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Asymptomatic *Leishmania* infection in blood donors from a major blood bank in Northeastern Brazil: a cross-sectional study

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ABSTRACT

This study has estimated the risk of *Leishmania* transmission via blood transfusion in one of the largest blood banks in Northeastern Brazil, where visceral leishmaniasis is endemic. Five hundred blood samples from donors were tested for circulating *Leishmania* spp. DNA by real-time PCR. Positive samples were tested by a species-specific conventional PCR targeting *Leishmania infantum*. Overall, 6.2% (95% CI: 4.1–8.3%) of the samples carried *Leishmania* DNA and in one sample the species was confirmed as *L. infantum*. No statistically significant differences were found in relation to gender, sex, education level, incomeas well as the place of residence between positive and negative blood donors. Our results confirm the presence of asymptomatic *Leishmania* carriers among blood donors in a large blood bank in Northeastern Brazil. Considering the studied population, we estimate that for every 1,000 blood donors screened, 41 to 83 will be positive for *Leishmania* DNA. This finding reinforces the urgent need for elaborating specific Blood bank guidelines to allow the early detection of asymptomatic *Leishmania* carriers among blood donors before their blood products are transfused to uninfected individuals.

KEYWORDS: Blood donors. *Leishmania*. Asymptomatic infection. Blood bank. Surveillance.

INTRODUCTION

Visceral leishmaniasis (VL) is a severe neglected disease caused by protozoa belonging to the *Leishmania donovani* species complex, which affects approximately 200,000 to 400,000 people annually, with most of the cases being reported in Brazil, India, Sudan, South Sudan, Ethiopia and Bangladesh[1]. The clinical manifestations of VL are directly related to the patient's immune response and also to virulence factors of the infecting *Leishmania* species[2]. Hence, even though many infected people remain asymptomatic, the disease is often severe and potentially fatal[3,4]. Indeed, it is estimated that 20,000 to 40,000 people die from VL every year in endemic areas[1].

In the Mediterranean Basin, Middle East, Central Asia, South America and Central America, VL is a zoonosis caused by *Leishmania infantum*[5]. Although primarily transmitted by female phlebotomine sand flies[6], *L. infantum* may also be transmitted by other routes, including blood transfusion[7]. Cases of transfusion-transmitted leishmaniasis have been reported in several countries such as Belgium,

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France, Brazil, Sweden and the United Kingdom[7]. In spite of that, blood donors' screening for *L. infantum* infection is not routinely performed in several endemic countries[7,8], including Brazil[9]. Although the risk of *L. infantum* transmission via blood transfusion is eminent, it remains largely underestimated in most countries. A screening in blood banks of different Brazilian cities (Montes Claros, Fortaleza, and Teresina) reported that among 615 clinically eligible donors, seven (1.1%) were positive in the screening procedure, a rapid immunochromatographic test[10]. Considering the total of 608 eligible donors, 37 (6.1%) were positive to *Leshmania* spp. by a commercial ELISA. Furthermore, among 296 patients who were transfused with red blood cells or platelet concentrates from the above-mentioned donors, 23 (7.8%) were seropositive prior to blood transfusion and 13 (4.4%) received blood from positive donors. Six patients were followed-up and two of these six seroconverted between 60 and 90 days after blood transfusion[10]. This study illustrates the tip of the iceberg regarding *L. infantum* transmission by blood donors in Brazil.

Evidence accumulated in recent decades indicates that many individuals infected with *Leishmania* living in endemic areas may remain asymptomatic and become blood donors. Nonetheless, the risk of *Leishmania* transmission via blood transfusion remains largely underestimated. In this context, the aim of the present study was to estimate the risk of *Leishmania* transmission via blood transfusion in one of the largest blood banks in Northeastern Brazil, where VL is endemic.

METHODS

Study population

This cross-sectional study was carried out in the Fundacao de Hematologia e Hemoterapia de Pernambuco (Hemope), from August to September of 2017 (Figure 1). Before blood collection, blood donors were invited to participate in the study. After signing the informed consent, blood donors answered a questionnaire regarding age, schooling, income and place of residence. Routinely, all

