Major Article

Presence of *Mycobacterium leprae* genotype 4 in environmental waters in Northeast Brazil

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Abstract

Introduction: This study quantified *Mycobacterium leprae* bacilli in environmental water samples from five municipalities in the State of Ceará by quantitative polymerase chain reaction (qPCR) and compared the identified genotypes with those obtained from leprosy patient biopsies. **Methods:** We collected five replicas from each of the 30 selected reservoirs and skin lesion biopsies from 25 new leprosy cases treated at a reference center in Fortaleza, Ceará from 2010 to 2013. The 16S rRNA gene region of *M. leprae* was amplified by qPCR and a standard curve was created with the pIDTBlue 16SrRNAMlep plasmid. The Juazeiro do Norte water samples and the biopsies were genotyped (single nucleotide polymorphism [SNP] 1 to 4) and the SNP 4 genotypes were subtyped. **Results:** Of the 149 water samples analyzed, 54.4% were positive for the *M. leprae* DNA. The *M. leprae* bacilli copy number ranged from 1.42×10^{-1} to $1.44 \times 10^{+2}$. Most biopsies showed SNP type 4 (64%), while all samples from Juazeiro do Norte were SNP type 4, with subtype 4-N appearing at the highest frequency. **Conclusions:** We suggest that environmental waters containing *M. leprae* bacilli play an important role in disease transmission, justifying PGL-1 seropositivity in individuals living in areas where there is no reported case, and in leprosy cases individuals who report no previous contact with other case. Therefore, further investigation is needed to clarify disease transmission in this region and to explore the role of the environment. We also suggest that in this area surveillance for leprosy cases should be intensified.

Keywords: *Mycobacterium leprae*. Leprosy. Real-time polymerase chain reaction. Water. Biopsy.

INTRODUCTION

Leprosy is a chronic granulomatous infectious disease that primarily affects the skin and peripheral nerves, leading to nerve function impairments. The causative agent of leprosy, *Mycobacterium leprae*, is an obligatory intracellular bacterium, not cultivable in axenic media. Comparative analysis of the genomes of *M. leprae* strains has demonstrated that the disease originated in East Africa with subsequent migration to occidental Africa and the Americas[1].

In 2014, 213,899 new cases of leprosy were detected worldwide, distributed in 121 countries[2]; in that same year, 31,064 cases, 14.5% of the world's total, were detected in Brazil. A small number of countries account for approximately 90% of all leprosy cases detected worldwide, including India, Brazil, Indonesia, Ethiopia, Bangladesh, The Democratic Republic of the Congo, and Nepal[3]. In the State of Ceará, located in Northeastern Brazil, leprosy is considered endemic, with a reported new leprosy case detection rate (CDR) of 23.7/100,000 inhabitants in 2014. In the same year, the occurrence of the disease in children under 15 years in Ceará was 6.1/100,000 inhabitants, demonstrating the existence of active disease transmission foci[4]. In 2015, the state reported new leprosy cases in 148 (80.5%) municipalities; 18 of these had a CDR > 40.0 and were considered hyperendemic.

The main means of leprosy transmission occurs through prolonged and continuous person-to-person contact. The existence of clinical disease cases in which patients did not report being in contact with other patients, suggests human infection from environmental sources[5,6]. A study conducted by Desikan and Sreevatsa[7] demonstrated the viability of *M. leprae* outside the human body under several unfavorable environmental conditions. In addition, the presence of *M. leprae* in water[8], soil from villages where the disease is endemic[9],

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and in the soil from the peridomicile of patients have also been reported[10]. Similarly, the viability of the bacillus within protozoan *Acanthamoeba* spp. has also been demonstrated[11]. The environment has been proposed as a reservoir allowing the bacillus to remain infectious following long periods outside the human body and consequent continual contamination and transmission of the disease[6].

In this study, we detected and quantified the presence of *M. leprae* deoxyribonucleic acid (DNA) in environmental water samples obtained from five municipalities in the State of Ceará by quantitative polymearase chain reaction (qPCR). In addition, we compared the environmental water genotypes with those of clinical samples from leprosy cases. Bacilli shed by the untreated leprosy cases in environmental waters, if remains viable then it could possibly have implications in transmission of the disease contributing to its prevalence. Therefore, surveillance implementation of these waters harboring the *M. leprae*, lead to prevent potential new cases and implementation of early detection. In this way, implementation of public health programs with new intervention strategies to prevent leprosy cases are urgent needed.

