Am. J. Trop. Med. Hyg., 98(1), 2018, pp. 126–133
doi:10.4269/ajtmh.16-0883

Human Competence to Transmit *Leishmania infantum* to *Lutzomyia longipalpis* and the Influence of Human Immunodeficiency Virus Infection

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Abstract. Visceral leishmaniasis (VL) caused by *Leishmania infantum* is a lethal disease transmitted by sand flies. Although, considered a zoonosis with dogs held as the main reservoirs, humans are also sources of infection. Therefore, control policies currently focused on dog culling may need to consider that VL and human immunodeficiency virus (HIV)/VL patients may also be infectious, contributing to transmission. Reservoir competence of patients with VL without and with HIV infection and of persons asymptomatically infected with *Leishmania* was assessed by xenodiagnosis with the vector *Lutzomyia longipalpis*. Parasites in sand fly's guts were identified by using optical microscopy and by conventional polymerase chain reaction (PCR). *Leishmania infantum* blood parasite burden was determined by quantitative PCR. Among the 61 participants, 27 (44%) infected sand flies as seen by microscopy or PCR. When infectiousness was assessed by microscopy, xenodiagnosis was positive in five (25%) patients not infected with HIV, whereas nine (45%) of those harboring HIV were positive. Among the 19 asymptomatic patients four (21%) infected sand flies only demonstrated by PCR. One (50%) asymptomatic patient with HIV had a positive xenodiagnosis by microscopy. 9/372 (2.4%) and 37/398 (9.2%) sand flies were infected when feeding in patients without and with HIV, respectively. Infectiousness was poorly correlated with quantitative PCR. The study shows that asymptomatic humans are capable of transmitting *L. infantum*, that ill persons with HIV infection are more infectious to sand flies, and that humans are more important reservoirs than previously thought. This fact may be considered when designing control policies for zoonotic VL.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a parasitic disease caused by protozoa of the genus *Leishmania*. In the Indian subcontinent and in East Africa the disease is caused by *Leishmania donovani*, whereas in Central Asia and in the Middle East, as well as in the Mediterranean Region and Latin America, it is a zoonotic disease caused by *L. infantum*.[1] In Brazil, the main VL vector is *Lutzomyia longipalpis*, which is an opportunistic feeder; humans are an important blood source for this species in urban areas.[2] Dogs (*Canis familiaris*) are held as the main domestic reservoir for infections, but the true role of humans in the transmission of the disease is not clearly defined.[3–5] People with VL were able to infect *Lu. longipalpis*[3] and individuals coinfected by the human immunodeficiency virus (HIV) and *L. infantum* infected *Phlebotomus perniciosus*.[6] However, no comparative study on the infectiousness of individuals not infected versus those infected with HIV has ever been performed.

VL, once restricted to rural areas and small urban sites,[7] has expanded to larger cities over the past decades,[8–10] becoming an important public health issue, intensified by the geographic overlap with the acquired immune deficiency syndrome (AIDS) pandemic. HIV infection increases the risk of progression from asymptomatic infection to symptomatic VL, and inversely, VL accelerates the immunodeficiency progression caused by the HIV.[11] In Brazil, the proportion of patients with VL and HIV coinfection has varied from 7.6%[12] to 36%,[13] increasing the chances of complications and reducing the chances of survival.[14]

HIV and *Leishmania* coinfected patients, in spite of treatment, can develop chronic infections with continuous parasite multiplication for several years.[15] Although protease inhibitors used for HIV infection treatment can reduce in vitro growth of *Leishmania* sp.[16] HIV/VL patients seems to be long-lasting and important sources of *L. infantum*. Therefore, this study was developed to assess human competence as *L. infantum* reservoirs, comparing patients with and without HIV, symptomatic and nonsymptomatic individuals, to measure their potential impact on the protozoa transmission in endemic areas.[17]

POPULATION, MATERIAL, AND METHODS

Study population. Patients were recruited from: 1) Infectious diseases reference hospital, where patients diagnosed with VL were tested for HIV infection, and those reactive for HIV were tested for *Leishmania*. 2) The local Test and Advisory Center for HIV, where individuals reactive for anti-HIV were asked to perform anti-*Leishmania* serology tests (Figure 1) shows the algorithm followed to select the study population. Finding study participants depended basically on the availability of patients, asymptomatic *Leishmania*- and HIV-infected individuals, and on having sufficient numbers of sand flies.

