Visceral Leishmaniasis in Hospitalized HIV-Infected Patients in Pernambuco, Brazil

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Abstract. Common in four continents, visceral leishmaniasis (VL) is an important but neglected disease. Human immunodeficiency virus (HIV) infection increases the risk of developing VL in people from leishmaniasis-endemic areas, with worse prognosis when there is coinfection. We conducted a cross-sectional study to determine the prevalence of HIV/VL coinfection in patients admitted in three referral hospitals for HIV/acquired immunodeficiency syndrome (AIDS) in Pernambuco, Brazil, and to compare epidemiological, clinical, and laboratory characteristics among HIV/VL coinfecting and HIV mono-infected individuals. The sample consisted of HIV patients aged 18 years or more, in a period of data collection of 6 months. We performed four *Leishmania* tests—polymerase chain reaction (PCR), direct agglutination test, rK39, and latex agglutination test—and individuals with at least one positive test were considered coinfecting. The HIV/VL coinfection prevalence we found was 16.9%. We observed large variation in prevalence according to the *Leishmania* test used, with low coincidence of positive tests. The most frequent symptoms found were weight loss (75.6%), fever (67.6%), and cough (55.3%). When we compared HIV/VL coinfecting and HIV mono-infected groups we did not observe statistically significant differences. Low educational level ($P = 0.004$) and pallor ($P = 0.009$) were more frequent in the coinfecting group. Serum albumin level was higher in coinfecting individuals ($P = 0.009$). It is important to follow-up these individuals to understand the dynamics of VL in people living with HIV. New tests are necessary, ideally differentiating active from latent infection. Testing for VL in people with HIV is important and should be considered as part of the initial investigation in these individuals.

INTRODUCTION

Visceral leishmaniasis (VL) is the clinical form of leishmaniasis more associated with HIV, and it is considered a public health problem.¹ Although it is widespread in 88 countries on four continents,² visceral leishmaniasis is one of the most neglected diseases.³ Six countries are responsible for 90% of all cases—Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan.⁴ It is known that in endemic areas, the risk of developing VL in people living with HIV rises from 100 to 2,320 times.² Because this coinfection has a potentially poor prognosis, it deserves special attention.^{2,4}

Infection with *Leishmania* spp. in people living with HIV produces pronounced activation of cells from the immunological system and higher production of pro-inflammatory cytokines, turning these cells into targets of the virus and these accelerate the progression to AIDS.^{5,6} In some cases, it was observed activation or re-activation of latent infections by *Leishmania* spp., which occurs in a more immunocompromised stage caused by HIV.^{7,8}

Visceral leishmaniasis may be diagnosed from classic symptoms such as fever, pallor, and hepatosplenomegaly, associated with a positive serological or parasitological test. However, this classic presentation is not always observed in people living with HIV, leading to a delay in diagnosis and often making it a challenge.^{9,10}

No one method currently available to diagnose VL in people with HIV has optimal sensibility and specificity associated with low cost and ease of use. Because of this, it is suggested

to combine different methods to better positivity ratios and make more precise diagnoses.¹¹

This research aims to investigate the prevalence of this coinfection in hospitalized HIV individuals in Pernambuco, Brazil, and to compare HIV/VL coinfecting with HIV mono-infected individuals regarding epidemiological, clinical, and laboratorial aspects.

MATERIALS AND METHODS

This was a cross-sectional study with comparison of groups. It was conducted in Recife, capital of the state of Pernambuco, Brazil, with inpatients from three referral public hospitals for infectious diseases—Hospital Correia Picanço, Hospital Universitário Oswaldo Cruz, and Hospital das Clínicas. These three hospitals together represent about 70% of all hospital admissions related to HIV/AIDS in Pernambuco.

There was no sample calculation. The sample consisted of individuals aged 18 years or older, diagnosed with HIV at any time, and who agreed to participate in the study. The researchers went to the hospitals once a week, during a pre-established period (June 2014–November 2014 at hospital #1; July 2014–December 2014 at hospital #2; and October 2014–January 2015 at hospital #3), to collect data. We included anyone with HIV who agreed to participate in the survey. There was no exclusion criterion. The data were obtained from questionnaires, physical examination, and blood and urine samples.

The definition of HIV/VL coinfection was the presence of at least one positive test for leishmaniasis. Thus, the patients were divided into two comparison groups: those with at least one positive test for VL (HIV/VL coinfecting group) and those with negative results for all tests for leishmaniasis (HIV mono-infected group).

