Frequency of *Leishmania* spp. infection among HIV-infected patients living in an urban area in Brazil: a cross-sectional study

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Abstract

Background: There is little information about the frequency of Leishmaniasis infection in asymptomatic HIV infected individuals and about the performance of laboratory diagnostic methods in coinfected patients in Latin America. The main objective of this study is to evaluate the frequency of Leishmaniaspp. infection in HIV infected patients living in an urban area from Brazil.

Methods: To detect Leishmaniasis infection, were performed diagnostic tests to detection of antibodies anti-Leishmania(ELISA using Leptomonas seymouriantigens; ELISA using rk39 antigens; ELISA using rK28 antigens; indirect fluorescent-antibody test (IFAT); direct agglutination test (DAT) and detection of Leishmania DNA by polymerase chain reaction (PCR) with the target genes kDNA and ITS-1.

Results: Frequency, considering at least one positive test, was 15%. For ELISA using Leptomonas antigens and IFAT, there was an association between CD4+ T-lymphocyte counts and test positivity, with a higher positivity of these tests in more immunosuppressed patients (T CD4+ cells count < 200/mm3).

Conclusions: According to our data, there was a high prevalence of Leishmaniaspp. in this population living with HIV. Although there is the possibility of cross-reaction, some tests considered highly specific for the diagnosis of Leishmania infection were positive. There was also an association between the positivity of some tests studied and lower values of T CD4+ lymphocytes.

Background

Leishmaniasis is one of the most common neglected tropical diseases and about 350 million people are at risk to acquiring it [1]. In HIV-infected patients, leishmaniasis may have an opportunistic behavior and has become a problem in several parts of the world, especially in east Africa, Brazil and India. Although atypical cases and more severe cutaneous and mucocutaneous leishmaniasis have been reported in HIV-infected patients, visceral leishmaniasis (VL) is responsible for the major burden of morbidity and mortality in the context of HIV infection [2]. The spread of HIV and VL has produced an overlap between transmission areas of both diseases; therefore, an increase in the number cases of coinfection has been observed. Both HIV infection and VL produce immunological disturbances that can enhance their effects if they occur concomitantly, contributing to the unfavorable outcome of
both diseases [3, 4]. HIV infection dramatically increases the risk of progression from asymptomatic infection towards VL and in the presence of coinfection, VL tends to be more severe and manifest atypically. *Leishmania* infection also drives HIV replication, inducing the proliferation and differentiation of HIV-infected human monocytes and inhibiting apoptosis of infected cells [3, 5–7]. In Brazil, a progressive increase of HIV/Leishmania coinfection has been reported since the beginning of the 1990s. There is a projection of continuous raise, mainly because of the overlapping of geographical areas of both diseases, with urbanization of visceral leishmaniasis and the propagation of HIV transmission to regions with lower urbanization rates and to small and medium-sized areas [8]. These data are related to full-blown symptomatic visceral leishmaniasis, and there are few studies including asymptomatic patients evaluating the prevalence of *Leishmania*/HIV coinfection in Brazil, with values ranging from 16% to 20.2% in some endemic areas [9, 10]. Increased lethality and therapeutic failure are frequent in patients coinfected and most of HIV-associated visceral leishmaniasis represents reactivation of a prior subclinical infection [11, 12]. Therefore, it is important to evaluate the magnitude of this problem in other areas, where asymptomatic immunosuppressed individuals can progress to symptomatic disease more frequently, with poor therapeutic outcomes and a high rate of relapse [11, 13–15]. Another important point is HIV-infected could be asymptomatic carriers of *Leishmania* and develop symptoms in the presence of severe immunosuppression or it could be reservoir to *Leishmania*, maintained transmission cycle of transmission in area without other mammals reservoir [16, 17].

This study aimed to estimate the frequency of *Leishmania* infection among HIV-infected patients from a major national reference center for HIV in an urban area according to detection of antibodies anti-*Leishmania* and *Leishmania* DNA detection.

