

Survey of Bancroftian filariasis infection in humans and *Culex* mosquitoes in the western Brazilian Amazon region: implications for transmission and control

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ABSTRACT

Introduction: The aim of this work was to identify possible lymphatic filariasis foci in the western Brazilian Amazonian that could be established from the reports of Rachou in the 1950s. The study was conducted in three cities of the western Brazilian Amazon region - Porto Velho and Guajará-Mirim (State of Rondônia) and Humaitá (State of Amazonas). **Methods:** For human infection evaluation thick blood smear stained with Giemsa was used to analyze samples collected from 10pm to 1am. Polymerase chain reaction (PCR) was used to examine mosquito vectors for the presence of *Wuchereria bancrofti* DNA. Humans were randomly sampled from night schools students and from inhabitants in neighborhoods lacking sanitation. Mosquitoes were collected from residences only. **Results:** A total 2,709 night students enrolled in the Program for Education of Young Adults (EJA), and 935 people registered in the residences near the schools were examined, being 641 from Porto Velho, 214 from Guajará-Mirim and 80 from Humaitá. No individual examined was positive for the presence of microfilariae in the blood stream. A total of 7,860 female *Culex quinquefasciatus* specimens examined were negative by PCR. **Conclusions:** This survey including human and mosquito examinations indicates that the western Amazon region of Brazil is not a focus of Bancroftian filariasis infection or transmission. Therefore, there is no need to be included in the Brazilian lymphatic filariasis control program.

Keywords: Lymphatic filariasis. Bancroftian filariasis. Amazon Region. *Wuchereria bancrofti*. *Culex quinquefasciatus*.

INTRODUCTION

Lymphatic filariasis, caused by *Wuchereria bancrofti*, is considered a neglected disease that affects the lives of people in many endemic regions of Ásia, Latin America, and África. The parasite is transmitted by *Culex quinquefasciatus*, especially in socially and economically depressed communities with no sanitation systems or treated water.

Currently, more than 1.3 billion people are at risk of the disease in 72 endemic countries. Approximately 65% of those people live in southeastern Ásia and 30% in Africa, with the remainder in other tropical areas of the world¹⁻³. In the Americas there are active foci in Brazil, Guyana, Haiti and the

Dominican Republic, with 12.4 million people requiring mass drug administration. Haiti accounts for the highest proportion².

The only focus of lymphatic filariasis known in Brazil is the metropolitan area of Recife (State of Pernambuco)³. Cities such as Belém (State of Pará) and Manaus (State of Amazonas), in the Amazon region, and Maceió (State of Alagoas), in Northeast Brazil, have been described as endemic areas in the past. However, these foci have been extinguished and are presently kept under surveillance^{3,4}.

In 1953, a hemoscopic survey was performed in Porto Velho, State of Rondônia, Brazil, in which 1,400 individuals were examined (12% of the population) and 6 (0.4%) were positive for *W. bancrofti* microfilariae⁵. However, none of these individuals was autochthonous but were, rather, migrants from areas known to be endemic at the time, such as Belém (PA) and Manaus (AM), who had been living in Porto Velho for a shorter time than the parasite's prepatent period⁵.

In 1953, in State of Rondonia, another hemoscopic survey was performed in Guajará-Mirim. A total of 603 individuals were examined, and only one (0.2%) was positive for *W. bancrofti* microfilariae; however, this case was reported as not being autochthonous⁶. Simultaneously, a hemoscopic survey was

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performed in Humaitá (AM), but none of the 435 individuals examined was found infected⁶.

Porto Velho has never been an endemic area of lymphatic filariasis, unlike other Amazonian cities such as Manaus and Belém, which were foci of active filariasis transmission.

The existence of many unconfirmed reports of elephantiasis cases in Porto Velho, the lack of further epidemiological surveys after those performed by Rachou et al.⁵ in the 1950s, and the high density of *C. quinquefasciatus* prompted the present research, which was conducted to examine the actual occurrence of this endemic disease in that region.

METHODS

Survey of human infection/inclusion and exclusion criteria

Individuals under the age of five years old were excluded, as the hypothesis considered that a short time of exposure to infection would not justify the examination. Individuals who refused to participate in the survey were also excluded. All remaining individuals aged five years and over participated in the survey.

The criteria used to select the places to be evaluated included areas populated earlier in the studied cities, exhibiting urban poor characteristics with respect to their infrastructure, basic sanitation, open-air sewage and houses built on wood pilings, which met the conditions required for vector growth.