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Data about gender, age, educational level, historical facts related to VL, and clinical signs were collected from questionnaires and from medical records. In addition, results from leukocyte count, hemoglobin, hematocrit, platelets, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, serum albumin, HIV viral load, and CD4 cells count were obtained from medical records.

We used four diagnostic tests for VL: two serological, rK39-immunochromatographic test (rK39-ICT) and direct agglutination test (DAT); one for detection of antigens in urine, using the latex agglutination test (KAtex); and one molecular test (PCR) in peripheral blood.

For the rK39-ICT, we used the DiaMed-IT LEISH kit (InBios International Inc., Seattle, WA). For DAT we used the DAT kit from Biomedical Research, Amsterdam, The Netherlands, and we considered a positive test when the result was equal to or greater than 1:6,400.¹² The search for antigens in urine was made using KAtex kit (Kalon Biological Ltd., Guildford, United Kingdom).

For the PCR from peripheral blood, DNA was extracted with the commercial kit QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). The process was performed according to the conditions described by Schönian et al.,¹³ targeting the internal transcript spacer 1, which produces a fragment with 300 pb–350 pb, depending on the *Leishmania* species, and primers LITSR (5'-CTGGATCATTTCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'). The polymorphism analysis of the restriction fragment was performed with the products from PCR (15 µL) digested with 1 µL of *Hae*III at 37°C for 90 minutes, according to recommendations from the supplier (Invitrogen, Carlsbad, CA). Fragments from restriction were submitted to electrophoresis on a 3% agarose gel at 100 V in 1× Tris-acetate-EDTA buffer and visualized and photographed by using a transilluminator after they were colored with ethidium bromide (0.5 µg/mL). The band patterns were compared according to those described by Schönian et al.¹³ for each species.

For patients with one or more positive tests, we offered a bone marrow aspirate for parasitological analysis to clarify whether the leishmanial infection was active. From this aspirate we also performed PCR.

Data were stored on a Microsoft Excel® 2013 spreadsheet (Microsoft Corp., Redmond, WA) in double entry, with subsequent verification of the quality of the information entered using Epi Info™ 3.5.4 software (Centers for Disease Control and Prevention, Atlanta, GA). For data analysis, we used Stata® SE 12.0 (StataCorp, College Station, TX) for Windows.

For the general characterization of the sample, simple frequencies and means of the variables of interest were obtained. To compare the categorical variables such as presence of fever, origin, and schooling, the χ^2 test was performed, with significance level $P < 0.05$, in a univariate analysis. For all continuous variables, such as age and CD4 cells count, we applied the Skewness/Kurtosis normality test. To compare those with normal distribution, we used the *t* test. To compare the others with not normal distribution, we used the Kruskal–Wallis test.

The ethics committee on research from the Aggeu Magalhães Research Center approved this study with the number 13197313.6.0000.5190. All subjects were adults and provided written, informed consent.

RESULTS

The sample was composed of 207 participants (Figure 1). None of the individuals refused to participate in the study after signing the consent form. Three patients did not want to undergo bone marrow aspirate but remained in the study. According to our criteria, which was to have at least one positive test for *Leishmania*, the prevalence of HIV/VL coinfection in our study was 16.9%. Table 1 shows the positivity for each *Leishmania* test we used. All participants that were not tested with Katex (22) and rK39 (3) were investigated with PCR for *Leishmania* test and had negative results.

FIGURE 1. Flowchart with the sample and the positivity for each *Leishmania* test used.

TABLE 1

Prevalence of HIV/VL coinfection according diagnostic test for *Leishmania* spp.

VL test	Positivity	Prevalence (%)
rK39-ICT	3/204	1.5
Direct agglutination test	23/204	11.3
Latex agglutination test	2/185	1.1
PCR in peripheral blood	8/207	3.9
Total (at least one positive test)*	35/207	16.9

rK39-ICT = rK39-immunochromatographic test; VL = visceral leishmaniasis.

Note: from 18 investigated with bone marrow aspirate, two were positive (11.1%).

* Only one participant had two positive tests.

More than half of the sample was composed of young males with low education. The coinfecting group had a higher frequency of individuals with less than eight years of schooling ($P = 0.004$). Among the participants, 1.45% reported cases of VL in the family and 1.93% reported cases of VL in the neighborhood (Table 2). The most frequent symptoms in the study population were weight loss (75.6%), fever (67.6%), and cough (55.3%). The Table 3 shows the results of the comparison between HIV/VL coinfecting and HIV mono-infected individuals regarding clinical and laboratory characteristics. In univariate analysis we found statistically significant difference between groups with regard to presence of pallor, more frequent in coinfecting individuals ($P = 0.009$), and serum albumin, higher in coinfecting individuals ($P = 0.009$).