Diagnosis of VL, asymptomatic *Leishmania*, and HIV infection. VL was diagnosed if fever, anemia, weight loss, and hepatosplenomegaly were present in a patient with laboratory evidence of VL. Laboratory evidence of VL was indicated by reactive serology or parasite demonstration by direct examination or culture. Asymptomatic infection was

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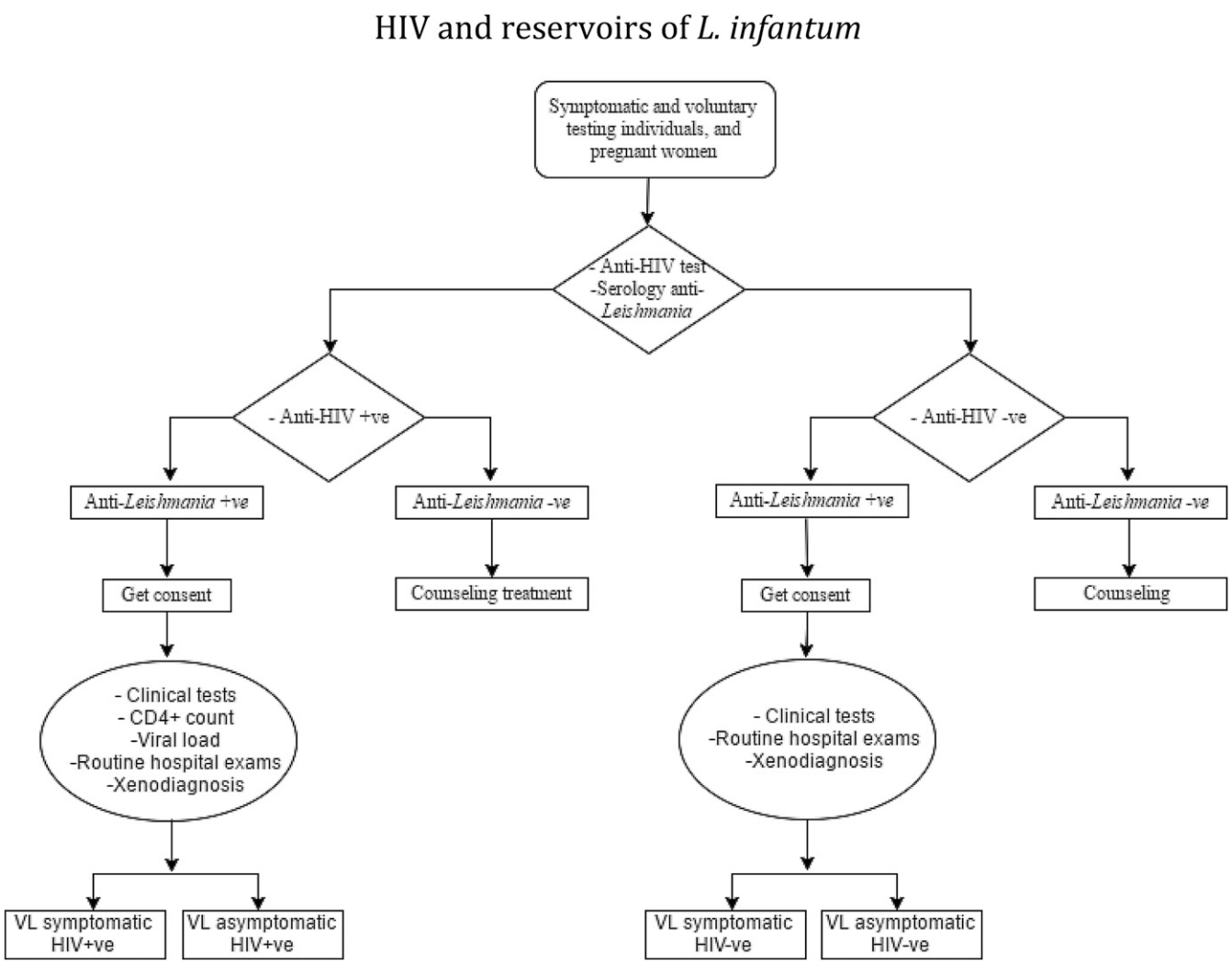

FIGURE 1. Algorithm used for selecting the study population. Patients with kala-azar, asymptomatic persons looking for diagnosis, and asymptomatic pregnant women were tested for human immunodeficiency virus (HIV). Those with and without (HIV) were separated between symptomatic and asymptomatic individuals.

defined by serological reactivity to *Leishmania* among individuals with no signs or symptoms of VL at the moment of the tests and had no prior records of the disease. People more than 18 months old were considered infected by HIV if they had at least two reactive anti-HIV tests and children under 18 months old if they presented detectable viral load.

Direct parasitological assessment of *Leishmania* infection. Smears recovered by bone marrow aspiration were stained by Giemsa and examined at 100× objective during at least 20 minutes before considered negative, as previously described.[18]

Culture for *Leishmania*. Bone marrows were cultured in dual-phase medium (Novy-MacNeil-Nicolle-NNN/Schneider's). The interpretation of the supernatant for promastigote identification was performed after 2, 3, 7, 14, 21, and 28 days after culturing. The cultures were taken as negative if after 28 days parasites were not identified.