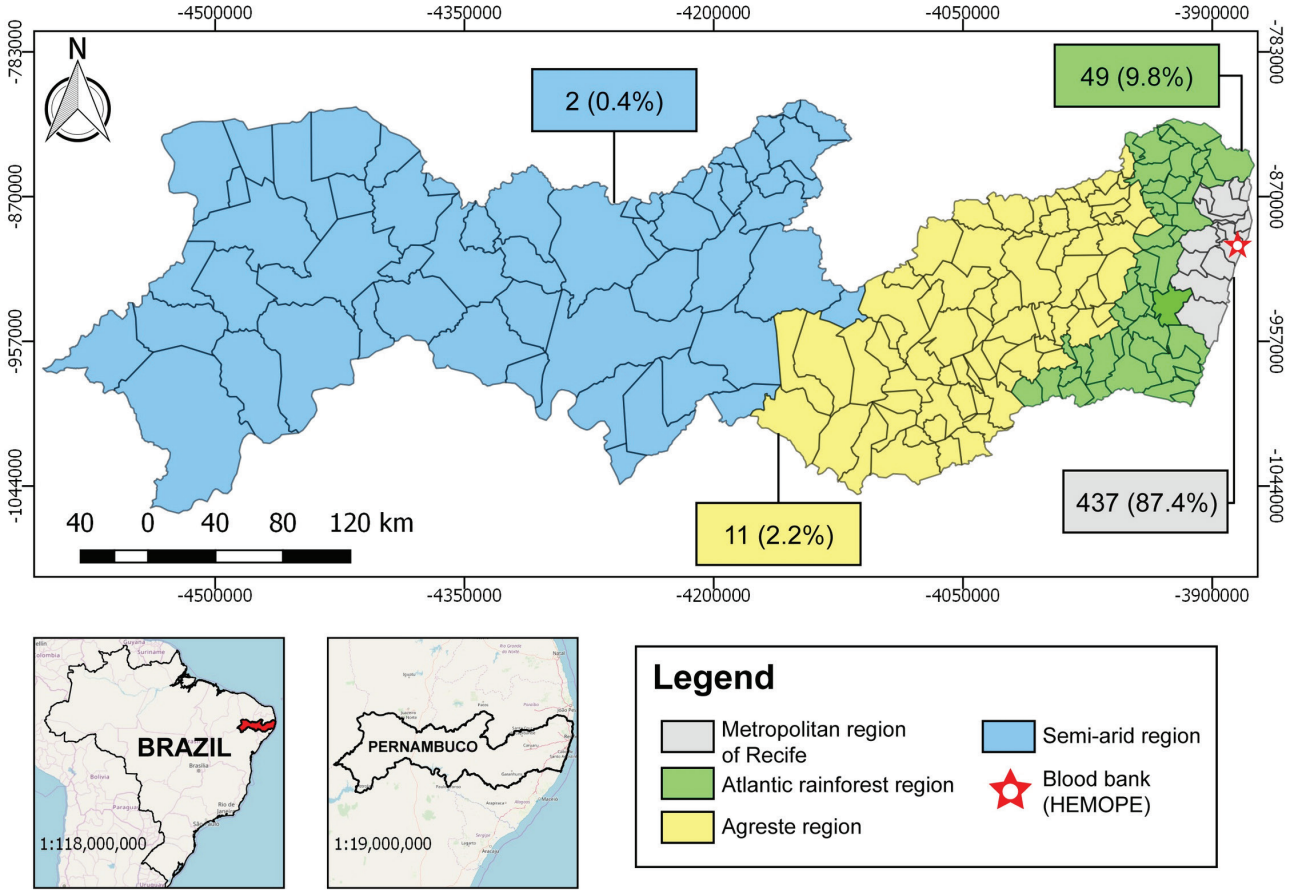

Figure 1 - Location of the Fundacao de Hematologia e Hemoterapia de Pernambuco (Hemope) and the geographic regions of Pernambuco State, from where blood donors came from. Numbers and percentages (in parentheses) refer to the number of positive patients and the positivity in each region. The maps were produced using the QGIS software version 2.18.28 (Free Software Foundation, Boston, Massachusetts, USA) and based on public geographical data obtained from OpenStreetMaps[30].

blood donors are subjected to a clinical screening (i.e., heart rate, blood pressure, temperature, weight, height, hemoglobin and hematocrit levels) and only those classified as clinically healthy (asymptomatic) are considered eligible for donation. In this blood bank, blood samples from donors are also routinely screened for syphilis, Chagas disease, hepatitis B and C, human immunodeficiency virus (HIV), human T-lymphotropic virus (HTLV) I and II, in addition to blood typing and Rh factor.

This study was approved by the ethics and research committees of the Aggeu Magalhaes Institute (Fiocruz-PE) (CAEE Nº 65770317.9.0000.5190) and the Hematology and Hemotherapy Foundation of Pernambuco (Hemope). All subjects were adults and voluntarily signed an informed consent before their enrolment in the study. All procedures were carried out in accordance with relevant guidelines and regulations.