There was no statistically significant difference between groups regarding aspects of HIV infection, such as CD4 cells count or HIV viral load (Table 4).

Bone marrow aspirate was performed in 18 of the 35 cases, with two positive results for *Leishmania*. One of them was positive by DAT and the other was positive by PCR in peripheral blood. Three participants refused the bone marrow aspirate. The other 14, given their clinical condition, were discharged before the bone marrow aspirate had been offered.

Three (8.6%) of the 35 coinfecting patients had all the classic signs and symptoms of VL: fever, loss of weight, hepatomegaly or splenomegaly, and some cytopenia. These three patients had anemia and thrombocytopenia, and two of them had leukopenia. From these three patients, one had 11 CD4 cells/mm³, 118,999 copies/mL of HIV-RNA, and positive DAT; another had 209 CD4 cells/mm³, 6,317 copies/mL

of HIV-RNA, and positive PCR for *Leishmania* in peripheral blood; and the third had 406 CD4 cells/mm³, 173,209 copies/mL of HIV-RNA, and positive rK39-ICT. Bone marrow aspirate was performed in two of these three patients with negative results for *Leishmania* in both.

From the 207 individuals in the sample, 40 (19.3%) died during the period of the study. The mortality was 14.3% in the HIV/VL coinfecting group, whereas in the HIV mono-infected group it was 20.3% ($P = 0.408$).

DISCUSSION

The prevalence we found in this study (16.9%) was similar to the 16% observed by Carranza-Tamayo et al.¹⁴ in the city of Brasília and approximated to the 20.2% found by Orsini et al.¹⁵ in Minas Gerais, Brazil. It is important to emphasize that despite the similarity in prevalence, there seem to be differences in the populations of each study. Our findings represent the prevalence of HIV/VL coinfection in patients hospitalized in referral services for infectious diseases in Pernambuco, which may imply the possibility of finding symptomatic and asymptomatic individuals for VL, whereas for Orsini, and probably also for Carranza-Tamayo, the study population consisted of asymptomatic individuals.

The prevalence found in our study and by two other Brazilian authors is comparable with that reported in the European Mediterranean region, which varies from 10% to 30% in asymptomatic individuals.¹⁶ In East Africa, however, prevalence ranged from 11.1% to 48.5% as shown in different studies in Ethiopia.¹⁷

Our study did not aim to evaluate the sensitivity and specificity of tests for VL. However, we observed a wide variation of positivity, depending on the diagnostic method used. Furthermore, the coincidence of positivity in the tests was low. Similar findings were reported by Carranza-Tamayo et al. and Orsini et al.^{14,15} We observed a considerable difference in positivity between the serological tests used in this study—DAT and rK39. This difference can be explained by the fact that DAT uses a crude antigen, which gives greater sensitivity to the test, whereas rK39 uses a recombinant antigen, with lower sensitivity and higher specificity, as suggested by Cota et al.¹¹

TABLE 2

Epidemiological characteristics of the sample of individuals seropositive for HIV investigated for VL in three referral hospitals of Pernambuco, Brazil, and results from the univariate analysis of the comparison between the HIV/VL coinfecting and HIV mono-infected groups

Variables	Total (N = 207)	HIV-VL (N = 35)	HIV (N = 172)	P value
Gender				
Male	140/207 (67.63%)	22 (62.86%)	118 (68.60%)	0.508
Female	67/207 (32.37%)	13 (37.14%)	54 (31.40%)	
Age				
18 to 39 years	115/207 (55.56%)	21 (60.00%)	94 (54.65%)	0.562
40 years or older	92/207 (44.44%)	14 (40.00%)	78 (45.35%)	
Mean (in years)*	39.66 ± 11.03	39.54 ± 11.27	39.68 ± 10.99	0.946
Educational level				
0–8 years	134/207 (64.73%)	30 (85.71%)	104 (60.47%)	0.004†
9 years or more	73/207 (35.27%)	5 (14.29%)	68 (39.53%)	
Prior leishmaniasis disease	2/207 (0.97%)	1 (2.86%)	1 (0.58%)	0.173
Leishmaniasis cases at home	3/207 (1.45%)	1 (2.86%)	2 (1.16%)	0.230
Leishmaniasis cases in the neighborhood	4/207 (1.93%)	1 (2.86%)	3 (1.74%)	0.365
Presence of domestic animals at home	97/207 (46.86%)	14 (40.00%)	83 (48.26%)	0.372

VL = Visceral leishmaniasis.

* Mean ± standard deviation.

† Statistically significant difference ($P < 0.05$).

