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Presence of *Mycobacterium leprae* DNA and PGL-1 antigen in household contacts of leprosy patients from a hyperendemic area in Brazil

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ABSTRACT. Leprosy is a highly infectious disease endemic to underdeveloped countries. In Maranhão State, Northeastern Brazil, the hyperendemic rate of 56.11 cases/100,000 inhabitants increased the necessity of better understanding the epidemiological profile of this population, particularly regarding efficient methods for evaluating individuals residing with diagnosed patients to understand disease transmission and the risk of infection. In

this study, we examined the percentage of contacts with positive indices for *Mycobacterium leprae* DNA and phenol-glycolipid-1 antigen (PGL-1). PGL-1 was analyzed by an enzyme-linked immunosorbent assay, the ML-Flow test, and polymerase chain reaction of oral and nasal secretions of 808 leprosy contacts from Maranhão. PGL-1 was detected in 14.0% of patients and differed by operational classification of the index case ($P < 0.05$). Seropositive results of ML-Flow were 15.0% and identified individuals with and without Bacillus Calmette-Guérin vaccine scars. Molecular diagnosis detected *M. leprae* DNA in 5.6% of oral samples and 4.6% of nasal tissues, and 87% of subjects resided with high bacillary load patients. This study reinforces the efficacy of combining molecular and serological techniques to identify potential bacillus carriers in the asymptomatic stage of infection, such as in household contacts, highlighting the importance of these methods for monitoring hyperendemic populations.

Key words: Asymptomatic infection; Hansen bacilli; Molecular detection; Serological diagnosis

INTRODUCTION

Leprosy is a chronic infectious disease caused by an obligate intracellular parasite, the bacillus *Mycobacterium leprae*. Its clinical manifestations include various immune response patterns, which can range from vigorous to absent cellular immune responses at the tuberculoid and lepromatous poles, respectively (Modlin, 2010).

Leprosy is transmitted between humans. The upper airways of untreated patients are the primary site of bacillus entrance and exit. Thus, household contacts of infected individuals are at the highest risk of becoming ill in the short term (Goulart and Goulart, 2008; Sales et al., 2011). According to the World Health Organization (2011), active searching and monitoring of patients' household contacts are the most effective strategies for limiting the spread of leprosy (Smith and Aerts, 2014).

The combination of molecular and serological techniques, such as polymerase chain reaction (PCR), phenol-glycolipid-1 antigen (anti-PGL-1) enzyme-linked immunosorbent assay (ELISA), and the ML-Flow test, have shown good efficacy in leprosy diagnosis (Santos et al., 2007; Job et al., 2008; Stefanni, 2008; Martinez et al., 2011). Additionally, contacts of leprosy patients that are positive for anti-PGL-1 have a 6-fold higher risk of illness compared to healthy individuals (Goulart et al., 2007). This reinforces that contacts carrying DNA sequences of *M. leprae* in high endemic areas may be involved in the disease transmission chain (Goulart et al., 2008; Job et al., 2008; Martinez et al., 2011; Reis et al., 2014).

The endemism of leprosy persists mainly in socio-economically underdeveloped countries. Brazil, as a developing country, ranks 2nd worldwide in the number of new leprosy cases per year (WHO, 2011). More specifically, Maranhão State, located in the northeast region of the country, has one of the highest national leprosy rates with a prevalence of 56.11/100,000 inhabitants in 2011 (Ministério da Saúde, 2011). However, there has been no substantial public effort for a laboratorial routine establishment regarding significant surveillance in the population.

Because of the current hyperendemic board and lack of efficient serological and molecular diagnosis in Maranhão State, we examined the absolute positivity frequency for DNA and specific antibodies against *M. leprae* on apparently asymptomatic household contacts.

MATERIAL AND METHODS

Study area, population, and disease classification

We analyzed intra-domiciliary (residing together) and extra-domiciliary (residing in the same neighborhood) contacts of leprosy treatment-naive patients. All contacts presented unaltered dermatological and neurological exams. All subjects who agreed to participate in this study answered a personal questionnaire including gender, age, number of Bacillus Calmette-Guérin (BCG) vaccine scars, likely source of infection, and clinical form and operational classification of their index case.