**Methods**

1. **Patients**

The study was conducted at the Institute of Infectious Diseases Emilio Ribas, Sao Paulo, Brazil, from April 2015 to March 2016. This institute is a reference for the treatment of HIV infection in Brazil and infectious tropical diseases and assists 8500 HIV/aids patients yearly. Until 2017, Sao Paulo city was
not considered an autochthonous VL area. Two hundred and forty-five patients were included in the study. Five patients were excluded because of problems in the storage of samples. Inclusion criteria were: the patient had to be older than 18 years old and have a definitive diagnosis of HIV infection according to criteria established by the Ministry of Health of Brazil. Severe immunodeficiency was recorded if the patient had an AIDS-defining illness or lymphocyte T CD4+ count <200 cells/mm³ at the time of inclusion.

All patients were questioned about where they were born and about all the cities they had lived for more than one year during their lives. Exposure to an endemic area of VL was recorded if the patient had been born in or lived for more than one year in a municipality with autochthonous transmission of VL, as reported by the National Surveillance System of the Ministry of Health. According to the World Health Organization, splenomegaly, prolonged fever and weight loss are the main characteristics of visceral leishmaniasis disease [1]. The presence of these signal/symptoms were evaluated throughout the clinical records. Relevant personal, epidemiological, clinical and laboratorial data were obtained by the analysis of clinical records. Written, free-and-informed consent to participate in the study was obtained from all individuals.

2. **Sample collection and laboratory tests**

After inclusion in the study, 8 ml venous blood were collected in two different tubes, one to be used in serological tests and one for DNA extraction containing EDTA. The first was centrifuged at 1800 g for 10 min, the serum was separated and then frozen at –20 °C for serological analysis. The other tube was frozen at –20 °C for molecular assays.

The following tests were used to detect *Leishmania* infection in 240 patients evaluated for HIV-infected: ELISA using *Leptomonas seymori* antigen (ELISA *Leptomonas*); ELISA using recombinant K39 antigen (ELISA rK39); ELISA using recombinant K28 antigen (ELISA rK28); indirect fluorescent antibody test (IFAT) and direct agglutination test (DAT). All samples were also tested to *Leishmania* kinetoplastid DNA (kDNA) amplification by polymerase chain reaction (kDNA PCR) and *Leishmania* internal transcribed spacer 1 (ITS-1) amplification by polymerase chain reaction (ITS-1 PCR).

2.1. **Serological methods**
**ELISA Leptomonas.** *Leptomonas seymouri* antigenic extract was prepared as previously described [18, 19], with modifications. Polystyrene plates (Corning Incorporated, New York, USA) were coated overnight at 4 °C with 50 μL of parasite antigens (4 μg/mL) diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6). Plates were washed with PBS-T, and blocked with 0.01 M PBS (pH 7.2) with 0.05% Tween-20 and 5% skim milk for 30 min at room temperature. Fifty microliters of sera per well diluted in PBS-T and 1% skim milk (1:200) were added to the plates and incubated for 1h at 37 °C. Plates were washed five times and incubated with anti-IgG immunoglobulin conjugated with peroxidase diluted in PBS (1:2.000) for 1 hour and washed again. After incubation with a solution containing 7.5 μl H2O2, 5mg O-phenylenediamine dihydrochloride (OPD-tablets, Sigma Co) and 12.5 ml citric acid-sodium citrate buffer, the assay was stopped by adding 25 μL of 4N HCl. The ELISA assay results were read at 492nm using a spectrophotometer (Titertek Multisplan Plus, Helsinki, Finland). The cutoff was determined by the value that demonstrated better sensitivity and specificity in the receiver operating characteristic (ROC) curve including the positive and negative controls. All experiments were independently repeated at least twice.