METHODS

Study setting

This study was conducted from November 2010 to December 2013. The municipalities were selected according to the epidemiological characteristics of leprosy (hyper, high, medium, and low endemicity), as well as geological and climatic conditions. The studied water sources are located in the following municipalities of the State of Ceará: Boa Viagem, Crato, Juazeiro do Norte, Mulungu, and Sobral, with average temperatures ranging from 24 to 29°C. These municipalities are located 217, 567, 491, 117, and 240km, respectively, from the state capital. The municipalities of Juazeiro do Norte, Sobral, and Crato were considered hyperendemic in 2012, with CDRs of 114, 91 and 53, respectively[12]. In contrast, the City of Boa Viagem reported only nine new cases in the same year, while no cases were reported in Mulungu[12].

Water samples were collected from natural sources, such as ponds, rivers, streams, recreational habitats, and wells. The sampling points were designated by the local health agents and were chosen based on their use by the local population for recreational and/or household purposes (drinking, bathing, washing dishes and clothing, and animals, including livestock, domestic and wild animals). Samples were collected from five sites in Juazeiro do Norte, eight sites in Sobral, four sites in Crato, seven sites in Boa Viagem, and six sites in Mulungu.

Collection of water samples

At each site, five replicates were collected at different depths, designated by the letters *A* to *E*. The collected samples were identified by the initial of the municipality [Boa Viagem (B); Crato (C); Juazeiro do Norte (J); Mulungu (M) and Sobral (S)] in numerical order (B1 to B7; C1 to C4; J1 to J5; M1 to M6; and S1 to S8) and the replicates were assigned in alphabetical order (B1a, B1b, B1c, B1d, B1e, B2a ... B7e). The *A* sample was

collected from the surface of the reservoir, while the *B*, *C*, and *D* samples were from an intermediate depth and the *E* sample was from the bottom of the reservoir. During transport of samples to the laboratory in Fortaleza, a sample from Boa Viagem (B7d) was damaged. All water samples were conditioned in sterile amber bottles with a 1,000-mL capacity, totaling 149 environmental water samples. The samples were transported within 24 hours of sampling in styrofoam boxes containing ice cubes to the mycobacteria laboratory of the Federal University of Ceará, in Fortaleza, Ceará State.

Biopsy samples

Biopsies were collected from 25 new leprosy cases prior to commencing treatment; punches 6 mm in diameter were removed from the edge of the skin lesions. The procedure was conducted at Dona Libânia National Reference Centre for Sanitary Dermatology (CDERM) in the City of Fortaleza, Ceará. The collected material was transferred into sterile 1.5-mL microtubes and maintained at -20°C until DNA extraction using the DNeasy Blood & Tissue kit (Qiagen Sciences Inc, Germantown, USA).

Quantitative polymerase chain reaction

Deoxyribonucleic acid extraction from water samples was performed using the PowerWater DNA Isolation kit (Mo Bio Laboratories, Carlsbad, USA) according to the manufacturer's instructions. The obtained DNA was stored at -20°C. qPCR reactions were conducted using an ESCO 96 Spectrum Real Time Thermal Cycler (Esco Micro Pte Ltd, Changi, Singapore) with primers 16S3-Forward: 5′ - TCG AAC GGA AAG GTC TCT AAA - 3′ and 16S3-Reverse: 5′ - CTT TCC ACC ACA AGA CAT GC - 3′ that amplify a region of the ribosomal RNA gene specific to *M. leprae*.

Absolute quantification by qPCR was conducted using the SYBR Green PCR kit Power Master Mix (Applied Biosystems Inc, Foster City, USA); each individual reaction consisted of 12.5µL Master Mix, 1µL of each primer, 5.5µL ultra-pure water, and 5µL of DNA from the environmental sample (total volume of 25µL). The reaction conditions were as follows: initial denaturation at 95°C for 10 minutes; followed by 45 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds; followed by a final extension at 72°C for 10 minutes. *M. leprae* copy numbers were calculated by absolute quantification using a standard curve for each run. To evaluate the Cycle Threshold (C_t), the obtained data were analyzed using the Swift Spectrum Real Time Software 96 (Esco Micro Pte Ltd, Changi, Singapore).