A total of 808 leprosy contacts and 200 patients from Maranhão were recruited for the study. Subjects were evaluated by dermatologic and neurologic examinations and bacilloscopy, and then placed on the leprosy spectrum based on Ridley and Jopling (1966), according to the following criteria: indeterminate, tuberculoid, borderline tuberculoid, borderline-borderline, borderline lepromatous, and lepromatous forms. All subjects were classified into operational classifications based on the number of cutaneous lesions, considering multibacillary (MB) as those with more than 5 lesions and paucibacillary as those with less than 5 lesions.

Ethical aspects

All individuals or their legal guardians were educated about the study and signed an informed consent form. The consent form and research design were approved by the Ethics Committee of Universidade Federal do Maranhão (Permission No. 23115 003005/2009-36).

Serological diagnosis

Anti-PGL-1 ELISA and ML-Flow test were used for immunological evaluation. The antibody titers were expressed as the ELISA index (EI) according to the formula: EI = optical density sample/optical density cut-off, as described previously by Lobato et al. (2011). EI ≥ 1.1 was considered to be positive. The ML-Flow test was carried out according to Bührer-Sékula et al. (2003).

Molecular diagnosis by PCR

Nasal and oral mucosa were sampled to extract DNA for molecular analysis. The samples were suspended in 500 µL nuclear lysis buffer (400 mM NaCl, 50 mM EDTA, pH 8.0, and 25 mM Tris-HCl, pH 8.0); DNA was extracted using the phenol-chloroform method as described by Sambrook et al. (1989) and used to amplify a 130-bp fragment of the RLEP3 region (X17153) of *M. leprae* (5'-GCA CGT AAG CAT GTC GGT GG-3' - forward and 5'-CCG CGG CGC TAA CAA CTA TC-3' - reverse). The natural resistance-associated macrophage protein-1 gene (forward: 5'-CTC GCA TTA GGC CAA CGA-3'; reverse: 5'-TTC TGT GCC TCC CAA GTT AGC-3') was used as an endogenous control (Goulart et al., 2007).

For amplification, the 25-µL reaction mixture contained: 2 µL genomic DNA, 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 1.5 mM $MgCl_2$, 200 µM each of dNTP (Invitrogen), 1 U Taq DNA polymerase (Invitrogen), and 10 pM of each primer. The PCR thermal cycling conditions were: denaturation at 95°C for 5 min; followed by 34 cycles of denaturation at 95°C, primer annealing at 58°C for 40 s, extension at 72°C for 40 s, and a final extension at 72°C for 10 min. Electrophoresis

was performed on a 2% agarose gel for 40 min, and the amplicons were visualized under UV transillumination (Figure 1).

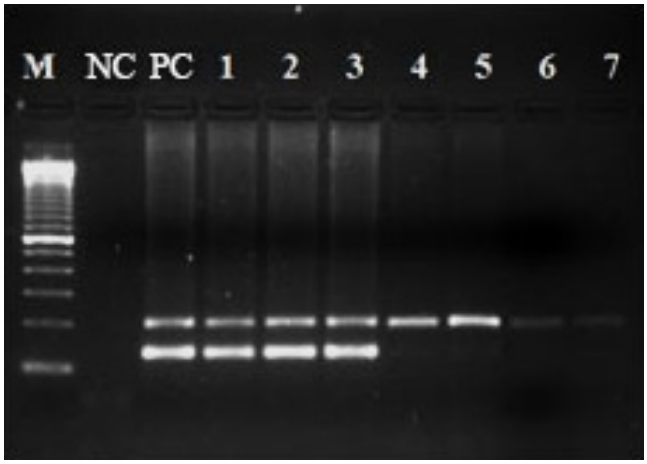

Figure 1. Agarose gel showing the amplification of *Mycobacterium leprae* DNA in oral mucosa and nasal samples. *Lane M*: 100-bp molecular weight ladder; NC: negative control; PC: positive control; 200-bp fragment: constitutive gene (NRAMP-1); 130-bp fragment: *M. leprae*. *Lanes 1*, *2*, and *3* correspond to the amplification of the RLEP3 region of the *M. leprae* genome. *Lanes 4-7* correspond to samples presenting amplification only to NRAMP-1 (200 bp).