***Leishmania* serology.** Indirect immunofluorescence assay for *Leishmania* (IFI-Leishmaniose Humana-Bio-Manguinhos, Rio de Janeiro, Brazil) and the immunochromatographic assay with rK39 antigen—Kalazar Detect (InBios International, Seattle, WA) were used for the VL diagnostic, in accordance to the manufacturer's recommendations.

HIV Serology. Serological diagnosis of HIV infection was performed according to the current national recommendations at the time data collection was performed. They consisted of a immunochromatographic rapid test (Rapid Check HIV 1 e 2, Fundação Apoio Hospitalar Universitário Cassiano Antonio Moraes, Espírito Santo, Brazil) followed by two confirmatory tests by indirect immunofluorescence (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) or by western blot available on the market.

Sand flies. The *Lu. longipalpis* specimens were cultured in a colony held at the Animal Sanity Laboratory at the Federal University of Piauí. Insects that originated the colony were captured in Teresina (05°05°20″S 42°48°07″W) and kept as the method described by Modi and Tesh (1983).[19] Insects originated from the field were fed on hamsters and first generation (F1) was kept in a separate environment, for xenodiagnosis.

Measurement of infectiousness. Approximately 40 F1 females, 4 days after hatching from pupal state were used for xenodiagnosis. Fed insects were maintained in a plastic cage covered with a net, at an air conditioned environment at ±26°C

and ±86% humidity, with 30% honey diet. All insects alive in the fifth or sixth day after blood meal were dissected and, immediately, their guts were examined at 40× objective. Dissected guts were stored in cryogenic tubes and frozen at –190°C for further conventional polymerase chain reaction (PCR).

DNA extraction. DNA extraction from the insects' guts and from peripheral blood of individuals followed the recommendations by the manufacturer QIAamp DNA Blood Mini Kit 250 (Sample and Assay Technologies; Qiagen, Chatsworth, CA). Concentration and purity were measured in a spectrophotometer (NanoDrop Thermo Scientific Inc., Wilmington, DE) in 260 and 280 nm wavelengths.

Primers. The primers used for conventional PCR were Lin R4 (5-GGG GTA GTT GGT AAA TAG GG-3′) and Lin 19 (5 CAG AAC GCC ACC CCT CG-3′)[20] that are specific for the *Leishmania*'s minicircle region and amplify a fragment of about 720 base pairs for *L. infantum* kDNA detection.

Conventional PCR. PCR conditions were denaturation at 94°C for 5 minutes and 94°C for 30 seconds, followed by 52°C for 30 seconds for annealing and 72°C for 30 seconds for extension, after a 35× cycle, 72°C for 10 minutes for final extension. Electrophoresis took place in 2% UltraPure™ Agarose° Gel (Invitrogen, Carlsbad, CA), died with GelRed™ (Biotium, Hayward, CA). Ultrapure water was used as negative control and *Leishmania* DNA from pure cultures as positive control, both for conventional and quantitative PCRs.

Quantitative PCR (qPCR). The amount of *Leishmania* kDNA in peripheral blood was measured according to the methodology described by Silva and others.[21] This kDNAemia measured as amastigote equivalents (AE) was interpreted as a representation of the total body parasite load.

Statistical analysis. Statistical analysis was performed using Stata/SE® 10.0 for Windows (College Station, TX). Means and 95% confidence intervals (CI) of the variables with normal distribution were calculated as well as the medians of the other distributions. Wilcoxon test was applied for kDNAemia comparison. Infectiousness was measured as 1) the proportion of individuals infecting at least one sand fly and 2) the proportion of infected insects fed on the studied groups. Spearman test was applied to verify the correlation between parasitic load and infectiousness. Logistic regression was applied to assess the interference of age, gender, symptoms, and HIV coinfection over parasitic load and infectiousness. Kappa test was applied to assess the matches between microscopy and PCR and χ^2 test was used to verify the association between dichotomic variables. The estimation of the probability of transmission to *Lu. longipalpis* was inferred by the Poisson distribution model according to the model described by Miller and others,[22] considering that *Lu. longipalpis* ingests a volume of 0.5 μL of blood.[23] The Poisson model was applied to infer the expected infectiousness from the observed kDNAemia and the expected kDNAemia from the observed infectiousness.

Ethical considerations. This study was approved by the Research Ethics Committee from the Federal University of Piauí (23111.011334/2010-93). All patients or their legal representatives signed informed consent form.