TABLE 3

Clinical and laboratorial finds of HIV seropositive individuals investigated for VL in three referral hospitals in Pernambuco, Brazil, and results from the univariate analysis of the comparison between the HIV/VL coinfectd and HIV mono-infected groups

Variables	Total	HIV-VL	HIV	P value
Symptoms				
Fever	140/206 (67.63%)	24 (70.59%)	116 (67.44%)	0.719
Time of fever (in days)*	11.69 ± 11.44	11.47 ± 12.08	11.74 ± 11.37	0.948
Shortness of breath	101/206 (49.03%)	16 (47.06%)	85 (49.42%)	0.801
Bleeding	30/206 (14.56%)	6 (17.65%)	24 (13.95%)	0.577
Cough	114/206 (55.34)	18 (52.94%)	96 (55.81%)	0.758
Diarrhea	97/206 (47.09%)	16 (47.06%)	81 (47.09%)	0.997
Loss of weight	152/201 (75.62%)	24 (70.59%)	128 (76.65%)	0.453
Loss of weight (kg)*	8.11 ± 4.95	7 ± 5.67	8.33 ± 4.80	0.110
Hepatomegaly	25/139 (17.99%)	3 (11.54%)	22 (19.47%)	0.342
Splenomegaly	14/139 (10.07%)	1 (3.85%)	13 (11.50%)	0.242
Rarefaction of hair	49/189 (25.93%)	6 (20.00%)	43 (27.04%)	0.419
Pallor	84/187 (44.92%)	20 (66.67%)	64 (40.76%)	0.009†
Laboratorial results*				
Leukocytes	5,993 ± 4,321	5,720 ± 3,484	6,054 ± 4,494	0.956
Neutrophils	4,054 ± 3,344	3,762 ± 2,760	4,121 ± 3,468	0.607
Lymphocytes	1,049 ± 825	1,072 ± 747	1,044 ± 843	0.499
Hemoglobin	10.03 ± 2.32	10.51 ± 2.53	9.92 ± 2.26	0.214
Hematocrit	29.89 ± 6.78	31.57 ± 7.41	29.51 ± 6.59	0.114
Platelets (×10 ³)	235.591 ± 135.871	244.9 ± 139	233.4 ± 135	0.627
AST	68.39 ± 103.74	54.72 ± 54.84	71.28 ± 111.3	0.367
ALT	58.60 ± 88.90	46.96 ± 45.42	61.00 ± 95.38	0.359
Urea	35.68 ± 27.21	32.47 ± 18.09	36.36 ± 28.80	0.987
Creatinine	0.97 ± 1.05	0.87 ± 0.53	0.98 ± 1.12	0.618
Albumin	3.13 ± 0.88	3.57 ± 0.79	3.01 ± 0.87	0.009†

VL = Visceral leishmaniasis.

* Mean ± standard deviation.

† Statistically significant difference ($P < 0.05$).

In coinfectd HIV/VL individuals, molecular tests are described as having better accuracy than serological tests such as DAT and rK39-ICT.¹⁸ We believe that this difference between prevalence based on DAT (11.2%) and PCR (3.8%) occurred because the study was performed with individuals hospitalized for any reason, not only with clinical suspicions of active VL. Therefore, positive serological tests may mean active disease or latent infection. Differences in prevalence values according to diagnostic tests also were observed in other studies.^{14,15}

Mortality rate is high in HIV/VL individuals.^{16,17,19} The lethality found in our study in the coinfectd group was 14.3%, being between the 8.7% observed by Cota et al.²⁰ and the 23.5% by Nascimento et al.,²¹ and approaching the 16.8% described by Lima et al.⁹ We did not find a statistical difference

when we compared the HIV/VL coinfectd group with the HIV mono-infected group. However, it is important to emphasize that the HIV mono-infected group were composed of hospitalized people with diseases in different levels of severity. On the other hand, we do not know the proportion of individuals who had active VL disease among the coinfectd, which, in fact, could result in higher lethality in the group.

The largest part of the sample were males with a mean age of 39 years. There was no difference from other studies and it probably reflects the proportion of HIV cases between men and women in the general population.^{9,15,22,23} Gil-Prieto et al.,²⁴ in Spain, observed that 90% of the HIV/leishmaniasis coinfectd individuals were aged between 25 and 49 years, not so far from the 75% in our study in the same age group. This is an important aspect because that is an economically active age.