Statistical analysis

The results were analyzed using STATA 10.0 (StataCorp. LP, College Station, TX, USA). Qualitative variables are reported as absolute and relative frequencies. The Pearson chi-squared test was used to evaluate the association between operational classification of patients, along with DNA and serological analysis of contacts. Additionally, the chi-squared test was performed to compare the proportion of positive results regarding the presence of *M. leprae* DNA or anti-PGL-1 and the presence of 2 BCG vaccine scars in contacts. For all tests, statistical significance was set at 5%.

RESULTS

The mean age of patients was 37 ± 17.5 years (10%; <15 years), and most patients had only 1 or no BCG vaccine scars (94.0%). Moreover, the numbers of MB (61%) and borderline tuberculoid (32%) patients were significant. Additionally, the mean age of leprosy contacts was 27.5 ± 18.3 years. Most of these subjects had 1 BCG scar (61.7%) and were intra-domiciliary contacts (70.0%) of the MB leprosy index case. All information obtained regarding population characteristics are described in Table 1.

Serological and molecular results are summarized in Table 2. The IgM response to PGL-1 antigen, specific to *M. leprae*, was observed in 14.0% of subjects (113/808) using ELISA.The ML-Flow test, performed in 200 contacts, showed that 15% (30/200) were positive for IgM anti-PGL-1 serum antibodies. Among the positive samples, 63% presented a weak reaction (+). ML-Flow revealed greater positivity in contacts of MB patients (Table 3).

M. leprae DNA was detected in 8.5% (69/808) of the contacts' samples by PCR. Of this number, 5.6% (45/808) were oral tissues and 4.6% (37/808) were nasal specimens. Among the

overall contacts with successful DNA detection, 21.7% (N = 15/69) had positive PCR results for both nasal and oral samples. In this subgroup, 87% (N = 13/15) resided within MB patients (67.0%; 10/15).

Table 1. Characterization of leprosy patients and their household contacts from a high endemic area in Maranhão, Brazil.

Characteristic	Categories	Patients % (N_{total} = 200)	Contacts % (N_{total} = 808)
Gender	Male	47.5	37.5
	Female	52.5	62.5
Age	<15 years	10.0	33.8
	≥15 years	90.0	66.2
Operational classification*	Paucibacillary	39.0	33.4
	Multibacillary	61.0	66.6
Clinical form*	Indeterminate (I)	13.0	8.7
	Tuberculoid (T)	25.0	23.6
	Borderline tuberculoid (BT)	32.0	25.6
	Borderline-borderline (BB)	15.5	23.1
	Borderline lepromatous (BL)	10.0	10.1
	Lepromatous (LL)	4.5	8.9
Number of BCG scars	0	47.5	19.9
	1	46.5	61.7
	2	6.0	18.4
Probable source of infection	Intradomiciliary	21.5	70.0
	Intradomiciliary-intimate	5.0	15.6
	Extradomiciliary	13.5	13.8
	Extradomiciliary-intimate	41.0	0.6
	Unknown	19.0	0.0

*Including patients and index cases of contacts.

Table 2. Frequency of *Mycobacterium leprae* detection through molecular analysis and serology of contacts according to the Clinical form and Operational classification of the index case in a high endemic area.

Operational classification	Clinical form	Oral swab PCR [N (%)]	Nasal swab PCR [N (%)]	Anti-PGL-1 ELISA [N (%)*]
Paucibacillary	I	4 (5.6)	2 (2.8)	7 (9.9)
	T	12 (6.3)	10 (5.7)	32 (16.7)
Multibacillary	BT	11 (5.3)	9 (4.3)	30 (14.5)
	BB	6 (3.2)	10 (5.3)	25 (13.4)
	BL	6 (7.3)	3 (3.7)	10 (12.2)
	LL	6 (8.5)	3 (4.3)	9 (12.9)
Total		45/808 (5.6)	37/808 (4.6)	113/808 (14.0)

*P < 0.05. For abbreviations, see Table 1.

Table 3. Frequencies of IgM response anti-PGL-1 in contacts of leprosy patients according to the Clinical form of the index case.