RESULTS

The 61 people who joined the study were split into four groups: 1) 20 patients diagnosed with VL without HIV; 2) 20 patients diagnosed with VL with HIV reactive serology; 3) 19 asymptomatic patients with reactive serology for *Leishmania* and nonreactive test for HIV; and 4) two asymptomatic patients with reactive serology for *Leishmania* and HIV. From all, 35 (57%) were males and the age varied from 1 to 65 years old, with a median of 31.

Sixty percent of the VL patients without HIV were males and were more than 18 years old. Only six (30%) were under 4 years of age. All patients with VL infected by HIV were adults and 85% were males. Most asymptomatic patients without HIV infection were females (79%) and were more than 16 years old (95%) ($P < 0.05$). Both asymptomatic patients with HIV were adult males (Table 1). $CD4^+$ lymphocyte count was available for 16 of the 20 coinfected patients and varied from 11 to 268 cells/μL with a mean of 117 cells/μL and median of 120 cells/μL (data not shown).

Xenodiagnosis. *Results per person.* From the 61 individuals 27 had positive xenodiagnosis by microscopy or by PCR; 15 by microscopy and 23 by PCR. Among the patients with VL and without HIV, xenodiagnosis was positive for seven by microscopy or PCR, microscopy only yield five positive results and PCR alone, seven.

Among the VL patients coinfected by the HIV, 15 were positive by one or the other methods, nine by microscopy only, and 12 by PCR only. From the 19 asymptomatic individuals, xenodiagnosis was positive for 4, only by PCR. From the two asymptomatic patients with HIV, one had positive xenodiagnosis by microscopy. Despite being asymptomatic and having normal physical examination at the first clinical evaluation, he was anemic and a few days after the test, a slight spleen enlargement was observed, a bone marrow smear evidenced the presence of parasites and a successful amphotericin B treatment was carried out (Table 2).

Results by insects. From the 2,385 exposed insects, 1,288 survived until the dissection day and 128 sand flies were found with *Leishmania*, evidenced by microscopy or by PCR. From the group of VL patients without HIV, 778 insects were allowed to feed and 372 survived and were dissected. Promastigotes or kDNA were found in 42 insects, from which nine by microscopy and 40 by PCR. From the group of VL/HIV coinfected patients, 767 insects were fed. Out of these, 398 survived and were dissected and 58 were observed and found to be positive, 37 by microscopy and 38 by PCR. Additionally, 760 insects were fed on asymptomatic individuals without HIV infection. From these, 495 survived and were dissected, resulting in 27 infected insects, all confirmed by PCR. At last, 80 insects were fed on 2 asymptomatic VL patients with HIV, from which 23 survived and were dissected resulting in one positive insect, confirmed by microscopy. However, since only one patient was infectious and only five insects fed on him

TABLE 1
Distribution of the study population by gender and age

Characteristic	VL symptomatic without HIV *n* (%)	VL symptomatic with HIV *n* (%)	VL asymptomatic without HIV *n* (%)	VL asymptomatic with HIV *n* (%)
Gender				
Male	12 (60)	17 (85)	4 (21)	2 (100)
Female	8 (40)	3 (15)	15 (79)	0 (0)
Age (years)				
<4	6 (30)	0 (0)	0 (0)	0 (0)
4 ≤ 18	2 (10)	0 (0)	1 (5)	0 (0)
>18	12 (60)	20 (100)	18 (95)	2 (100)

HIV = human immunodeficiency virus; VL = Visceral leishmaniasis.

TABLE 2

Distribution of infectiousness (xenodiagnosis) of humans to *Lutzomyia longipalpis* accordingly to the presence of signs and symptoms of visceral leishmaniasis and HIV-1 infection status

Study groups according to VL clinical status and HIV status	No. of individuals	No. of *Lu. longipalpis* allowed to feed in the group	Mean of fed *Lu. longipalpis* per person	No. of dissected *Lu. longipalpis* (%) (95% CI)	No. of infectious persons by microscopy or PCR (%) (95% CI)	No. of infectious persons by microscopy (%) (95% CI)	No. of infectious persons by PCR (%) (95% CI)	No. of infected *Lu. longipalpis* by microscopy or PCR (%) (95% CI)	No. of infected *Lu. longipalpis* by microscopy (%) (95% CI)	No. of infected *Lu. longipalpis* by PCR (%) (95% CI)
VL symptomatic without HIV	20	778	39	372 (47,8) (44–51)	7 (35) (15–59)	5 (25) (8–49)	7 (35) (15–59)	42 (11,2) (8–14)	9 (2.4) (1–4)	40 (10.7) (7–14)
VL symptomatic with HIV	20	767	38	398 (51.8) (48–55)	15 (75) (50–91)	9 (45) (23–68)	12 (60) (36–80)	58 (14,5) (11–18)	37 (9.2) (6–12)	38 (9.5) (6–13)
VL asymptomatic without HIV	19	760	40	495 (65.1) (61–68)	4 (21) (6–45)	None	4 (21) (6–45)	27 (5,4) (3–8)	None	27 (5,4) (3–8)
VL asymptomatic with HIV	2	80	40	23 (28.8) (19–39)	1 (50) (1–98)	1 (50) (1–98)	None	1 (4,3) (0–21)	1 (4.3) (0–21)	None
Total	61	2385	39	1288 (54) (51–56)	27 (44) (31–57)	15 (25) (14–37)	23 (38) (26–51)	128 (9,9) (8,3–11,7)	48 (3,7) (2,7–4,9)	105 (8,1) (6,7–9,7)