The standard curve was generated using a ten-fold dilution series (ranging from 10 to 10^9 copies/reaction) of plasmid pIDTBlue16SrRNAMleprae (Integrated DNA Technologies, Inc., San Diego, USA), which contains a 171-bp fragment of the *M. leprae* 16S ribosomal ribonucleic acid (rRNA) gene[10]. Other mycobacteria species and non-mycobacterial (*Mycobacterium tuberculosis* H37Rv, *Mycobacterium* sp., *Streptococcus pneumoniae* ATCC 49619, *Staphylococcus aureus, and Escherichia coli*) were also included as controls. These strains were obtained from the bacterial culture collection

of the Mycobacterial Laboratory of the Federal University of Ceará, Fortaleza, Ceará and the DNA samples were adjusted to 10µg/mL prior to being used for qPCR.

Genotyping of *Mycobacterium leprae* isolates

qPCR-positive samples were subjected to single-nucleotide polymorphism identification (SNPs 1 to 4), using two pairs of primers targeting nucleotides at positions 14676 and 2935685 of the *M. leprae* genome[1,13]. The Taq PCR Master Mix kit (Qiagen Sciences Inc, Germantown, USA) was used for amplification with the following conditions: 94°C for 3 minutes; followed by 45 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes; with a final extension at 72°C for 10 minutes[14]. Following amplification of the 2935685 genomic fragments, samples were subjected to enzymatic digestion with 10U/µL of *Bst*UI (New England Biolabs, Ipswich, USA) at 60°C for 1 hour. While the PCR products of the 14676 region were digested with 10U/µL of *Sml*I at 55°C for 1 hour.

The samples identified as genotype 4 were subjected to subtyping, from 4-N, 4-O, to 4-P[14]. The amplification reactions were performed with the Taq PCR Master Mix kit (Qiagen Sciences Inc, Germantown, USA), using 1.5µL of primers Ins-978589-F, Ins-9789589-R, Del-1476519-F, and Del-1476519-R[14].

Statistical analysis

Graphics and descriptive tables (means, standard deviation, and proportions) were used to present the data (DNA positivity, Ct, and copy number). Bacilli copy number range in the water samples and SNP typing were compared by chi-square and Fisher's exact two-tailed tests. Crude and adjusted Poisson regression models with robust variance and estimated incidence rate ratios (IRR) and 95% confidence intervals (95% CI) were generated to assess the associations between *M. leprae* genotyping positivity (water and biopsy samples) and genotype (SNP typing 1 or 2, 3, and 4). The level of significance was $p < 0.05$. STATA statistical software version 13.1 was used for the analyses.

Ethics considerations

All patients were aware and gave written informed consent for the collections of biopsy samples. The study was approved by CDERM Ethical Committee (protocol 011/07) and performed in compliance with the guidelines of the Ethical Committee.

RESULTS

Of the 149 analyzed water samples from the five municipalities surveyed, 81 (54.4%) were positive for *M. leprae* DNA (**Table 1**). The municipality of Mulungu had DNA positivity in 22 of the 30 M1 to M6 replicates (73.3%). Juazeiro do Norte had 16 positive samples out of 25 (64%) for sampling points J1, J2, J3 J4 and J5. The town of Boa Viagem had 19 positive samples out of 34 (55.9%), only collection point B7 (dam) was negative. The municipality of Sobral, with the greatest number of collected samples (40 replicates), had 18 (45%) replicates positive for *M. leprae* (points S1, S2, S4, S5, S6, S7, and S8). The Sobral samples collected in site S3 (lake) were negative.

TABLE 1

Mycobacterium leprae 16S rRNA gene DNA positivity in environmental water reservoirs from the municipalities of Boa Viagem, Crato, Mulungu, Juazeiro do Norte, and Sobral, Ceará, Brazil, 2010-2013 determined by qPCR.