**ELISA rK39 and ELISA rK28.** The recombinants proteins of *L. infantum*, rK39 and rK28 were provided by the Infectious Disease Research Institute, Seattle, WA. Polystyrene plates (Corning Incorporated, New York, USA) were coated overnight at 4 °C with 50 μl of recombinant antigens (0.5 μg/ml) diluted in carbonate-bicarbonate buffer (0.05M, pH 9.6). Washing was done three times using PBS 0.01M with 0.05% Tween-20 (PBS-T). Plates were blocked with a solution containing PBS-T and 5% skim milk for 2h at 37°C. Fifty microliters of sera per well diluted in PBS and 5% skim milk (1:100) were added to the plates and incubated for 30 min at 37 °C, washed and incubated with anti-IgG immunoglobulin conjugated with peroxidase (1:30.000) for 30 min and washed again. After incubation during 7 min with 3,3’5,5’-Tetramethylbenzidine (TMB, Sigma Co) the assay was stopped by adding 25 μl of 2N H2SO4. The ELISA assay results were read at 450nm using a spectrophotometer. Each plate had negative and positive controls. For both antigens, the cutoff point was determined using a ROC curve. The reactivity index (RI) was calculated for each sample by dividing the sample absorbance value by the cutoff. Samples were considered positive if the RI value was ≥ 1.
**IFAT.** The test was performed according to the protocol described by Guimaraes and others, 1974 [20]. Briefly, the slides were prepared with promastigotes of *L. major*-like antigens (MHOM/BR/71/49). Sera were diluted in PBS (0.01M, pH 7.2) (1:40), applied to the slides and incubated for 30 min, at 37 °C. After three washings with PBS, slides were incubated with anti-IgG fluorescein isothiocyanate conjugate (1:150) (BioMerieux, Marcy l’Etoile, France) and Evans Blue 4 mg%. Samples scored positive when fluorescent microscopy showed clear evidence that they produced a cytoplasmic or membranous fluorescence. For each test standard, *Leishmania*-positive and *Leishmania*-negative human sera were included as controls.

**DAT.** In brief, samples were diluted in physiological saline (0.9% NaCl) containing 0.78% β-mercaptoethanol. Dilution series of the sera were performed in a V-shaped microtiter plate, starting at a dilution of 1:50 and going up to a maximum serum dilution of 1: 51,200. Column 1 was used as a blank control whereas rows G and H were used as positive control. Fifty microliters of DAT antigen (Royal Tropical Institute, Amsterdam, Netherlands) were added to each well containing 100 μl-diluted serum, and the results were read after 18 hours of incubation. The cut-off value of the DAT was set at >1:6,400.

### 2.2 Molecular methods

**DNA extraction**

Extraction of DNA from blood was performed using the Minikit QIAamp (QIAGEN, Chatsworth, CA, USA), according to the manufacturer’s instructions.

**kDNA PCR.** The entire conserved kDNA region (720 bp) was amplified using primers (LINR4 and LIN19) described elsewhere, according to Aransay *et al*, 2000, with modifications [21]. Each reaction set included one negative PCR control (no DNA) and one positive control extracted from *Leishmania* (*L.*) *infantum chagasi* promastigotes in culture. An isolated area, irradiated with UV light before reaction setup and free of DNA, biological samples or amplification products, was used to prepare the reactions.

The reaction mixture consisted of 31.0 μl of water DNAsé free, 5.0 μl of buffer 10x PCR Rxn®, 2.0 μl 50mM MgCl2, 2.0 μl of each primer (LINR4 and LIN19) (10 pM/μl), 1.0 μl deoxynucleoside
triphosphates (dNTPs), 2.0 µl of Taq polymerase (Invitrogen® - 5 U/µl) and 5.0 µl of DNA extract, in a total reaction volume of 50.0 µl. PCR was performed under the following conditions: 1 cycle of 2 min at 35 °C; 33 cycles of 30s at 95 °C, 30s at 58°C, 1 min at 72°C and 10 min for final extension at 72°C. After amplification, 5µl of the samples were separated by electrophoresis on a 1% agarose gel, and the amplified sequences were visualized by ethidium bromide staining.