CI = confidence interval; HIV = human immunodeficiency virus; PCR = polymerase chain reaction; VL = Visceral leishmaniasis.

were dissected, the proportion of infectiousness was 20% (Table 2).

At the univariate analysis, VL patients coinfected by the HIV significantly infected more sand flies than those with VL but without HIV infection, when considering microscopic examination only ($P < 0.001$). However, when the PCR results were analyzed, no statistically significant difference was found. When comparing individuals without HIV, symptomatic and asymptomatic, those symptomatic were more infective by microscopy ($P < 0.001$) as well as by PCR ($P = 0.003$). At the multivariate logistic regression of infectiousness, after controlling gender, age and HIV infection status, and analyzing the results both by microscopy, infectiousness increases with HIV infection ($P = 0.04$) and with the presence of symptoms ($P = 0.01$). When considering both microscopy and PCR results, HIV infection and the presence of symptoms also increases infectiousness, but not when including only the PCR results, due to collinearity.

The correlations between xenodiagnosis results by microscopy and by PCR were weakly concordant between each other, both when results were plotted by infective people (kappa statistic = 0.32; $P = 0.003$) as well for infected insects (kappa = 0.27; $P < 0.001$). However, the association between the two methods was significant when the results were given both for people ($P = 0.008$) and for insects ($P = 0.001$) (Table 3).

Parasite load. The presence of kDNA in the blood varied from none to 11,143.9 AE/mL, with a mean of 746 AE/mL and median 70.9 AE/mL. The mean of VL patients without HIV was 268.2 AE/mL and the median 67.7 AE/mL. Patients with VL and HIV coinfection had a kDNA count much larger than those without HIV, with a mean of 1,876.1 AE/mL and median 682.2 AE/mL (P =0.002). Asymptomatic individuals without HIV infection had a mean of 41.5 AE/mL and median 3.8 AE/mL. Only the single asymptomatic individual with VL and HIV had a detectable kDNA count, of 1,866.7 AE/mL. The mean for both individuals was 933.3 AE/mL (Table 4). Symptomatic patients had a kDNA count much larger than those without symptoms ($P < 0.001$) (Figure 2). The multivariate regression model, which included age, gender, symptoms, and HIV infection status, evidenced that the presence of symptoms (odds ratio 37.4; $P = 0.003$) and HIV infection (odds ratio 6.5; $P = 0.04$) are the risk factors for high kDNA count.

There was a weak correlation between the amastigote equivalent count and the proportion of infected *Lu. longipalpis*, whether the insects were examined by microscopy (correlation coefficient $r = 0.47$), PCR ($r = 0.32$), or by both ($r = 0.44$) methods, although the association was statistically significant ($P < 0.001$) for all.

The Poisson model for infectiousness estimation from the results of kDNAemia showed the expected infectiousness was always higher than when observed by microscopy, and the reverse was, obviously, true. For the mean of 268 AE/mL in the group of patients with VL and no HIV, the expected estimate of infected insects was 13%, above the percentage observed when the insects were analyzed by microscopy (2.4%). In the group of symptomatic patients with HIV infection, for a mean of 1,879 AE/mL, a percentage of 62% infected insects were expected, whereas the percentage measured by microscopy was 9.2%. In the group of asymptomatic patients without HIV infection, the kDNA count was 41 AE/mL and the estimate of infected insects was 2%; however, no insects were infected when analyzed by microscopy. In

TABLE 3

Concordance between xenodiagnoses performed with *Lutzomyia longipalpis* in subjects with symptomatic and asymptomatic infection with *Leishmania infantum*, infected or not with HIV, according to the method of infectiousness assessment, by microscopy and by PCR

	PCR				
Microscopy	Negative (%)	Positive (%)	Total (%)	Kappa statistic	*P* value
Analysis per patient					
Negative (%)	33 (86)	13 (56)	46 (75)	0,32	0.03
Positive (%)	5 (14)	10 (44)	15 (15)		
Total (100%)	38 (100)	23 (100)	61 (100)		
Analysis per insect					
Negative (%)	1,158 (98)	80 (78)	1,238 (96)	0,27	< 0.001
Positive (%)	23 (2)	23 (22)	46 (4)		
Total (100%)	1,181 (100)	103 (100)	1,284 (100)		

HIV = human immunodeficiency virus; PCR = polymerase chain reaction.

the group of asymptomatic patients with HIV, the mean of parasites was 933 AE/mL and the estimate of infected insects was 38% but the observed proportion of infected sand flies was 4.3% (Table 5).