Sampling

Venous blood samples (approximately 4 mL) were withdrawn from 500 blood donors and immediately placed into EDTA tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), which were previously labelled with a unique bar code for each donor. Then, 600 µL aliquots were transferred to a 1.5 mL microtube (Eppendorf, Hamburg, Germany) and frozen at –80 °C until further analysis.

Molecular diagnosis

Extraction of genomic DNA from blood samples and from cultured *Leishmania* promastigotes [reference strains: *L. infantum* (MHOM/BR/2016/DBA) and *Leishmania braziliensis* (MHOM/BR/2016/FAL)] were performed using the Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The quality of the extracted DNA was assessed using a NanoDrop-2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA).

DNA samples were screened for the presence of *Leishmania* spp. kinetoplast DNA by a real-time PCR (qPCR), using the primers LEISH-1 (5'-AACTTTTCTGGTCCTCCGG GTAG-3') and LEISH-2 (5'-ACCCCCAGTTTCCCGCC-3'), and the TaqMan-MGB probe (FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent quencher-MGB), as described elsewhere[11]. This assay detects different *Leishmania* spp., not only *L. infantum*[12]. The reaction mixture contained 7.5 µL of TaqMan Genotyping master mix (2X), 1.35 µL of each primer (final concentration of 900 nM each), 0.3 µL of probe (final concentration of 200 nM each), 2.5 µL of sterile water (DNase and RNase free) and 2.0 µL of genomic DNA, in a final volume of 15 µL. The thermal cycling conditions (QuantStudio 5 Real-Time PCR machine, Applied Biosystems, Foster City, CA, USA) were set as follow: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Samples were initially screened one by one and them positive samples were rested in duplicate. A standard curve with six serial dilutions (100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg per reaction) of genomic DNA from *L. infantum* was used as positive controls and also to estimate the parasite load in blood samples. DNA-free water was used as non-template control (NTC).

Positive samples by qPCR were further tested by a species-specific conventional PCR assay, using the primers FLC2 (5'-GTCAGTGTCGGAAACTAATCC GC-3') and RLC2 (5'-GGGAAATTGGCCTCCCTGAG-3'), which amplify a 230 bp fragment of *L. infantum* kDNA[13]. The final reaction volume was 25 µL, consisting of 1 X buffer, 0.2 mM dNTP (dATP, dCTP, dGTP, and dTTP), 1.5 mM $MgCl_2$, 1.5 U Taq Polymerase (Invitrogen,Thermo Scientific, Waltham, MA, USA), 0.4 pmol of each primer and 2 µL of genomic DNA. Genomic DNA from *L. infantum* and *L. braziliensis* were used as positive and negative controls, respectively. PCR assays were carried out in a LifeECO Thermal Clycler Bioer (Bioer Technology, Hangzhou, China), using the following thermal cycling conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were subjected to 1% agarose gel electrophoresis with 1 X tris-borate-EDTA buffer (pH 8.0) at 150 V and 400 mA for 1 h and 20 min. Gels were stained with ethidium bromide (0.5 µg/mL) and visualized under ultraviolet light.

Statistical analyses

The minimum sample size (n = 377) was calculated considering a 5% margin of error, 95% confidence level, unknown population size and expected positivity of 50%, using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). The positivity rate was calculated as the percentage (%) of positive individuals among all screened individuals. The 95% confidence interval was also calculated. The positivity rate was analyzed according to sociodemographic characteristics of the study population, using Chi-square (χ^2) or G-test (G). Differences were considered statistically significant when the P value was equal or less than 5. Statistical analyses were conducted using the BioEstat software, version 5.3 (Mamirauá Institute, Tefé, Amazonas, Brazil).

RESULTS

Among the total of 500 blood donors enrolled in this study, 42.6% (n = 213) were female and 57.4% (n = 287) male (Table 1). Regarding the skin color, 35.0% (175) were of mixed ethnicity, 30.4% (n = 152) white, 25.4% (n = 127) black, with other self-declared skin colors accounting for the remaining 9.2% (n = 46). Regarding age, 50.2% (n = 251) of the donors were 18-25 years old, 27.4% (n = 137) 26-33 years old, 12.2% (n = 61) 34–41 years old, 8.2% (n = 41) 42–50 years old and 2.0% (n = 10) 51–60 years old. The age groups 18–25 and 26–33 years old corresponded to 77.6% of the total. In respect to the education level, most donors had completed superior education (n = 479; 95.8%), with 4.2% (n = 21) of the donors presenting with only the elementary education.