**ITS-1 PCR.** The ITS-1 region was amplified using primers (LITSR and L5.8S) described elsewhere, according to [22]. Each reaction set included one negative PCR control (no DNA) and one positive control extracted from *Leishmania infantum* (MHOM/BR/1974/PP75) promastigotes in culture. An isolated area, irradiated with UV light before reaction setup and free of DNA, biological samples or amplification products, was used to prepare the reactions. PCR was performed according to manufacturer’s instructions using a GoTaq® Hot Start Green Master Mix Protocol (Promega Corporation, Madison, USA), in a total reaction volume of 50.0 µl. PCR was performed under the following conditions: 1 cycle of 3 min at 95 °C; 35 cycles of 40s at 95 °C, 45s at 53°C, 1 min at 72°C and 6 min for final extension at 72°C. After amplification, 5µl of the samples were separated by electrophoresis on a 2% agarose gel, and the amplified sequences were visualized by ethidium bromide staining.

**Results**

From April 2015 to March 2016, 245 patients were included in these study; however, five of them were excluded due to problems related to storage of samples. Therefore, 240 individuals were included in the analysis. Patients’ ages ranged from 24 to 80 years, with mean age of 45.5 years, and men comprised the majority of the samples (172, 71.6%). Mean time between HIV infection diagnosis and study inclusion was 13.3 years. Viral load ranged from non-detectable to 3.206.948 copies/mL, and mean TCD4+ was 564.4 cells/mm³ (ranged from 2 to 1.724 cells/mm³). Regarding the use of HIV therapy, 87.9% (211/240) were on regular highly active antiretroviral therapy (HAART). Ninety-two (38.3%) individuals had a history of exposure to endemic area of VL. Baseline characteristics of the sample are shown in Table 1.

No individual had visceral or cutaneous leishmaniasis previously, and one patient had Chagas’
disease (undetermined form). Twenty-eight patients were in treatment for at least one disease and 17 of them had at least one signal/symptom considered also compatible with visceral leishmaniasis diagnosis, as described above (fever and/or splenomegaly and/or weight loss). The diseases being treated at the moment of inclusion and the number of times that each one was present were the following: tuberculosis (9), cytomegalovirus disease (5), cerebral toxoplasmosis (3), lymphoma (3), Kaposi sarcoma (2), cryptococcal meningitis (2), neurosyphilis (2), muscular abscess (1), CNS demyelinating disease (1), pulmonary aspergillosis (1), hepatic cirrhosis (1) and pneumocystosis (1). It is noteworthy that four patients had more than one disease concomitantly. More than one patient had splenomegaly, totaling 18 individuals with at least one signal/symptom considered compatible with VL. At the discretion of the attending medical staff, two of them were submitted to bone marrow aspiration to search for *Leishmania* and, in both cases, the result was negative. Therefore, no patient had clinically confirmed leishmaniasis. Table 2 shows the positivity of each diagnostic test. Among 240 patients included, nine were positive by ELISA *Leptomonas* (3.75%), three by ELISA rK39 (1.25%), six by ELISA rK28 (2.5%), eleven by IFAT (4.5%), four by kDNA PCR (1.66%) and 10 by ITS–1 PCR (4.16%). No sample was positive by DAT. Considering as infected those patients who presented at least one positive test, the estimated prevalence of asymptomatic infection was 15%. Concordance between tests was poor (Kappa test < 0.20) comparing all tests. No patient had two positive molecular methods, but one ITS–1 PCR positive patient had positive serological methods: positive by ELISA *Leptomonas* and IFAT.