Study areas

Domicile survey in the Municipality of Porto Velho: The peripheral area and older neighborhoods of Porto Velho (8° 45' 42.57" S, 63° 54' 07.06" W) were the target areas of the study alongside the Madeira River, where there are slums and houses built on pilings. The neighborhood and residences of Porto Velho were randomly sampled. The sample size was calculated after mapping and a census of the neighborhoods by taking each dwelling as a sample unit, using an empirical prevalence of 1%, a design effect for cluster surveys of 1.8 and a confidence interval of 95% calculated by OpenEpi⁷. In this case, the sample size was estimated as a total of 557 randomly selected dwellings and 974 individuals older than five years old. To perform the study, the selected neighborhoods were divided into seven sectors, and 1/3 of the houses in each section were randomized and selected.

Domicile survey in the Municipality of Guajará-Mirim: All dwellings in the old neighborhood of Triângulo in Guajará-Mirim (10° 47' 27.33" S, 65° 19' 55.98" W) were selected because they shared the same poor urban infrastructure characteristics as the areas of Porto Velho. The population of Triângulo was estimated to comprise 506 individuals of all ages, and a total of 214 (31.7%) blood samples were collected. Individuals more than five years old living in the selected area participated in the survey. The sample was not randomized and was intended to comprise all the inhabitants and houses.

Domicile survey in the Municipality of Humaitá, State of Amazonas: All dwellings of the old neighborhood of Santo Antonio in Humaitá (7° 30' 22.20" S, 63° 01' 37.82" W) were

selected, with 80 houses featuring a similar infrastructure as those in the previously described areas and with a population of 240 inhabitants. The sample was not randomized and was intended to comprise all houses and inhabitants.

Figure 1 shows the areas of research for *W. bancrofti* in the western Amazonian region and the old and present foci of lymphatic filariasis in Brazil (**Figure 1**).

Survey of night students

Since evening students include mainly adolescents and young adults, the highest-risk group for bancroftosis⁸, and because of the parasite's nocturnal periodicity in the region⁹, the survey was also performed in public night schools in Porto Velho, Guajará-Mirim, and Humaitá. All 23 schools, which have a total of 3,601 students in the target area, were located within the oldest risk areas of the three cities involved in the Program for Education of Young Adults (EJA). These schools provide education to any student, youth and adult older than 18. All the students who wanted to participate were included in the sample.

Blood samples and diagnosis

The blood samples were collected from 10pm to 1am in the dwellings and from 10 to 11pm in the schools due to the nightly periodicity of the microfilariae in the peripheral blood stream of the human hosts⁹. The thick blood smear (TBS) method was used for the diagnosis according to the following protocol: blood collection was performed by finger prick using a disposable lancet and used for the preparation of TBS equivalent to 0.06-0.08mL of blood, which were dried at room temperature. Twelve hours after being dried, the TBSs were dehemoglobinized, fixed with methanol, stained with eosin at 0.05%, and then stained with Giemsa. The slides were examined under an optical microscope with an objective lens with magnifications ranging from 10 to 40X in a blinded manner by two independent examiners.

Survey of mosquito infection

Vector collection: The Culicidae captures were performed in Porto Velho, Guajará-Mirim and Humaitá in the neighborhoods of the humans who participated in this study. All the dwellings were visited and registered except locked dwellings and those whose owners refused to participate in the survey. The number of visited dwellings exceeded the originally sampled dwellings to increase the number of mosquitoes captured. An aspirator was used to collect adult mosquitoes (Castro aspirator). Ingurgitated insects were captured inside the houses from 7 to 10am. The insects were placed inside special fine mesh cages, separated according to the collection location and date, and then taken to the laboratory. The captured *C. quinquefasciatus* females were selected, placed inside test tubes (five mosquitoes/tube), and frozen at -20°C for further polymerase chain reaction (PCR).

Detection of W. bancrofti in mosquitoes by polymerase chain reaction (PCR): Briefly, genomic DNA was isolated from blood-engorged mosquitoes using the method described by Vasuki et al.¹⁰ with minor modifications. Dried mosquitoes (five per tube) were macerated with 100µL of TE buffer (10 mM Tris-HCl, 0.1mM EDTA, pH 8.0) and incubated at 100°C for 10min. After incubation, the DNA was isolated