Analyzing the place of residence, 437 (87.4%) resided in the metropolitan region of Recife, 49 (9.8%) in the Atlantic

Table 1 - Sociodemographic characteristics of blood donors in relation to their positivity to *Leishmania* DNA by real-time PCR, Recife, Brazil.

Variables	*Leishmania* DNA-negative *n* (%)	*Leishmania* DNA-positive *n* (%)	Statistics
Gender			
Female	199 (93.4)	14 (6.6)	$\chi^2 = 0.06$, $df = 1$, $P = 0.812$
Male	270 (94.1)	17 (6.0)	
Skin color			
White	142 (93.4)	10 (6.6)	$G = 2.41$, $df = 4$, $P = 0.661$
Black	117 (92.1)	10 (7.9)	
Yellow	44 (97.8)	1 (2.2)	
Mixed Ethnicity	165 (94.3)	10 (5.7)	
Indigenous	1 (100.0)	0 (0.0)	
Age (years)			
18-25	234 (93.2)	17 (6.8)	$G = 2.60$, $df = 4$, $P = 0.626$
26-33	128 (93.4)	9 (6.6)	
34-41	59 (96.7)	2 (3.3)	
42-50	38 (92.7)	3 (7.3)	
51-60	10 (100.0)	0 (0.0)	
Schooling			
1-5 years	5 (100.0)	0 (0.0)	$G = 6.49$, $df = 3$, $P = 0.090$
6-9 years	16 (100.0)	0 (0.0)	
High school	239 (95.6)	11 (4.4)	
Higher education	209 (91.3)	20 (8.7)	
Income*			
< 1 minimum wage	6 (100.0)	0 (0.0)	$G = 3.43$, $df = 4$, $P = 0.488$
1-2 minimum wages	251 (94.0)	16 (6.0)	
3-4 minimum wages	127 (91.4)	12 (8.6)	
5-6 minimum wages	48 (96.0)	2 (4.0)	
≥7 minimum wages	35 (97.2)	1 (2.8)	
Place of residence			
Metropolitan region of Recife	408 (93.4)	29 (6.6)	$G = 2.36$, $df = 4$, $P = 0.670$
Atlantic rainforest	47 (95.9)	2 (4.1)	
Agreste region	11 (100.0)	0 (0.0)	
Semi-arid region	2 (100.0)	0 (0.0)	
Other State	1 (100.0)	0 (0.0)	

*In the income variable, two participants did not respond. G-test = G; degrees of freedom = df

rainforest region, 11 (2.2%) in the agreste region, two (0.4%) in the semi-arid region, one (0.2%) in another state.

When asked about the water supply, 99.6% (498) of the donors reported access to potable water. Regarding the type of sewage system, 87.2% (n = 436) reported having access to basic sanitation, 5.2% (n = 26) had no access and 7.6% (n = 38) did not know.

Upon qPCR testing, 31 out of 500 (6.2%; 95% CI: 4.1-8.3%) blood samples were found to carry *Leishmania* DNA. Most positive donors were males and individuals with age ranging from 18 to 25 years (Table 1). Almost all *Leishmania*-positive donors were residents of the Metropolitan region of Recife (n = 29), with only two donors coming from the agreste region. The parasite load ranged from 2.2 to 201.0 parasites per mL of blood (average, 31.6 parasites/mL). Only two positive blood donors presented with more than 100 parasites per mL of blood (Figure 2).

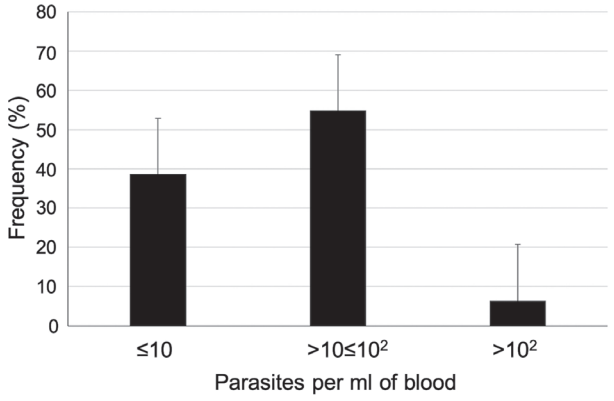

Figure 2 - Frequency of different parasite load ranges detected among the 31 positive blood donors for *Leishmania* spp. DNA detection by quantitative PCR.

When qPCR-positive samples were tested by the conventional PCR targeting *L. infantum* DNA, only one was positive.