Infection by *Leishmania* spp., defined as positivity by any test, showed similar distribution by patient, sex, presence of signal/symptoms, exposure to endemic areas to VL, presence of current clinical intercurrence undergoing treatment, with no statistical significant association between the positivity of the tests and these factors (data not shown). Regarding TCD4+ counts, there was a significant association between the positivity of ELISA *Leptomonas* and IFAT and the presence of TCD4+ count less than 200 cells/mm³ (Table 3), when they were analyzed separately. For the other methods, this association was not demonstrated (data not shown).

**Discussion**
There are few studies evaluating the prevalence of *Leishmania* asymptomatic infection among HIV-infected individuals, and there are no studies analyzing this frequency in an urban area with no reports of autochthonous transmission of VL in Brazil. Taking into account the overall prevalence (at least one positive test) observed in this study, the results were smaller than those reported by Orsini *et al.*, 20.2% [10]. In another study performed in Brazil, Carranza-Tamayo *et al.* showed similar data, with an overall prevalence of 16.0%, [9]. Considering studies in other parts of the world, the frequency is lower than that found by Garrote *et al.*, 64.0%, in Spain [23]. These discrepancies are probably explained by the use of different diagnostic tests, including different antigens and molecular targets. Recently, in a systematic review about asymptomatic visceral leishmaniasis in the Indian subcontinent, Hirve *et al.* [24], including 31 articles and using different tests (rk39 immunochromatographic test, rk39 ELISA, DAT, PCR or Leishmanin skin test) found a prevalence from 0.25% to 36.9%, depending on the method used. Recently, Echchakery *et al.* [16], in Marroco, found prevalence of 5% in HIV-infected patient, by indirect immunofluorescence. Clearly, there is no consensus about the best diagnostic tool to estimate the frequency of asymptomatic *Leishmania* infection in the population and, in the context of HIV infection, even in full-blown symptomatic disease, there is little data in Latin America regarding the performance of diagnostic methods [25].

Data also demonstrated poor agreement between serological tests. This fact has been shown by other prevalence studies involving HIV infected patients and immunocompetent individuals [9, 10, 26, 27], which can be accounted for the use of different antigens and molecular targets (surface, soluble or recombinant antigens) that can detect many stages of the asymptomatic infection.

ELISA using *Leptomonas seymori* antigen was performed for the first time in patients with HIV infection. It has already been demonstrated that this antigen had a good performance for the diagnosis of visceral leishmaniasis; in fact, its performance is comparable to *L. chagasi* antigen. Similarly to other crude antigens, there is the possibility of cross-reactivity with *T. cruzi* and other *Leishmania* species [18]. Recently, Kesper *et al.* [28], while performing ELISA using *Leptomonas seymouri* and *Crithidia fasciculata* antigens, showed 100% reactivity with sera from visceral leishmaniasis (VL) cases, and no reactivity with American tegumentary leishmaniasis (ATL).
Nevertheless, these patients did not have HIV-infection. We support strongly that further studies are necessary to determinate the sensitivity and specificity for VL diagnosis in individuals with HIV infection, using *Leptomonas* antigen.

IFAT using *L. major*-like antigen is a test recommended by the Ministry of Health of Brazil for the diagnosis of leishmaniasis. In this study, IFAT showed greater positivity compared to ELISA *Leptomonas*. In other studies evaluating the prevalence of *Leishmania/HIV* coinfection, ELISA using crude antigens demonstrated greater positivity compared to IFAT [9, 10], and this was attributed to the fact that IFAT detects mainly antibodies against surface antigens, while crude antigens ELISA detect a wider variety of antibodies directed against soluble antigen components. In our study, this difference was not significant and probably related to divergence between specificity of these tests than to the immunological characteristics of antigens.