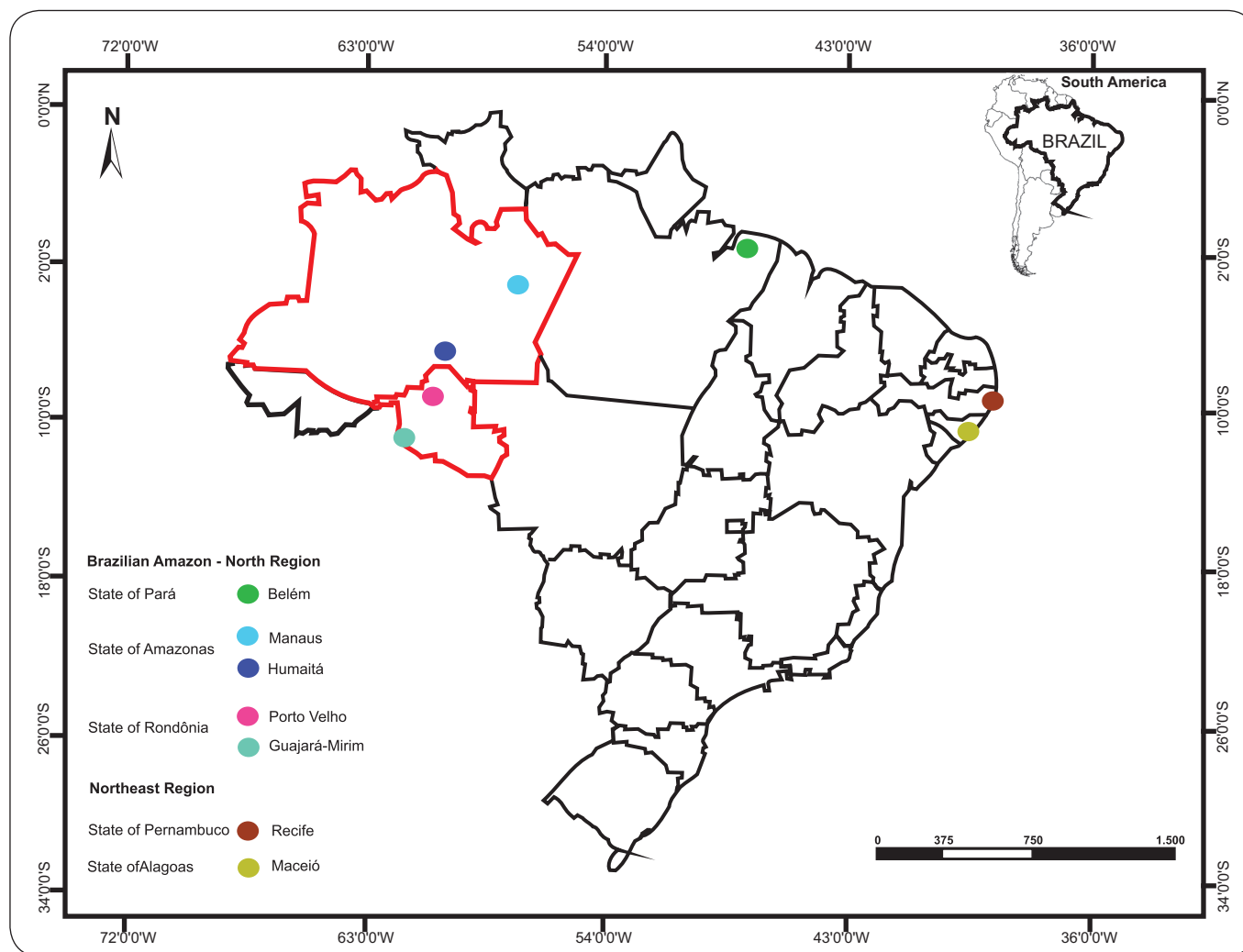


FIGURE 1 - Map showing the survey locations (Porto Velho, Guajará-Mirim and Humaitá), the old foci of lymphatic filariasis transmission where the disease has already been eliminated (Belém, Manaus and Maceió), and the only area where lymphatic filariasis transmission is still present (Recife and its metropolitan area).