When estimating the expected kDNAemia from the results of infectiousness, the proportion of 2.4% infected sand flies in the group of symptomatic patients without HIV generated the expected mean of 48 (22; 92) AE/mL. Among the symptomatic patients coinfected by the HIV, for an observed infectiousness of 9.2%, a mean of 193 AE/mL (CI 136–267) was expected, whereas the kDNA count was 1,876 AE/mL. Since no positive insect was found among the asymptomatic HIV negative individuals, the expected kDNAemia was zero. In the group with asymptomatic patients co-infected by the HIV, the expected parasite load was 87 AE/mL (Table 5).

DISCUSSION

This is the first study to compare the infectiousness of humans with infected with *L. infantum* but infected or not with HIV. The study demonstrates that HIV-infected patients are more infectious to the sand fly *Lu. longipalpis*. The study also showed the infectiousness of asymptomatic patients, with and without HIV, revealing the potential of this large portion of the general population as reservoir of *L. infantum* in endemic areas. Besides, a comparison between microscopy and PCR to detect infection in sand flies was performed and revealed a poor correlation between both methods, raising questions about the meaning of PCR positive insects. The study also demonstrated that qPCR from blood is not a reliable proxy of infectiousness to sand flies.

The most infectious individuals were the symptomatic ones. As in previous studies, a significant proportion of infectious humans with VL are infectious. Here, one-quarter of the HIV-negative and symptomatic patients infected at least one sand fly, but only a small proportion of sand flies became infected. These results are about the same as those obtained in the past, also in Teresina. Both findings demonstrate the stability of xenodiagnosis over time when examined by microscopy in previously immunocompetent individuals. However, the results differ from those from Deane and Deane[7] and Sherlock.[24] Although the first study found a little higher proportion of infectious patients, both studies found more infected sand flies. These differences shall be attributed to the different study populations, comprised only by children in those studies, since children seem to be to be more infectious.[3] In reality, only six out of the present series of 20 non-HIV infected patients were children under 4 years old, and none was infectious. This small sample of children had a lower parasite load (data not shown), which could help explain their low infectiousness, albeit parasite load is not a refined predictor of infectiousness. However, difference across studies might be due to different sand fly susceptibility to infection due to several factors like insect's gut microbiota or sugar diet. Another possible factor could be different skin area infectiousness, where xenodiagnosis is applied.

In a report by Molina and others, in Spain, six of six people, coinfected with *L. infantum* and HIV, were infective to sand flies, and as many as 89% of flies that fed on patients became infected.[25] These numbers are much higher than those in the present study. One possible explanation is that the Spanish patients were not under the highly active antiretroviral therapy (HAART) and were at the end stage of AIDS, since their CD4 count was much lower than the patients here described. The presence of *L. infantum* infectious patients with HIV seems to be quite important due to prolonged disease in some of these patients. Indeed, a study by Bourgeois and others[15] showed a high proportion of patients with HIV and VL remaining chronically ill, with frequent relapses and persistently with viable circulating parasites. Incidentally, an infectious patient with post-kala-azar dermal leishmaniasis from Teresina was found by our group infectious to *Lu. longipalpis,* years after HAART and after several courses of prophylactic amphotericin B.

The results of infectiousness of asymptomatic individuals are puzzling. Although roughly one-fifth of the non-HIV infected patients were infective to at least one sand fly and a portion of sand flies were infected, none was detected by microscopy, but only by PCR. Several studies have shown that asymptomatic individuals infected with *L. infantum* may

TABLE 4

Parasite kDNA load estimated in peripheral blood in individuals with symptomatic and asymptomatic infection with *Leishmania infantum*, infected or not with HIV

Study group	Mean*	SD	Median*	IQ (25; 75)
VL symptomatic without HIV	268.2	465.9	68	10; 316.8
VL symptomatic with HIV	1876.1	2858.3	682.2	340; 2125.7
VL asymptomatic without HIV	41.5	136.7	3.8	2.3; 11.6
VL asymptomatic with HIV	933.3	1319.9	933.3	0; 1866.6

HIV = human immunodeficiency virus; IQ = interquartile interval; SD = standard deviation; VL = Visceral leishmaniasis.