Considering only serological methods, we found that ELISA *Leptomonas* and IFAT had positivity associated with T CD4+ counts less than 200 cells/mm³. This data can be, at first, considered surprising, given that serological methods have worse sensitivity for the visceral leishmaniasis diagnosis in HIV infected patients compared to the immunocompetent individuals [6, 25, 29]. This concept is applicable to other infectious diseases, especially to the advanced stage of aids, in which severe T and B lymphocyte dysfunction triggers decreased production of specific antibodies [3]. It is important to highlight that all this information is related to the performance of diagnosis tests in subjects with full-blown symptomatic visceral leishmaniasis and, herein, we are discussing asymptomatic individuals. Another important point is the possibility of cross-reaction of these tests. Although there was no association between current disease in treatment and test positivity, it is possible that these individuals with lower values of T CD4+ counts had subclinical or latent diseases, which may have interfered with the results. Another question is the possible variability of humoral response in HIV infected subjects. Gradoni et al [30], comparing serological IFAT titles of HIV infected and non-HIV infected individuals with visceral leishmaniasis, showed worse sensitivity of this method in those infected with HIV, but a greater variability of titles in this group, with some patients with IgG levels far above the non-HIV infected subjects. These findings may reflect the temporal sequence of
acquisition of the two infectious agents (HIV and *Leishmania*). Individuals that acquiring *Leishmania* infection before HIV infection should produce high levels of IgG directed against antigens recognized before T cell impairment due to the HIV. This response is probably related to the increased IgG production because of non-specific polyclonal B cell activation, which occurs more frequently in patients with severe immunosuppression [3, 30]. This abnormal humoral response, more evident in severe immunosuppressed patients, can explain a greater proportion of positive serological tests in the group, considering that all samples are from individuals currently living in a non-endemic area for VL that probably acquired *Leishmania* infection before HIV infection.

ELISA rK39 had the lowest positivity comparing all serological methods. This antigen has been used both in ELISA assays and in rapid diagnostic tests [31–33], including in HIV infected patients [34, 35]. As it is characteristically associated with active disease [36, 37], a sample composed predominantly by asymptomatic individuals is expected to have a lower positivity. Furthermore, even considering the diagnosis of full-blown symptomatic disease, the sensitivity of ELISA using rK39 antigen is considerably worse in HIV-infected individuals [38]. Therefore, although it can be possible to detect this antigen in asymptomatic individuals [32], these characteristics can limit its use in this context. In the general population, the sensitivity and specificity of recombinant antigen k28 (99.6% and 95–100%, respectively) is similar to ELISA rK39 for the diagnosis of VL [39]. This antigen can be used in rapid tests with a good performance, showing high specificity (near 100%) and sensitivity (92%) to the diagnosis of visceral leishmaniasis [40]. However, it has not been used in studies including patients in Latin America, and there is few studies discriminating its performance in HIV-infected subjects. Silva et al [41] using two immunochromatographic tests to detection antibodies anti-rK 39 and anti rK28 found sensitivity of 67.74 and 61.29% respectively, in patients with active visceral leishmaniasis and HIV-infection, showing poor performance when compared with active visceral leishmaniasis without HIV infection.

In our study, in a group of asymptomatic individuals, its positivity was greater than ELISA rk39, which can suggest a good perspective of its use in HIV infected Latin American asymptomatic patients, maybe as an early marker of *Leishmania* infection.
In our sample, no individual had a positive DAT. This test is one of the most widely used diagnostic tests for the diagnosis of VL around the world [42] and, in comparison with other serological methods, it has a good performance in HIV infected patients [25]. DAT can be used to diagnose active forms of the disease and can diagnose infections before the clinical presentation, with titers declining to negative values one year after the cure [43]. There are two important points to highlight in the population included in this study. Firstly, the individuals included were predominantly asymptomatic and nobody had a confirmed leishmaniasis diagnosis or a previous history of this disease. The performance of this test considering this scenario had never been evaluated, probably because there is no gold standard to compare this one with other tests and to establish sensitivity and positivity. Moreover, although a part of cohort had lived in endemic areas in the past, all subjects were currently living in a non-endemic area for VL. We believe the fact that we are analyzing a sample composed by asymptomatic HIV-infected patients not continuously exposed to *Leishmania* can explain this result as the positivity of test depends on the presence of active disease, recent cure or exposition to antigen.

