

## Anti-PGL1 salivary IgA/IgM, serum IgG/IgM, and nasal *Mycobacterium leprae* DNA in individuals with household contact with leprosy



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### SUMMARY

**Objectives:** Leprosy household contacts represent a group at high risk of developing the disease. The aim of this study was to detect *Mycobacterium leprae* subclinical infection in this group through serological and molecular parameters.

**Methods:** Serum anti-PGL1 IgG/IgM and salivary anti-PGL1 IgA/IgM was investigated using an ELISA, and nasal carriage of *M. leprae* DNA was detected by PCR, in leprosy household contacts of paucibacillary (PB) and multibacillary (MB) household leprosy patients ( $n = 135$ ), their index cases ( $n = 30$ ), and in persons living in a low endemic city ( $n = 17$ ).

**Results:** Salivary anti-PGL1 IgA and serum anti-PGL1 IgG showed good correlation comparing contacts and index cases ( $p < 0.01$ ,  $p < 0.005$ , and  $p < 0.0001$ , respectively). This was not observed for serum anti-PGL1 IgM ( $p > 0.05$ ). A high frequency of anti-PGL1 IgM positivity was found in IgG-negative samples ( $p < 0.0001$ ). For IgG-positive samples, IgM antibodies were also positive in most of the samples. None of the 17 volunteers living in a low endemic city presented seropositivity for IgG; however, two of them showed positivity for anti-PGL1 IgM. *M. leprae* DNA was found in the nasal swabs of nine out of the 85 MB household leprosy contacts (10.6%) and in three out of the 50 PB household leprosy contacts (6.0%).

**Conclusion:** We strongly suggest that serum IgG/IgM and salivary anti-PGL1 IgA/IgM measurements are used to follow leprosy household contacts.

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### 1. Introduction

Leprosy remains a public health challenge, with approximately 250 000 new cases being reported each year worldwide.<sup>1</sup> Brazil has the second highest number of cases in the world, with 33 955 new cases registered in 2011.<sup>2</sup> In 2012, a total of 2066 cases were reported in the state of Ceará, which represents a detection coefficient of 24 cases per 100 000 inhabitants.<sup>3</sup>

Strategies for leprosy control include the administration of multidrug therapy to the patients and vaccination of household

contacts with the bacille Calmette–Guérin (BCG) vaccine.<sup>4</sup> The World Health Organization (WHO) also recommends clinical evaluation of leprosy household contacts and health education as part of the disease control strategy.<sup>1</sup> Physical examination does not identify the early stages of the disease, when clinical manifestations are rarely present.<sup>5,6</sup> Therefore, it is necessary to employ more sensitive tools in order to investigate *Mycobacterium leprae* infection among household contacts.<sup>5</sup> Serological and PCR tests have been used in seroepidemiological studies.<sup>1,6</sup>

Phenolic glycolipid (PGL1) is an *M. leprae*-specific antigen,<sup>7</sup> and this dominant lipid in the cell wall is responsible for its immunological specificity.<sup>8</sup> The most studied antibody isotype is the serum anti-PGL1 IgM.<sup>9–12</sup> Few studies have evaluated salivary anti-PGL1 IgA and IgM,<sup>13,14</sup> or serum anti-PGL1 IgG.<sup>7,14–17</sup> Our previous work suggested that salivary anti-PGL1 IgA and IgM

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antibodies might be indicative of infection but not of disease.<sup>17</sup> It is known that individuals seropositive for anti-PGL1 antibodies have a 7.5-fold greater risk of acquiring leprosy compared to seronegative contacts.<sup>18</sup> A prospective study<sup>9</sup> demonstrated that two seropositive contacts progressed to clinical, borderline tuberculoid (BT) leprosy within 1 year of follow-up.

Several authors have described methods to detect *M. leprae* based on nucleic acid amplification techniques,<sup>6,19–23</sup> since direct bacterial detection tests have too low sensitivity.<sup>24</sup>

In the present work, we evaluated serum IgG/IgM and salivary IgA/IgM against PGL1 in addition to the detection of nasal carriage of *M. leprae* in leprosy household contacts and their index cases.

## 2. Materials and methods

### 2.1. Patients and contacts

Thirty leprosy patients (median age 45 years) and 135 household contacts (median age 26 years) living in the cities of Crato and Maracanaú, Ceará State, Brazil, were included in the study. Seventeen individuals living in a city with a low incidence of leprosy (city of Sao Paulo) who reported no contact with leprosy patients were also included in the project for serological evaluation (control group – CT group). In 2012, the detection rates of leprosy in the cities of Crato and Maracanaú were 42.8 and 30.5 per 100 000 inhabitants, respectively.<sup>3</sup> The detection rate of leprosy in the city of Sao Paulo was 2.21 per 100 000 inhabitants in 2011.<sup>25</sup>

Leprosy diagnosis and classification of the index cases were based on clinical assessment and on the detection of acid-fast bacilli in slit skin smears. The leprosy patients were classified as paucibacillary (PB) or multibacillary (MB), according to the lesion count.<sup>26</sup> Seventeen were classified as MB patients and 13 as PB patients. Fifty out of 135 (37.0%) were contacts of PB leprosy patients, and 85 out of 135 (63.0%) were contacts of MB leprosy patients.