Municipality	Type of reservoir	Dna Positivity n (%)
Boa Viagem (N= 34)		19 (55.9)
B 1	Dam	5
B 2	Dam	2
B 3	Dam	3
B 4	Dam	3
B 5	Dam	2
B 6	Dam	4
B 7	Dam	0
Crato (N= 20)		6 (30.0)
C 1	Recreational stream	4
C 2	Dam	2
C 3	Dam	0
C 4	Dam	0
Mulungu (N= 30)		22 (73.3)
M 1	Lake	5
M 2	Recreational stream	3
M 3	Well	4
M 4	Well	5
M 5	Stream	3
M 6	Well	2
Juazeiro do Norte (N= 25)		16 (64.0)
J 1	Dam	5
J 2	Dam	3
J 3	Dam	4
J 4	Dam	3
J 5	Stream	1
Sobral (N= 40)		18 (45.0)
S 1	Dam	4
S 2	Dam	3
S 3	Lake	0
S 4	River	2
S 5	River	1
S 6	Stream	3
S 7	River	3
S 8	River	2
Total		**81 (54.4)**

rRNA: ribosomal ribonucleic acid; **DNA**: deoxyribonucleic acid; **qPCR:** quantitative polymerase chain reaction.

The lowest positivity was detected in the municipality of Crato; 6 of the 20 collected replicates (30%) from sampling points C1 (recreational stream) and C2 (dam) were positive.

A 10-fg dilution of plasmid pIDT16SrRNAMleprae was defined as the analytical sensitivity or detection limit for the qPCR assay, showing a Ct value < 37 cycles in every standard

curve run. Titration of plasmid pIDT16SrRNAMleprae using 16S rRNA primers revealed a limit of detection of approximately 30 cells, based on a *M. leprae* chromosome size of ~3.27Mb.

To determine the specificity of the qPCR assay, two other mycobacterial species and three other bacterial species and control samples were also tested. The target region was only amplified by qPCR from mycobacteria species and with *M. leprae* DNA; however, the amplicons were obtained using a different Tm (77.5°C) compared to that for *M. leprae* (78.1°C). No DNA amplification was observed for bacterial species from other genera or the controls. The obtained standard curve (**Figure 1**) was linear with a Pearson correlation coefficient (r) of -0.996, a correlation coefficient (R^2) of 0.992, and an amplification efficiency of 99.51%. **Table 2** details the average C_t of the environmental water samples from each municipality, as well as the copy number range. The municipality of Mulungu showed the highest average C_t (29.55; 27.8 to 34.9 range); followed by Crato (29.48; 25.5 to 33.4 range) and Juazeiro do Norte average (28.96; 28.0 to 30.1 range). Lower C_ts were found in the water samples from Sobral (average C_t of 28.8) and Boa Viagem (average C_t of 27.4).

Of the 25 patient biopsy samples analyzed, six (24%) samples were characterized as SNP 1 or 2, three (12%) samples were SNP genotype 3, and 16 (64%) samples were classified as SNP 4 (**Figure 2**).

As expected, the Poisson regression model, based on crude and adjusted analysis, demonstrated that the positivity incidence rate ratio in the biopsy samples was 60% higher than that in the water samples (p = 0.0016; **Table 3**). No statistical difference in *M. leprae* genotyping was observed among biopsy and water samples from Juazeiro do Norte (p = 1,000). The majority of biopsy and water samples were characterized as SNP 4-N, with 16 (100%) and 14 (87.5%) samples, respectively. The other two water samples (2/16, 12.5%) were identified as SNP 4-O.