DISCUSSION

In this study, we investigated the presence of *Leishmania* DNA in blood samples from donors coming spontaneously to a large blood bank in Northeastern Brazil. We found that 6.2% of the donors were carrying *Leishmania* DNA in their blood, and all of them were asymptomatic. Although the majority of positive blood donors resided in municipalities belonging to the Metropolitan region of Recife, the possibility that the infection had been acquired elsewhere cannot be ruled out.

Similar studies reporting the presence of anti-*Leishmania* antibodies or *Leishmania* DNA in asymptomatic blood donors have been carried out in Brazil[10,14-21]. Altogether, our results confirm that asymptomatic *Leishmania* infections are relatively frequent among blood donors living in endemic areas, since the screening of *Leishmania* DNA in blood banks in Brazil is still not routinely performed.

Different methods have been tested to prevent contamination of blood bags or even to destroy *Leishmania* parasites present in contaminated blood bags. For instance, filtration systems can remove several pathogens (e.g., cytomegalovirus, human T-lymphotropic virus, *Orientia tsutsugamushi* and *Trypanosoma cruzi*) from blood components[22]. Leukodepletion filters used at the time of blood collection or bedside filters used after storage are reputed to be the best strategies to minimize the risk of blood transfusion transmission of *Leishmania* parasites[7,22,23]. The only issue related to the use of leukodepletion filters is the high costs, which may limit their large-scale use, particularly in low-income countries.

It is acknowledged that diagnosing asymptomatic *Leishmania* infections is not an easy task, due to the low parasite load and low to moderate humoral response in healthy, infected individuals[8]. In fact, we found a low parasite load among positive blood donors (mean of 31.6 parasites per mL of blood), highlighting the need of highly sensitive assays (e.g., qPCR) for detecting *Leishmania* infections in healthy individuals, as it is the case of blood donors. As a corollary, only one blood donor, with a parasite load of 201,01 parasites/mL of blood, was positive when tested by conventional PCR, reinforcing the superiority of qPCR for detecting *Leishmania* DNA in asymptomatic carriers, especially when using blood samples, usually containing less parasite DNA as compared to other samples, such as bone marrow. Furthermore, the qPCR assay used herein is not specific for *L. infantum*, as it also detects *L. braziliensis*, another species endemic in Pernambuco[24]. The fact that only one qPCR-positive sample was positive in the *L. infantum*-specific conventional PCR could be related to the lower sensitivity of this assay or even to the presence of *L. braziliensis*. In fact, it is known that *L. braziliensis* DNA can be detected in blood samples of animals and humans[25,26]. The possibility that *L. braziliensis* could be transmitted via blood transfusion has been speculated[19], but remains unproven.

It has been shown that individuals with higher parasite load are more infectious to phlebotomine sand fly vectors[27]. Nonetheless, the fact that blood donors examined herein presented a low parasite load and were asymptomatic does not necessarily mean that they are not infectious. A recent study suggested that not only VL patients, but also asymptomatic infected individuals could serve as a source of infection to phlebotomine sand fly vectors[28].

It is acknowledged that VL is a disease of the poorest of the poor. The disease affects disproportionally poor people,

living in rural areas or in suburbs, where the exposure to the vectors as well as the existence of predisposing factors (e.g., malnutrition and HIV coinfection) is frequent. In this study, no statistically significant differences were found in relation to gender, skin color, education level, income and place of residence. The absence of statistical differences may be partly explained by the low number of infected individuals, but may also indicates the lack of a well-defined epidemiological profile for *Leishmania*-infected blood donors[29].

The determinant factors for progression from asymptomatic infection to symptomatic VL in humans are still poorly understood, but may be related to genetic background, nutritional status, underlying disease and/or coinfections. As such, we encourage public health authorities to elaborate specific guidelines for diagnosing and managing *Leishmania* infections in asymptomatic carriers in Brazil. These guidelines should also deal with blood donors diagnosed as infected during routine testing in blood banks, as well as blood recipients that received blood from infected donors. Finally, blood banks should exclude donors that are positive for *Leishmania*, as emphasized elsewhere[22].

In conclusion, we report herein the finding of asymptomatic *Leishmania* carriers in 6.2% of asymptomatic blood donors in a large blood bank in Northeastern Brazil. This finding reinforces the urgent need for elaborating specific blood bank guidelines to allow the early detection of asymptomatic *Leishmania* carriers who show up to donate blood before their blood products are transfused to uninfected individuals. This would ultimately benefit both, asymptomatic *Leishmania* carriers themselves, but especially blood recipients by reducing the risk of VL in these individuals.

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