using a Microcon YM-100 centrifugal filter device (millipore) according to the manufacturer's instructions, a step introduced in the original technique in order to concentrate the extracted DNA. The PCR was conducted using the primer pair NV1 (5'- CGT GAT GGC ATC AAA GTA GCG - '3) and NV2 (5'- CCCTCACTTACCATAAGACAA - '3) to detect the 188bp target repeat fragment (SspI) of *W. bancrofti* (SspI; GenBank accession n. L20344)¹¹. Conventional PCR were performed in a volume of 25µL containing 20pmol of each primer (NV1/NV2), 200 µM deoxyribonucleotide triphosphates (dNTPs), PCR buffer (50 mM KCl, 10mM Tris-HCl, pH 8.4), 1.5mM MgCl₂, ultrapure DNA-free water, 1.5U/µL Taq DNA polymerase and 4µL of template DNA. All reagents were from Invitrogen®. The PCR thermal cycling (PCR Express ThermoHybaid™) conditions were 35 cycles of 92°C for 1min, 55°C for 1min, and 72°C for 1min. The PCR amplicons were run on a 1.5% (m/v) agarose gel at 90V for 1h, stained with ethidium bromide (4mg/mL), and visualized under ultraviolet illumination (Vilber Lourmat™ TFX-20.M). The gels were photographed using a video documentation system (Vilber Lourmat™ DP-001 FDC)

and then analyzed (photoCaptMW for Windows 10.01 Vilber Lourmat™).

Ethical considerations

Every person examined signed a consent form and was well informed before being engaged in the survey, and the participation of the minors was authorized by their parents or guardians. Every person examined was able to sign his/her name. The project was approved by the Internal Review Board (IRB) of São Lucas College on December 11, 2007, and registered under the number 143/2007 and by the REC of São Paulo University (USP).

RESULTS

Human Infection

Domicile surveys in the Cities of Porto Velho, Guajará-Mirim and Humaitá: In Porto Velho, 493 houses were visited (88.5% of the total houses selected for inclusion), and 641

people were surveyed (65.8% of the individuals in the defined area). In Guajará-Mirim, 169 houses were visited (100% of the houses in the defined area), and 214 dwellers were examined (42.3% of the total individuals). In Humaitá, all 80 houses of the old neighborhood of the Santo Antonio Community were visited, and 80 individuals were surveyed (33.3% of the total individuals). In the three surveyed areas, all examined samples were negative for microfilariae (**Table 1**).

Night students survey: Out of 3,601 students, 2,709 were examined in the three research areas. In Porto Velho, the visits involved 11 schools in the previously defined area and included 1,684 (71.1%) students. In Guajará-Mirim, all five schools in the previously defined area were visited, and 272 (81.2%)

students were examined. In Humaitá, all seven night schools in the previously defined area were visited, and 753 (83.6%) students were examined (**Table 2**). All results were negative for microfilariae in the students blood. This study found no evidence of microfilarial infection in any of the individuals studied in all three areas (**Table 2**).

Survey of the infection of mosquito vectors: A total of 8,212 female mosquitoes were collected in the three target areas, with 95.8% (7,860) being *C. quinquefasciatus* and 4.2% belonging to other genera, including *Aedes*, *Psorophora* and *Anopheles*. PCR was performed to detect the DNA of *W. bancrofti* in all *C. quinquefasciatus* females, but none of the mosquitoes was positive (**Table 3**).

TABLE 1 - Results of thick blood smear examination for *Wuchereria bancrofti* infection carried out among the population of three cities in the Amazon region of Brazil - 2008/2009

Municipalities	Number of houses in the surveyed area	Visited houses		Number of dwellers in the surveyed area*	Examined dwellers**		Result positive exams
		n	%		n	%	
Porto Velho	557	493	88.5	974	641	65.8	0
Guajará-Mirim	169	169	100.0	506	214	42.3	0
Humaitá	80	80	100.0	240	80	33.3	0
Total	806	742	92.0	1,720	935	54.4	0

*All ages, including children under 5 year old; **People who accepted to participate in the survey over 5 years old.

TABLE 2 - Results of thick blood smear examinations for *Wuchereria bancrofti* infection carried out among students older than 18 years in schools, in three cities in the Amazon region of Brazil - 2009

Municipalities	Number of schools enrolled in the study	Total number of students	Students examined		Result positive exams
			n	%	
Porto Velho	11	2,368	1,684	71.1	0
Guajará-Mirim	5	333	272	81.7	0
Humaitá	7	900	753	83.7	0
Total	23	3,601	2,709	75.2	0

TABLE 3 - Results of the screening of *Culex quinquefasciatus* mosquitoes for *Wuchereria bancrofti* DNA examined by polymerase chain reaction (PCR), collected in three cities of Brazilian Amazon region - 2008/2009

Municipality	Number of dwellings*	Visited houses		Houses - mosquitoes collection		Total of female mosquitoes	<i>Culex</i>		Result positive PCR
		n	%	n	%		n	%	
Porto Velho	1,525	1,262	82.7	1,080	85.6	3,650	3,512	96.1	0
Guajará-Mirim	275	261	95.0	228	87.3	1,979	1,765	87.9	0
Humaitá	324	164	50.1	156	84.3	2,583	2,583	99.6	0
Total	2,124	1,687	79.4	1,464	86.8	8,212	7,860	95.8	0

PCR: polymerase chain reaction; *Some dwellings are not in the same area of the human survey, but in the neighborhood. In this case, the study area was amplified in order to increase mosquito capture.