* kDNA correspondence to the number of parasites/mL (amastigote-equivalents).

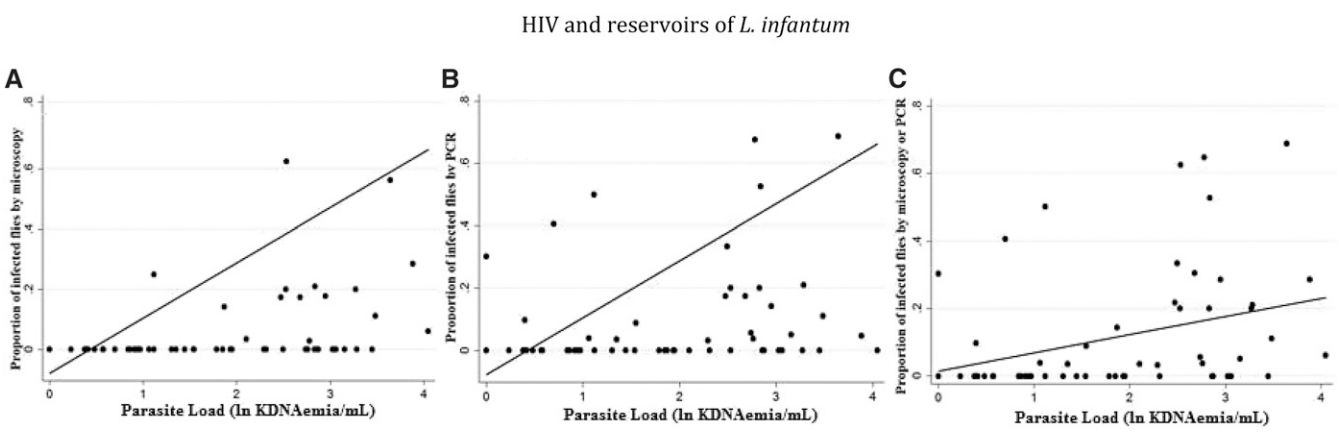

FIGURE 2. Correlation between the natural logarithm of parasite load (kDNA/mL, or amastigote-equivalents) as measured by quantitative polymerase chain reaction (PCR) from peripheral blood from each individual with visceral leishmaniasis or asymptomatically infected with *Leishmania infantum* and the infectiousness to *Lutzomyia longipalpis* as measured by the proportion of infected sand flies fed on that person. (**A**) by microscopy ($r = 0.47$; $P < 0.001$), (**B**) by PCR ($r = 0.32$; $P < 0.001$), and (**C**) by microscopy or PCR ($r = 0.44$; $P < 0.001$).

have circulating parasites, identified by culture or PCR,[4,26–29] indicating that the discovery of asymptomatic infectious persons is rather expected. However, the finding of infected sand flies only by PCR needs explanation. Although the direct observation of moving promastigotes in the insects' guts generates little doubt about the presence of *Leishmania* infection, the same cannot be stated for PCR.

After feeding from vertebrate hosts, sand flies synthesize a thick bag-like semipermeable structure that surrounds the blood meal, named peritrophic matrix. After 72 hours, *Lu. longipalpis* eliminates the enclosed meal.[30] Therefore, free *Leishmania* DNA taken from the host should not be present at the moment sand flies were dissected (120–144 hours after the meal), and the *Leishmania* DNA detected at this time should belong to viable living parasites. On the other hand, since identifying promastigotes with microscope is certainly dependent on the amount of parasites present in the midgut, a lower number of parasites may remain undetectable by microscopy, but not by PCR. This would explain why more people had positive results by PCR rather than by microscopy.

If these insects with low grade of infection would eventually transmit the infection to another host, however, does not sound likely. Schlein (1996) points out that the extrinsic incubation period in these low-grade infected sand flies would be longer, and therefore most of these insects would die before metacyclic promastigotes reach the insect's mouth parts to be transmitted during the next insect bites.[31] In opposition, however, since the peritrophic matrix is semipermeable, it cannot be ruled out that the parasite's DNA present in host blood could have crossed it in such a way that the detected DNA in *Lu. longipalpis* represents just DNA but not live promastigotes. Due to this last possibility, albeit unlikely, allied to the to the low concordance between microscopy and PCR, and also as a matter of prudence, only the microscopy results would ensure that a given insect has actually been infected while feeding. Hence, while the present findings strongly reinforce data supporting the existence of infectious asymptomatic humans with *L. infantum*, it still could not prove their existence.