DISCUSSION

The results of this study demonstrate high *M. leprae* DNA positivity in environmental waters. In addition, it reveals that the *M. leprae* genotype present in the collected water samples is the same as that found in the lesions of leprosy patients. Previous studies with environmental samples have also shown the presence of *M. leprae* DNA with varying positivity percentages: from 24 to 47.7% in water[8,15]; 33.3%, 34.3%, and 37.5% in soil samples from the peridomicile of leprosy patients[9,10,16]; and 21% in armadillos from endemic regions in Ceará[17].
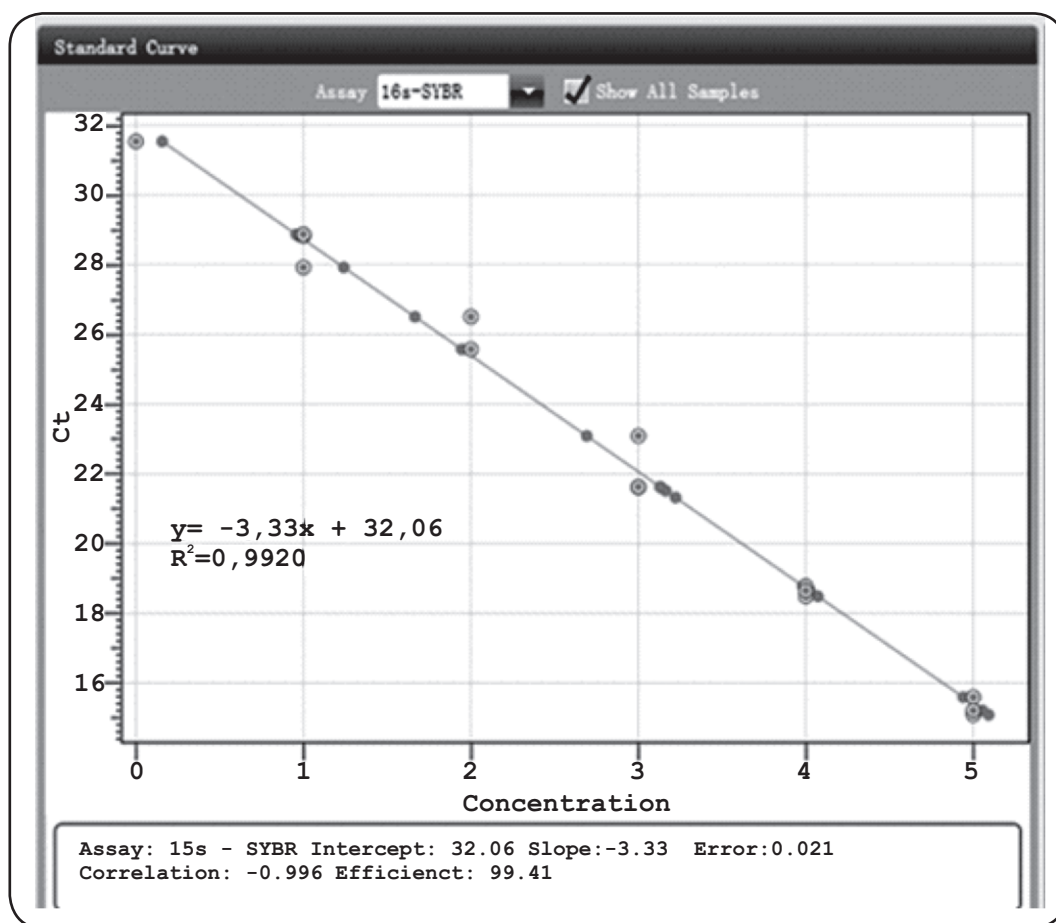

FIGURE 1 - Linear regression of the Cycle Threshold values vs. *Mycobacterium leprae* **plasmid DNA dilutions, 2010-2013. DNA:** deoxyribonucleic acid; **Ct:** cycle threshold; **y:** theoretical limit of detection of the reaction (Ct value); **R²:** correlation coefficient.
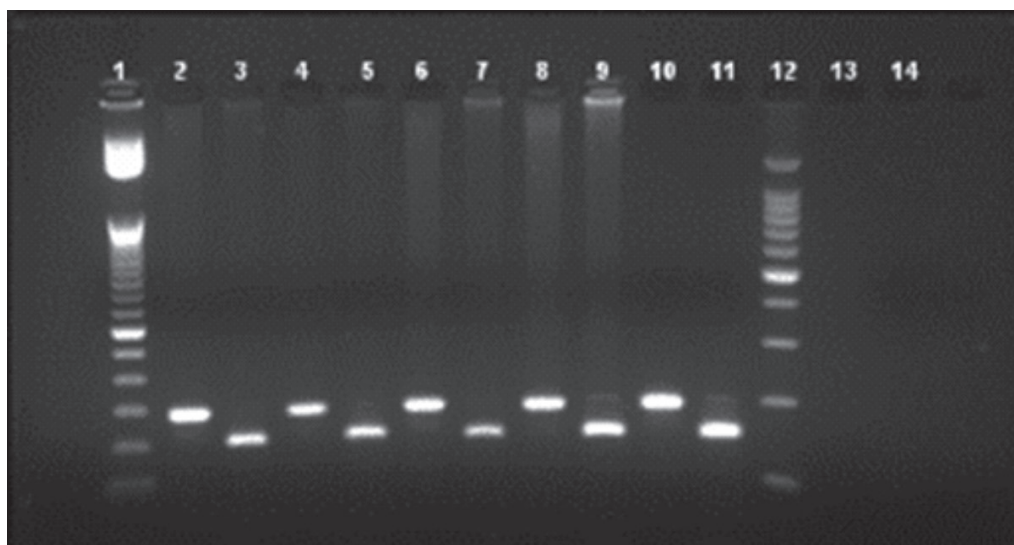
FIGURE 2 - Agarose gel analysis of the 14676 region RFLP-PCR *Sml*I-digested and undigested fragments. PCR products from the 14676 region of *Mycobacterium leprae* were digested 10U/μL of *Sml*I for 1 hour. The digested products were run on a 3% agarose gel for 1.5 hour at 80 volts. **Lane 1**: 50bp DNA ladder; **Lanes 2, 4, 6, 8,** and **10**: undigested 194bp fragments; **Lanes 3, 5, 7, 9,** and **11**: 149bp digested fragments; **Lane 12**: 100bp DNA ladder; **Lane 13**: undigested negative control; **Lane 14**: digested negative control. **RFLP-PCR:** restriction fragment Length polymorphism-polymerase chain reaction; ***Sml*I:** endonuclease I from *Stenotrophomonas maltophilia;* **bp**: base pair; **DNA**: deoxyribonucleic acid.