Regarding molecular methods, we used two different targets to perform the polymerase chain reaction (PCR). In a systematic review and meta-analysis, de Ruiter et al [44] found a pooled sensitivity and specificity of PCR in peripheral blood of 93.1% and 95.6%, respectively. There are studies demonstrating promising results in immunosuppressed patients as well [45, 46]. Although kDNA is more used for DNA amplification of *Leishmania*, because of the high number of copies per parasite [47], in this study, ITS–1 PCR had greater positivity (4.2%), compared to kDNA PCR (1.7%). Several different genomic targets are used for *Leishmania* sp. detection, and there is no consensus about the best one, especially due to the discrepancies of objectives and methods of each study [47]. The fact that no patient had both PCR positive and the discrepancy between two tests reinforces this limitation and the necessity of further studies about this theme, including symptomatic and asymptomatic subjects. Molecular methods have been developed with the purpose of complementing and creating alternatives for the diagnosis of leishmaniasis, as well as the possibility of follow-up and laboratory control of cure [47, 48]. Possibly, more immunosuppressed patients present a greater periodicity of parasitic circulation in their organism. The higher positivity of this method in this
context of patients may indicate that this target is more effective in identifying intermittent parasitemia in this group of patients, since it can be used in the future to screen individuals at risk of developing manifest leishmaniasis. This information would be of extreme importance for these patients with advanced immunosuppression, who are known to present with more severe forms of the disease, more complications and higher mortality [49, 50].

Social factors or the host may constitute a risk for the occurrence of Leishmania infection. Here, we evaluated aspects related to the host, as risk factors to Leishmania infection. Presence of signal/symptoms did not demonstrate association with test positivity. At first, the information about cities with autochthonous transmission was obtained through records of the Ministry of Health of Brazil showing that a considerable part of these cities is considered having autochthonous transmission due to the presence of just one or few cases of confirmed VL. Therefore, many patients may not have been exposed to VL, despite living in transmission areas. In the same way, it was not be possible to guarantee that, at the moment the individual lived in an endemic area, this place presented autochthonous VL. Most cases of HIV infection in Brazil are located in the south and southeast region, where Sao Paulo state is located. Regarding VL, between 1999 and 2013, 2,328 autochthonous cases of visceral leishmaniasis were confirmed in Sao Paulo state, corresponding to 80 cities with VL transmission. Of these, 202 evolved to death, corresponding to a lethality of 8.7%. Of note, Sao Paulo city has had no report of autochthonous VL transmission to date [51].

Injecting drug use is the major drive of HIV infections in many parts of the world, in Leishmania/HIV coinfection [1]. In Brazil, this pathway of HIV transmission is not important and sexual transmission corresponds to more than 95% of HIV infection acquisition [52]. Thus, although we do not have data about the percentage of injecting drugs users in the sample, we consider the possibility of Leishmania acquisition through this route to be very low. Concerning the presence of symptoms, 17 of 28 patients presenting with some symptom defined as suggestive of VL had other diseases, including tuberculosis as the most cited. Therefore, probably part of these symptoms was more attributable to these diseases than to diagnosis of VL or to other diseases not diagnosed yet. Due to that, there was not association with this variety and positivity of diagnostic tests.
Conclusions
We showed a prevalence of 15% of *Leishmania* spp. infection in HIV infected patients. This data should be approached with caution. It is important to highlight that serology at a given time may reflect a contact with parasite followed by sterile cure. Considering only molecular methods, the prevalence falls to 5.8%. Nevertheless, the natural history of *Leishmania* infection in humans is dynamic, with parasites present in peripheral blood at a given time and with immune responses acting to inhibit the parasite production in another defined moment, a fact that can be more evident in the context of HIV infection. Thus, indirect tests may reflect an asymptomatic infection, especially considering the possibility of reactivation in HIV immunosuppressed patients. The weak overlap observed here and in other studies indicates that the use of more than one diagnostic method may increase the proportion of cases detected. Taking into account the positivity of these tests, including molecular methods and very specific serological tests in individuals with HIV infection living in a city with no autochthonous cases of VL, but in a country with high transmission of VL, it is important to consider leishmaniasis infection as a cause of fever and other symptoms, especially in the context of advanced immunosuppression.