Operational classification	Clinical form	ML-Flow anti-PGL-1 (N = 200)			
		+ [N (%)]	++ [N (%)]	+++ [N (%)]	Negative [N (%)]
Paucibacillary	I	7 (36.8)	1 (0.2)	0 (0.0)	32 (18.8)
	T	2 (10.5)	1 (0.2)	0 (0.0)	23 (13.5)
Multibacillary	BT	6 (31.7)	2 (0.4)	3 (14.5)	41 (24.1)
	BB	2 (10.5)	1 (0.2)	1 (13.4)	41 (24.1)
	BV	2 (10.5)	0 (0.0)	2 (12.2)	14 (8.2)
	VV	0 (0.0)	0 (0.0)	0 (12.9)	19 (11.2)
	Total	19 (9.5)	5 (2.5)	6 (3.0)	170 (85.0)

For abbreviations, see Table 1.

No consistent association was observed between the presence of the bacillus DNA in contacts and the operational classification of the respective index case ($P > 0.05$). However, a significant difference was observed when comparing the positive results by anti-PGL-1 ELISA and the index case operational classification ($P < 0.05$). Additionally, in the entire spectrum of index case clinical forms, the presence of *M. leprae* DNA and positive anti-PGL-1 ELISA were substantially different.

The positive results for molecular and serological diagnosis of household contacts not only differed among vaccinated and non-vaccinated individuals, but also showed consistent contrast in subjects with 2 BCG vaccine scars. We found that 7.2% of vaccinated contacts were positive for ML-Flow compared to 5.8% in non-vaccinated subjects ($P = 0.015$; Table 4).

Table 4. Percentage of DNA presence or antibody detection for *Mycobacterium leprae* according to a BCG vaccination status in household leprosy contacts.

Test type	Vaccinated	%	Non-vaccinated	%	P value (χ^2)
Nasal swab PCR	9	3.2	28	4.7	0.560
Oral swab PCR	9	4.4	38	4.7	0.600
Anti-PGL-1 ELISA	23	12.1	114	13.2	0.335
ML-Flow	62	7.2	11	5.8	0.0015

DISCUSSION

The high detection rates of pathogenic DNA and antigen in human samples in contacts in a highly endemic region indicate the urgency of monitoring these individuals using highly specific diagnostic methods to determine the pathogenic potential for disease spread within the population. Leprosy diagnosis remains challenging because of the lack of a gold-standard method for detection of infection during early stages. Public health centers often subject patients to dermatologic and neurologic examination to determine whether bacilli are present. Although this method is accessible and inexpensive, it cannot efficiently diagnose asymptomatic individuals. This is one of the main factors contributing to the spread of leprosy, making it a worldwide public health problem. Therefore, the development of more sensitive and *M. leprae*-specific diagnosis methods is needed, particularly when applied for the early detection of bacillus in asymptomatic contacts, as a method for disease control (Goulart and Goulart, 2008).

IgM anti-PGL-1 were detected in 14.0% of contacts using anti-PGL-1 ELISA. This rate was similar to that reported by Araújo et al. (2012), in which 13.3% were seropositive subjects in an endemic region, but lower than the value observed by Barreto et al. (2011) in a hyperendemic area (35%). Nevertheless, leprosy contacts have a higher risk of developing the disease, regardless the proportion of seropositive contacts in endemic and non-endemic areas (Da Silva et al., 2008; Frota et al., 2010). Our results demonstrate that the same may apply when comparing the seropositive rate in endemic and hyperendemic areas.

The high seropositive index of contacts observed in this study may have resulted because of continuous bacillus exposure in a hyperendemic region. We found that approximately 3% of contacts showed a strong humoral response (positive +++) and were at the highest risk for developing the disease in its lepromatous forms. Although serological diagnosis is limited in the evaluation of the bacillus charge in paucibacillary patients, this method can be used in epidemiological analysis to identify sickness risk groups. Furthermore, the ML-flow test can support operational classification of individuals; this is a low-cost test requiring limited standard laboratory infrastructure, and thus, it is more accessible for application in public health services, mainly in areas with low financial support (Bührer-Sékula et al., 2003).