TABLE 2

Cycle threshold average, standard deviation, and copy number range of *Mycobacterium leprae* in environmental water samples from Boa Viagem, Crato, Mulungu, Juazeiro do Norte and Sobral, 2010-2013.

Municipality	Average C_t^*	Standard deviation	Copy range ($C_t \leq 37$)
Mulungu	29.55	2.18	$1.88 \times 10^1 - 1.42 \times 10^{-1}$
Crato	29.48	2.91	$9.11 \times 10^1 - 3.95 \times 10^{-1}$
Juazeiro do Norte	28.96	0.71	$1.66 \times 10^1 - 3.85 \times 10$
Sobral	28.83	1.31	$3.23 \times 10^1 - 1.81 \times 10$
Boa Viagem	27.41	1.11	$1.44 \times 10^2 - 4.27 \times 10$

C_t: cycle threshold. $*p = 0.0005$.

TABLE 3

Poisson regression model exploring the association between the *Mycobacterium leprae* genotypes identified in CDERM patient biopsies and environmental waters of the municipality of Juazeiro do Norte, Brazil, 2010-2013.

	Negative		Positive		Crude analysis			Adjusted analysis		
	n	%	n	%	IRR	95% CI	p	IRR	95% CI	p
Samples										
water	32	66.7	16	33.3	1			1		
biopsy	50	66.7	25	33.3	1.6	(1.1 - 2.2)	0.016	1.6	(1.1 - 2.2)	0.016
Genotyping										
SNP 4	9	22.0	32	78.0	1.0			1.0		
SNP 1 or 2	35	85.4	6	14.6	1.0	(0.8 - 1.2)	1,000	1.0	(0.6 - 1.5)	1,000
SNP 3	38	92.7	3	7.3	1.0	(0.8 - 1.2)	1,000	1.0	(0.6 - 1.5)	1,000

CDERM: Dona Libânia National Reference Centre for Sanitary Dermatology; **IRR:** incidence rate ratio; **CI**: confidence interval; **SNP:** single-nucleotide polymorphism.

A correlation was found between *M. leprae* DNA positivity in the water samples and the leprosy CDR of the municipality. Thus, the frequency of positivity obtained in Crato, Juazeiro do Norte, and Sobral could be associated with the high number of new cases detected annually in these cities, which are considered hyperendemic regions for leprosy. The high number of leprosy cases would be responsible for the excretion of the bacteria in the environment. The population of these municipalities, which most likely includes leprosy cases, makes use of the water reservoirs examined in this study for washing clothes, bathing, leisure activities, as well as direct consumption. Contamination of the water with *M. leprae* bacilli could be responsible for the continued transmission of the disease and would explain the high positivity values of these collection points. However, the Cities of Boa Viagem and Mulungu, which report few or no cases of the disease, are among the three municipalities (Mulungu 73.3%, Juazeiro do Norte 64%, and Boa Viagem 55.9%) with a higher frequency of DNA positivity. These results may also indicate a possible impoverishment of the local health systems or a very low rate of notification of leprosy cases in these municipalities. Mulungu and Boa Viagem have a low population density (estimated population of 11,485 and 52,498 inhabitants, respectively, in 2010[18]); these cities have deficient health services dependent on other developed cities. Historically, the official incidence of leprosy in Mulungu and Boa Viagem has been much lower than in other municipalities in Ceará. These cities have a poor disease notification system and are surrounded by other cities with a high reported prevalence of leprosy[4,19]. Our results suggest that there are unnotified cases of leprosy in these cities and these untreated cases release bacilli that might contaminate the water reservoirs.