In Teresina, the proportion of asymptomatically infected individuals yields a huge absolute number, but those symptomatic comprise just little proportion of them.[32] Therefore, those asymptomatic individuals may represent an important infection source of *L. infantum*, along with sick HIV-infected persons and children, and nonhuman reservoirs. Even though most of them harbor low parasite burden and might generate only low-infectious doses to sand flies, if only a small portion had higher parasite burden at the biting site and therefore gave higher infectious dose, these asymptomatic individuals may add greater value to *L. infantum* basic reproduction number and help parasite survival, as projected by Miller and others for *L. donovani* in Ethiopia.[22]

Direct xenodiagnosis is a poorly accepted method to assess infectiousness, but it is the only available. Besides, it depends on the maintenance of sand flies colonies, which have unpredictable yield. Additionally, examination under the

TABLE 5

Expected kDNAemia (amastigote-equivalents) and proportion of infected *Lutzomyia longipalpis* calculated by Poisson distribution* according to the presence of signs and symptoms of infection by *Leishmania infantum* and HIV infection

Study group	Observed mean kDNAemia (amastigote equivalents/mL)	Expected % of infected insects*	Observed % of infected insects by microscopy (CI)	Expected mean parasite load* (CI)
VL symptomatic without HIV	268	13	2.4 (1.1, 4.5)	48 (22, 92)
VL symptomatic with HIV	1876	62	9.2 (6.6, 12.5)	193 (136, 267)
VL asymptomatic without HIV	41	2	None	None
VL asymptomatic with HIV	933	38	4.3 (0, 21.9)	87 (0, 493)

CI = confidence interval; HIV = human immunodeficiency virus; VL = visceral leishmaniasis.

* Calculation of the expected parasite load was performed by using the Poisson distribution $P = -\frac{\ln(1-x)}{v}$, where P is the expected parasite kDNAemia, v is the assumed blood volume taken by the insect, considering that *Lu. longipalpis* takes 0.5 μL (Ready,[23] and x is the observed mean proportion of infected *Lutzomyia tzomyialongipalpis* in the group. The same equation was used to calculate the expected percent of infected insects. The underlying assumption are that at least one single amastigote would be able to determine infection to sand flies, that parasites are density independent, that sand flies are equally susceptible, and that once an infection is established it will progress (Miller and others).[22]

microscope of dozen or hundreds of sand flies is time consuming and may require several observers. These characteristics hamper larger epidemiological studies aiming to identify special groups of highly infectious individuals. Therefore, new technologies should be investigated for this. Conventional PCR for the identification of infected insects and qPCR for evaluating host infectiousness were both unsatisfactory. To our knowledge this is the first study to compare the infection in sand flies by using microscopy and PCR simultaneously, examining sand fly by sand fly. The results showed that the concordance between microscopy and conventional PCR as measure by Cohen's kappa statistic was poor, showing that the meaning of PCR for the identification of infected sand flies with *L. infantum* is still elusive. More detailed research is necessary before the method is able to trustfully substitute direct microscopy of dissected sand flies.

qPCR from blood of infected humans and animals has been used for parasite burden estimates.[33–37] These studies accept parasite burden as parasitemia, by assuming they measure the amount of living parasites in the blood. Following this thought, researchers dropped live parasites in peripheral blood in the laboratory, measured parasite burden by qPCR and then performed artificial xenodiagnosis in the blood.[38] This validated estimation was then applied to fieldwork to quantify parasitemia in humans and to evaluate the dynamics of transmission.[22] However, the present results do not support this idea since the correlation between qPCR and infectiousness among sand flies was not good, either by examining sand flies by microscopy, by PCR or by both. However, albeit not a reliable predictor of infectiousness, qPCR did apparently correctly estimate the total body parasite burden in the studied groups of patients, since the highest was found in those symptomatic with HIV, followed by asymptomatic with HIV, then by symptomatic without HIV, and finally those asymptomatic without HIV.

Recently, in Teresina, it was described a lack of correspondence between microscopy count of circulating amastigotes and the estimated amount by qDNA in the blood of patients with VL.[21] This finding led to conclusions that the parasite DNA present in the blood is derived from dead parasites rising from infected organs and that the minimal amount of circulating parasites could not justify the infectiousness to sand flies from the blood, but instead that infectiousness arises possibly from the skin. In fact, the mathematical model applied here to estimate the expected number of infected sand flies from qPCR found a meaningful higher estimate than the actual number of infected insects. Unfortunately, this research could not demonstrate the mostly needed substitute to xenodiagnosis, but, at least showed, for the first time, that conventional PCR, for sand flies, or qPCR, for hosts, are not the ideal ones.

Received November 8, 2016. Accepted for publication May 25, 2017.

Published online November 6, 2017.

Financial support: This study was funded by Brazil's Health Ministry, bid 779/2006, call 2/2006.

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