that 7.7% of HIV/ds-infected patients also had *Leishmania (Viannia)* DNA in their blood, detected by PCR. All the patients had negative results in serology and in direct search, culture and PCR of the skin samples. ACL is endemic in the northwestern region of the Paraná State, Brazil. From the total cases of the southern region of Brazil, 97% occurred in Paraná State (BRASIL, 2007). According to Silveira et al. (1999), 69.3% of the 804 ACL-positive patients were rural workers or inhabited the rural area; in this area *L.* (*V.*) *braziliensis* is the main agent of ACL. Current investigation, however, reported that only 5.3% of HIV/-infected patients lived in the rural areas. Moreover, 13 patients with DNA *Leishmania* in their blood were from the urban region. Only 10 infected patients having lesion, scars and skin spots, although direct research and PCR produced negative results in all of them.

Serological tests may be particularly useful in the diagnosis of visceral or mucocutaneous leishmaniasis (DENIAU et al., 2003). In this work IFA was employed to investigate anti-*Leishmania* serological reactivity even though in a previous investigation within the same region the technique had 75.7% sensitiveness (SILVEIRA et al., 1999). Since results show that infected patients did not have anti-*Leishmania* antibodies at detectable levels by IFA, it is necessary that more sensitive techniques must be employed. In current research, a single patient had positive serology for Chagas's disease.

Specific primers used in the PCR for *Leishmania (Viannia)* amplify the preserved region (69 bp) of the k-DNA mini-circle and detects 0.9 fg of the parasites' DNA (VELÁSQUEZ et al., 2006). Since dermatological manifestations in HIV-infected patients have atypical clinical traits, in current research every type of skin lesion, even tattoos and spots, were suspected of ACL. In fact, reports are extant with regard to amastigotes in health skin, tattoos, Kaposi's sarcoma lesions and herpes zoster (PUIG; PRADINAUD, 2003). However, only one out of 30 HIV-infected patients with tattoos, skin spots or piercing had PCR positive. Further, the use of injectable drugs was also taken into account since reports give a high predominance of *Leishmania infantum* in syringe-sharing intravenous drug users (CRUZ et al., 2002). Although 12.4% of patients declared themselves drug users, no one was ACL positive.

A significant decrease in HIV-*Leishmania* co-infection has been reported in France (DEL GIUDICE et al., 2002) and in Spain (DE LA ROSA

et al., 2002) after the introduction of anti-retroviral therapy, which may actually be interpreted as a reconstitution of the immune system. Besides, "*in vitro*" studies in *L. major* and *L. infantum* cultures showed that the proteases-inhibiting drugs (Indinavir and Saquinavir) have leishmanicidal activity (SAVOIA et al., 2005). Current research has shown that in the context of HIV-infected patients under analysis, 70.4% were undergoing treatment, of which 44.5% used protease inhibitors, including the four subjects who had had ACL. Although studies on the drugs' anti-*Leishmania (Viannia)* activity have not been undertaken, such possibility may not be disposed of and may be investigated later on.

It has been found that 58.0% of HIV/Aid-infected subjects had one or more opportunistic diseases, among whom 10 subjects that presented *Leishmania* DNA. Opportunistic infections may accelerate HIV infection by the microorganisms' capacity in stimulating the Th2 cytokines (IL-4, IL-10, among others) production which may trigger the progression of HIV/AIDS disease and it has already been demonstrated that HIV/*Leishmania* co-infection favors viral replication and worsens the immunosuppression state (FERREIRA; BORGES, 2002). The immunosuppression may cause an increase in viral load, decrease in the number of T $CD4^+$ cells and an uncontrolled multiplication of *Leishmania*. However, in current studies no significant association was found between any investigated opportunistic infections and PCR positiveness.

Conclusion

Results show that HIV-infected patients that inhabit endemic areas may have *Leishmania* DNA in their blood without triggering the humoral immune response and without any ACL symptoms. A possible explanation could be that the blood DNA detected by PCR methods in asymptomatic patients would be a fossil DNA, without the ability to trigger the immune system. On the other hand, the anti-retroviral treatment, which is able to control viral replication, can maintain the functionality of the immune system and control of *Leishmania* infection, or the drug may have activity against *Leishmania*. However, the reasons for these findings are not clear, but the evidence of *Leishmania* DNA in blood found in subjects with no history of ACL raises questions concerning their clinical consequences. Since that in Brazil anti-retroviral treatment is indicate for all HIV-infected patients, symptomatic or not, who have a T $CD4^+$ lymphocyte count lower than 350 cells μL^{-1}, it is very important that all HIV-infected patients living in the *Leishmania*-transmission area are analyzed for ACL.

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