List Of Abbreviations
ATL: American tegumentary leishmaniasis
DAT: direct agglutination test
ELISA: enzyme-linked immunosorbent assay
IFAT: indirect fluorescent-antibody test
ITS-1: internal transcribed spacer 1
kDNA: kinetoplastid DNA
PCR: polymerase chain reaction
RI: reactivity index
VL: visceral leishmaniasis

Declarations

**ETHICS APPROVAL**
All procedures performed in studies involving human participants were in accordance with the ethical
standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was assessed and approved by the Research Ethics Committee of the Faculty of Medicine of Sao Paulo University (protocol number: 12287213.2.3001.0065) and at the Institute of Infectious Diseases Emilio Ribas (protocol number: 12287213.2.0000.0061).

CONSENT FOR PUBLICATION
Not applicable.

AVAILABILITY OF DATA AND MATERIALS
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS
The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS
MAC, BJC, NK, MF, MAL, WLJ and JALL contributed to execution of laboratory tests. MAC, ASI, LMO, EASN, FFF contributed to subject recruitment. MAC and JALL analyzed the data. All authors were contributors in writing the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1
Distribution by age, sex, clinical, epidemiological and laboratory parameters of the 240 patients in the study

| Parameter                                      | Mean (range)        |
|------------------------------------------------|---------------------|
| Age (years)                                    | 46 (24-80)          |
| Time since HIV diagnosis (years)               | 13.3 (1 – 30)       |
| TCD4+ (cells/mm³)                              | 568.4 (2 – 1724)    |

| Parameter                                      | Frequency - n (%)   |
|------------------------------------------------|---------------------|
| Undetectable viral load                       | 195 (81.3)          |
| Male (%)                                       | 172 (71.6)          |
| On regular HAART (%)                          | 211 (87.9)          |
| Previous exposure to endemic area for VL      | 92 (38.3)           |

Table 2
Positivity of diagnostic tests for detection of *Leishmania* spp. infection in the 240 patients included in the study

| Test                        | Total n (%) | 95%CI    |
|-----------------------------|-------------|----------|
| ELISA Leptomonas            | 9 (3.8)     | 2.0 – 7.0|
| ELISA rK39                  | 3 (1.3)     | 0.4 – 3.6|
| ELISA rK28                  | 6 (2.5)     | 1.2 – 5.3|
| IFAT                        | 11 (4.6)    | 2.6 – 8.0|
| kDNA PCR                    | 4 (1.7)     | 0.6 – 4.2|
| ITS-1 PCR                   | 10 (4.2)    | 2.3 – 7.5|
| At least one positive test  | 36 (15)     | 10.7 – 20.2|

CI: confidence interval

Table 3
Positivity of ELISA *L. major*-like, ELISA *Leptomonas*, IFAT and ITS-1 PCR in relation to TCD4+ counts, categorized by values above or greater than/equal to 200 cells/mm³
|                      | Positive n (%) | Negative n (%) | p - value |
|----------------------|----------------|----------------|-----------|
| **ELISA Leptomonas** |                |                |           |
| TCD4 + <200          | 4 (14.8)       | 23 (85.2)      | 0.011**   |
| TCD4 + ≥200          | 5 (2.3)        | 208 (97.7)     |           |
| **IFAT**             |                |                | **      |
| TCD4 + <200          | 5 (18.5)       | 22 (81.5)      | 0.004 **  |
| TCD4 + ≥200          | 6 (2.8)        | 207 (97.2)     | **       |

* Chi-squared test applied; ** Fisher exact test applied.