DISCUSSION

Since the 1960s, no survey has been performed to determine whether the vicinities of Porto Velho, Guajará-Mirim and Humaitá were or could be silent areas of lymphatic filariasis transmission. The weather conditions of these areas in Amazonas

favor the transmission of *W. bancrofti*. In addition, because of its lack of basic sanitation, the known vector of the parasite was present, and autochthonous occurrences have been diagnosed in the area in the past. The aim of this study was to identify why filariasis did not develop in these areas, as found in the survey described above.

Using the TBS method, this work studied populations settled alongside the rivers in areas favorable for the development of this parasite as well as night students, whose environment conditions were similar to those reported in studies performed in extinct foci, such as Maceió and Belém³, and Recife metropolitan region, the only remaining area of filariasis in Brazil. No occurrence or evidence of the disease was found in the studied Cities of Rondônia and Amazonas.

In the present study, the diagnostic method was the TBS, and its main limitation is a reduced sensitivity to the identification of patients affected by mild microfilaremia¹². However, TBS is a rapid, practical and economic technique, commonly applied in large-scale field studies, and features good sensitivity when the parasitemia is greater than 10 microfilariae/mL of blood¹².

This study, as similar surveys performed in other states, endeavored to provide care to the populations that are undoubtedly subjected to higher social and environmental risk factors, that is, extremely poor areas with no basic sanitation systems and that are close to areas of higher vector proliferation. Notably, the investigated municipalities are relatively new, whereas the surveys were performed in contiguous, older areas of these cities. Poor basic sanitation conditions in habitational conglomerates are determining factors that could foster and maintain filariasis transmission. Thus, the populations exposed to higher social and environmental transmission risks were incorporated, and the likelihood of false-negative results for filariasis infection was reduced. In addition to the risk population, the present study included also participants of the Young Adult Students' program of the local State government involving 18 to 35 year old individuals enrolled in basic and mid-level schools. This categorization was chosen for operational reasons according to the studies performed previously in other areas of the country^{8,13}. As suggested by Rocha et al.¹⁴, the prevalence of bancroftosis may occur in young age brackets, which were emphasized in this survey. Furthermore, the surveyed schools were located near those places with a higher probability of the presence of the disease, which are the oldest settlements of the cities with poor urban infrastructure.

The assessment of filariasis transmission was performed by monitoring the infection in the insect vectors by PCR. The PCR has proven to be a powerful tool to evaluate the contamination intensity of the mosquitoes in endemic areas and, because of these advantages, has replaced the conventional dissection methods for the diagnosis of *W. bancrofti* in mosquitoes¹⁵. This method, in addition to being more sensitive than dissection, enables a large number of samples to be processed in a short period.

Other factors that may have contributed to the nonproliferation of the disease may be associated with the environment, the vector itself, and the cases diagnosed and treated in the 1960s.

Regarding the environment, filariasis is known to occur in tropical and subtropical hot and humid areas. Chandra et al.¹⁶ found that the density of *Culex* mosquitoes decrease during rainy periods, although periods of higher temperatures and humidity favor *W. bancrofti* transmission, shortening developmental period from L₁ to L₃ (infective larva) stages of the parasite in the vector.

The studied areas of Porto Velho, Guajará-Mirim and Humaitá exhibit the characteristics that facilitate rapid development of the parasite into its infectious larval form. However, during periods of increased heat and humidity, the number of vectors decreases, although there is no synchrony between the infecting larvae and the number of vectors, and the transmission risk decreases. One of the factors that may have contributed to the nonproliferation of the disease is the lack of synchronization between the number of vectors and the environmental conditions for the development of the agent that causes the disease¹⁷.

Several vector types are involved in filariasis transmission, including mosquitoes of the genera *Culex*, *Anopheles*, *Aedes* and *Mansonia*¹⁸. In Brazil, the only known vectors are the mosquitoes of the genus *Culex*, which are prevalent in the studied areas.