TABLE 4

Characteristics relative to the HIV infection in the sample of HIV seropositive individuals investigated for VL in three referral hospitals in Pernambuco, Brazil, and results from the univariate analysis of the comparison between the HIV/VL coinfectd and HIV mono-infected groups

Variables	Total	HIV-VL	HIV	P value
Time since HIV diagnosis (in months)*	60.34 ± 68.08	75.25 ± 87.49	57.45 ± 63.57	0.259
In use of antiretroviral (ARV)	151/207 (72.95%)	22 (62.86%)	129 (75.00%)	0.140
Time using ARV (in months)*	44.06 ± 66.75	54.44 ± 89.05	42.16 ± 62.10	0.609
Viral load				
Undetectable (< 50 copies)	24/169 (14.20%)	5 (16.67%)	19 (13.67%)	0.876
Up to 100,000 copies	85/169 (50.30%)	14 (46.67%)	71 (51.08%)	
More than 100,000 copies	60/169 (35.50%)	11 (36.67%)	49 (35.25%)	
CD4				
Up to 200	107/174 (61.49%)	16 (51.61%)	91 (63.64%)	0.308
200–500	46/174 (26.44%)	9 (29.03%)	37 (25.87%)	
500 or more	21/174 (12.07%)	6 (19.35%)	15 (10.49%)	
Mean CD4*	219 ± 267	255 ± 282	211 ± 263	0.819

VL = visceral leishmaniasis.

* Mean ± standard deviation.

Regarding educational level, we observed a similar distribution in a study from Tocantins, Brazil.²⁵ Low educational level, although a majority in both HIV/VL and HIV groups, was higher in positive cases of VL, maybe because this disease affects the poorest areas.

More than half of the study participants had weight loss, fever, and cough. When comparing the two study groups with respect to clinical findings, we observed no difference, except for pallor, which was more frequent in the coinfecting group. Pallor was the third most frequent symptom in coinfecting individuals after fever and weight loss. This result was similar to that described by Albuquerque et al.²⁵ in Tocantins, Brazil. In another study in Teresina, Brazil, pallor was the most frequent symptom in HIV individuals with active VL.⁹ Although in our study, pallor had been more frequent in coinfecting than in mono-infected individuals, we cannot say that this difference is due to active VL because this prevalence investigation does not differentiate active disease from latent infection.

There was a statistically significant difference in the serum albumin level between the two groups. In the mono-infected group, the mean value was just less than the lower limit of normality, whereas in the coinfecting group, the mean value was at the lower limit of normal. Although this result seems contradictory to what we expected, it is important to consider that hospitalized patients with AIDS in our region usually have advanced disease and comorbidities, which are responsible for nutritional impairment that may result in a fall in serum albumin levels. On the other hand, patients who tested positive for *Leishmania* in our study did not necessarily have active VL.

A potential limitation of this study is the possibility of false-negative results especially when serological tests are used because of the immunodepression of some participants, and the biases associated with the time between infection and the tests. To minimize this possibility, we used a combination of tests that included antigenic (KAtex) and molecular ones. Another potential limitation is the use of serological tests that may imply cross-reactions to malaria, Chagas' disease, and other infections. Considering that malaria is not endemic in Pernambuco, this possibility is minimal.²⁶ Pernambuco has endemic areas of Chagas restricted to a specific region.²⁷ Although they are distant from the study city, this should be taken into account in interpreting positive tests. To minimize the possibility of cross-reaction generating false positive results, we used a higher cutoff point for the DAT and a specific target for PCR. We also used another serological test, rK39, which, although has lower sensitivity, is more specific.

Another potential limitation of this study was the impossibility of differentiating active disease from latent infection. However, this was not really a limiting factor because the main objective of the study was to investigate the prevalence of VL, regardless of the status of the infection. To identify patients with active disease, we advised bone marrow aspirate for participants with at least one positive test.

Follow-up of these patients with positive tests for *Leishmania* could help understand the dynamics of VL in people living with HIV. In addition, new laboratory techniques or improvement of current techniques, especially molecular tests, are necessary for faster and more accurate diagnosis, preferably with discrimination of active disease from latent infection. Studies with biomarkers may prove useful in anticipating reactivation in coinfecting individuals.²⁸ Thus, severe infections and mortality would likely decrease with early

intervention, and the high cost and the discomfort of unnecessary treatments would be avoided.

The prevalence of coinfection was high. In the group of coinfecting, pallor and low schooling were more frequent than in the mono-infected group. As coinfecting individuals may reactivate a latent leishmanial infection, we suggest that testing for VL in people living with HIV should be considered as part of the initial investigation, especially for individuals from endemic areas. The care team should be attentive to the appearance of symptoms during the follow-up of coinfection for early antileishmaniasis therapy.

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