The bacterial DNA detection rate by PCR of nasal swabs (4.6%) among contacts agrees with those reported for other hyperendemic regions, which were 5.9% (Beyene et al., 2003) and 4.0% (Job et al., 2008), and endemic areas, which was 4.7% (Araújo et al., 2012). The PCR detection rate using oral swabs obtained in the present study was similar to previously reported values. Martinez et al. (2011) observed a positivity rate of 6.83% among 1288 contacts from an endemic area.

The detection percentages of *M. leprae* DNA can vary from 1 to 10%, likely because of the use of different primers, amplification techniques, and clinical form of the index case (Goulart et al., 2008; Martinez et al., 2014). In our study, no significant association was observed between the bacillary load of the index case and the detection of bacterial DNA. Thus, it is possible to detect contacts carrying the bacteria regardless of the operational classification of the index case.

The presence of IgM and *M. leprae* DNA in the household contact samples does not indicate illness, as there are biological and social factors that determine the development of leprosy (Sales et al., 2011; Dos Santos et al., 2013). Few studies have demonstrated association between DNA positivity and leprosy risk (Reis et al., 2014). However, it is necessary to monitor positive contacts for a minimum of 5 years over the incubation time of *M. leprae* in the host. Moreover, individuals with subclinical infection may be involved in the disease transmission chain (Moet et al., 2004; Goulart et al., 2008).

Most contacts and patients had only one or no BCG vaccine scar. The relationship between tests, positive results, and the occurrence of 2 vaccine scars was not substantial, with exception of testing using ML-Flow, for which there was a greater number of seropositive cases carrying 2 BCG scars. According to Matos et al. (1999), the vaccine may not be directly related to seropositive leprosy contacts, and it is probable that there was a higher number of infected among vaccinated than non-vaccinated subjects. Nevertheless, Düppre et al. (2012) examined a 10-year cohort and found more cases of leprosy among seropositive contacts in paucibacillary patients than in vaccinated contacts. A combination of PGL-1 tests and BCG vaccination should be used as major tools for leprosy control. BCG vaccine does not prevent disease progression, but can improve the immune response, playing a protective role.

BCG induces an increase in the production of gamma-interferon, and is thus highest in previously vaccinated individuals (Düppre et al., 2012). The absence of an immune response between exposed contacts was associated with an increased risk of sickness, suggesting the importance of 2nd doses to reinforce prevention (Goulart et al., 2008). Moreover, vaccination in intra-domiciliary contacts is a prevention measure suggested by the Brazilian Ministry of Health to fight leprosy. However, continuous surveillance remains the most effective method for reducing the number of cases.

Several other reports have observed a high concentration of leprosy cases in individual households (Sales et al., 2011; Düppre et al., 2012; Dos Santos et al., 2013) and extra-domiciliary contacts (Moura et al., 2013). This indicates the necessity of preventive measures against leprosy that are efficient in hyperendemic areas. According to Goulart et al. (2008), the prophylactic treatment for contacts with subclinical infection containing *M. leprae* DNA, positive anti-PGL-1 serology, and negative Mitsuda is a one dose of rifampicin, oflaxicin, and minocycline.

The rate of leprosy cases is high Maranhão State. In 2011, the prevalence was 56.11/100,000 inhabitants, resulting in the area being classified as hyperendemic. This rate reflects the deficient measures of disease control. The household contacts analyzed in this study were mainly exposed to high bacillary charges and had only 1 BCG vaccine scar, as well as shared residency with the non-treated patient over a long period. These variables have been identified in other reports as risk factors for the development of leprosy in contacts (Sales et al., 2011; Düppre

et al., 2012). Thus, it is mandatory to analyze the bacillary charge using reverse transcription-quantitative PCR in order to determine the number of viable bacilli and the risk of sickness.

In conclusion, we found that the frequencies of contacts carrying DNA or specific antibodies to *M. leprae* in hyperendemic areas are similar to those observed in endemic areas. Moreover, serological and molecular methods are useful for identifying household contacts with subclinical infection, which should be strategically monitored to evaluate the overall risk of sickness.

Conflicts of interest

The authors declare no conflict of interest.

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