Studies with different filariae worms show that the vector efficiency depends on the susceptibility to pathogen infection and the microfilaria density¹⁹⁻²¹. Vectorial competence can be affected by the densities of microfilariae ingested during blood meal, and not all the blood fed mosquitoes became infected. Furthermore, when the insect is exposed to a high concentration of parasites an increased vectorial mortality rate can occur.

The non occurrence of the disease may also be associated with the blood feeding in vectors of low vectorial competence. Not all larvae ingested by the mosquitoes will survive inside the gastrointestinal tract and some of the larvae are expelled by the vector itself, whereas others are injured by the oropharyngeal tract during the repast²². As a result, the transmission is reduced²².

Those intrinsic and extrinsic factors and the complex interactions between filarial parasites and mosquitoes, that influence vectorial competence, could be in part responsible for geographic distribution of filariasis.

Accordingly, the discussion also involves findings from the studies performed by Hairston and De Meillon²³, which clearly showed that immature *W. bancrofti* larvae do not survive inside their hosts, thereby largely decreasing the load of contaminative filariae (L3) and increasing the number of bites required for infected mosquitoes to cause a parasitic infection. An estimated 15,500 infecting bites are required to cause microfilaremia²³. Snow and Michael²⁴ in 2002 and Chandra¹⁷ in 2008 also showed that the filariae present in the vector may increase the likeliness of dissemination but may also be fatal to the vector when their presence is too high by increasing the lethality, which contributes to the reduction in transmission.

Regarding the number of bites by mosquitoes infected with larvae in this survey, no individual was found to have been infected with *W. bancrofti* microfilariae by the TBS method, and no infected mosquito was found by PCR in the areas surveyed, proving the lack of foci formation in the studied areas, such as in the allochthonous cases of *W. bancrofti* reported in the past. In the studied areas, no focus formation was detected, even with the presence of the parasite vectors and appropriate environmental conditions. The factors that influence the probability of the vector being infected and transmitting the infection include

the microfilarial infection of the vertebrate hosts. The mosquito infection rate is proportional to the microfilariae density in the peripheral blood stream of the patients. Brito et al.²⁵ found that the infection rate of mosquitoes fed with the blood of individuals with mild *W. bancrofti* microfilarial infections (1-10 microfilariae/mL) was 0.07%, whereas the efficiency of the vector was nearly 17%. That is, for every six microfilariae ingested, only one reaches the infecting larva stage.

Several studies have demonstrated that the persistence of this endemic disease depends on the occurrence of high natural infection rates of the mosquitoes. Nevertheless, there has been no consensus among researchers that can forecast the occurrence of a new case of patent microfilarial infection in humans²⁶. However, below a certain critical number of infecting bites, lymphatic filariasis has not been confirmed to be an endemic disease, which may be the case in this area. In places where the microfilariae density is maintained below five microfilariae/60mL blood, there is considered to be no transmission risk²⁷.

In a recent study performed in Maceió it was showed that the filarial load in a patient is one of the determining factors for maintaining transmission foci²⁸. Such a load may have been one of the factors that determined the disease dissemination in the studied areas. In Maceió, Leite et al.²⁸ found that an individual suffering from *W. bancrofti* microfilarial infection who came from an endemic area and had been living for more than 10 years in a nonendemic area, was not capable of inducing the formation of a new bancroftosis focus, even though the new area exhibited the appropriate environmental conditions. One of the possibilities considered by the researchers was the mild microfilarial infection of the infected individual (4 microfilariae/mL of blood)²⁸.

However, with the growing migration in the country, there is a risk that the disease might be introduced in areas free of the infection³. In Sri Lanka infected migrants have engendered lymphatic filariasis in areas where the disease was previously unknown²⁹. This occurred also in metropolitan Recife, where cases of the parasitosis appeared in previously unaffected areas^{30,31}.

The magnitude of the potential risk for the establishment of sustainable foci of lymphatic filariasis depends mainly on the number of *W. bancrofti* carriers, the microfilarial density in these individuals, the existence of potential vectors, and environmental conditions that favor transmission³²⁻³⁴.

Finally, the few people suffering from *W. bancrofti* microfilarial infections found in the Cities of Porto Velho and Guarajá-Mirim in the 1950s had allochthonous, mild microfilarial infections (out of the seven people infected with parasites, five had fewer than five microfilariae per slide examined)^{5,35}. It appears that the small number of people infected with the parasites and the mild nature of the microfilarial infection reported in the 1950s in Porto Velho and Guarajá-Mirim were not sufficient to establish and maintain the lymphatic filariasis in the examined areas, which were considered harmless and may or may not be associated with environmental and other factors related to the vectors.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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