A previous study conducted in Ceará[20] did not identify any differences between the anti-phenolic glycolipid-I (PGL-1) in seroprevalence among contacts of leprosy cases in Sobral and those with no known contacts in Mulungu. Therefore, the high seroprevalence frequency in Sobral and Mulungu might be explained by the presence of *M. leprae* in their environmental waters. Indirect contact with *M. leprae* harbored in environmental sources by individuals who had not been in contact with leprosy cases would lead to a specific immune response against the bacilli[21].

A number of previous studies conducted in Ceará have demonstrated a diverse distribution of leprosy in the state[4,22]; the cities with a high disease incidence are in the most developed and urbanized regions, but have a high level of social inequality. Moreover, factors, such as limited education, social inequality, poor personal hygiene, and nutritional deficiency, have been reported to be associated with the development of leprosy. In addition, a possible influence of environmental variables on the increased risk of developing leprosy has been identified[9]. Another study conducted in Ceará has reported a significant association between bathing in environmental water sources 10 years prior to the emergence of the disease and the high number of leprosy cases in regions near water sources used for the purpose of bathing and washing clothes[5]. However, it is important to note that the detection of *M. leprae* DNA does not indicate that the bacilli in the analyzed environmental water samples are viable. The detected DNA may originate from live or dead bacteria, or even constitute extracellular DNA[23]; detection of bacterial DNA derived from lysis by heating followed by ultraviolet exposure has been demonstrated[24,25].

The high proportion of biopsies and environmental samples with SNP type 4 originated from Western Africa during the slave trade period. Approximately four million slaves were brought to Brazil between the 17th and 19th centuries[26] and leprosy disease was widespread among the slaves. In the state of Ceará, slave labor from Western Africa was used by sugarcane plantations, which was the main activity during the colonial period, as already demonstrated in other genotyping studies[1,27]. Thus, the predominance of SNP type 4 in Ceará is directly related to the Western African slaves. Similar to previous studies conducted in an endemic village in India, we found a concordance between the SNP subtyping (subtype 4-N) of *M. leprae* from skin biopsies and those from water samples[9,28].

Although TaqMan is described as a more sensitive and specific method compared to SYBR Green[29-31], our assays using the SYBR Green dye were highly specific and sensitive because our primers were specific to *M. leprae* bacilli. Furthermore, SYBR Green is simple and low-cost method. Therefore, qPCR primer design and optimization were important for the success of the SYBR Green qPCR assay.

In addition, results with statistical significance were not obtained in our study because of the small number of genotyped clinical and environmental samples. The reason for this is the difficulty to amplify DNA from environmental samples, possibly due to the presence of inhibitory substances[32]. Future studies employing whole genome sequencing comparing biopsies of cases that live near the environmental water would address the role of water reservoirs in the transmission of this disease.

Detection of *M. leprae* DNA in environmental water samples and the genotypic concordance with strains from leprosy cases suggests indirect infection via waters containing leprosy bacilli. Therefore, we suggest that environmental waters containing the bacilli play a major role in the transmission of leprosy in the studied regions of the State of Ceará. These data could explain the PGL-1 seropositivity in people residing in municipalities without disease notification and may explain the diagnosis of new leprosy cases without previous contact with a patient. Our results also indicate that the individuals living nearby or have access to these waters containing the *M. leprae* bacilli should be monitored for early leprosy diagnosis. Improvements in living conditions in these areas should be implement and evaluated for the leprosy prevalence reduction. Further studies are necessary to investigate the role of the environment in the transmission dynamics of leprosy, including detection of *M. leprae* mRNA and the role of amoeba, fish and water plants that might harbor the bacteria and provide its viability.

Conflicts of interest

The authors declare that there is no conflict of interest.

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