The study was approved by the Ethics Committee of the Universidade Federal do Ceará, and each participant or his/her guardian provided written informed consent.

### 2.2. Samples

Serum samples were collected and stored at  $-20^{\circ}\text{C}$ . Unstimulated saliva was collected 30 min after water consumption and 2 h after solid food intake and kept at  $-20^{\circ}\text{C}$ . Using sterile swabs moistened in 0.9% saline solution, nasal secretions were collected from both nostrils by gently swabbing the outer nares. Each swab tip was placed into a tube containing 300  $\mu\text{l}$  of lysis solution and kept at  $-20^{\circ}\text{C}$ . The lysis solution was comprised of 100 mM Tris-HCl, pH 8.5, with 3% Triton X-100 and 1 mg/ml proteinase K.

### 2.3. Serum analysis

Serological analysis for anti-PGL1 antibodies was performed in accordance with the procedure described by Nagao-Dias et al.<sup>14</sup>

Polystyrene microplates (Costar, USA) were coated with 10 mg/l of native PGL1 in absolute ethyl alcohol, kindly donated by Dr John Spencer, Colorado State University, USA. The plates were incubated for 18 h at room temperature (RT). For serum IgG and IgM antibody measurements, the microplates were incubated with 1% bovine serum albumin (BSA, Sigma, USA) in phosphate buffered saline (PBS, pH 7.4) for 2 h at RT. After four washes with PBS–0.01% BSA, serum samples previously diluted 1:50 in PBS–0.5% BSA were added to the plates in duplicate. The plates were then

incubated overnight at  $4^{\circ}\text{C}$ . After washing, peroxidase-labeled anti-IgG or anti-IgM (Sigma, USA) previously diluted 1:1000 was added to the plates and incubated for 1.5 h at RT. After washing, the plates were incubated for 30 min with the substrate solution which contained 0.4 mg ortho-phenylenediamine per milliliter of 0.01 M citrate-phosphate buffer, pH 5.0. The reaction was interrupted by adding 25  $\mu\text{l}$  of 2.5 N sulfuric acid. The analysis was performed at 492 nm using an ELISA plate reader. An aliquot of pooled normal human serum was used as cut-off sample and tested in all assays. The results were expressed according to the following formula: optical density (OD) mean of the test sample (minus blank) divided by the OD mean of the normal human serum pool (minus blank). The cut-off was considered to be 1.0. Values above 1.3, which were 30% above the cut-off, were considered to be positive.<sup>14</sup>

### 2.4. Salivary analysis

For salivary IgA and IgM measurements, PGL1-coated plates were blocked with 1% BSA–Tris solution for 2 h at RT. Saliva samples, previously centrifuged at  $2600 \times g$  for 15 min at  $4^{\circ}\text{C}$ , were diluted 1:50 with 0.5% BSA–Tris, added to the plates, and incubated for 18 h at  $4^{\circ}\text{C}$ . After four washes with 0.01% BSA–Tris, alkaline phosphatase-conjugated anti-IgA or anti-IgM (Sigma, USA) was diluted 1:1000 in 0.5% BSA–Tris and added to the plates. The plates were then incubated for 2 h at RT. After washing, the plates were incubated with the substrate solution (1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine containing 0.5 mM  $\text{MgCl}_2$ , pH 9.8). After 100 min, absorbance readings were recorded at 405 nm using an ELISA plate reader. The results were expressed as the OD mean of the values (minus blank). The cut-off was considered to be the 97<sup>th</sup> percentile of normal controls.<sup>14</sup> Results considered to be 30% above the cut-off value were considered to be positive. Blank samples contained all reagents except for saliva or serum.

### 2.5. Molecular *M. leprae* detection

Detection of *M. leprae* DNA in nasal swabs was performed in accordance with the procedures described by Torres et al.<sup>21</sup> and De Wit et al.,<sup>27</sup> with some modifications.

The nasal swabs immersed in lysis buffer were incubated at  $55^{\circ}\text{C}$  for 18 h followed by incubation for 15 min at  $97^{\circ}\text{C}$ . After removing the swabs, 135  $\mu\text{l}$  of 5 M NaCl solution was added to each tube and incubated for 2 h at  $-20^{\circ}\text{C}$ . After centrifugation at  $8117 \times g$  for 5 min at  $4^{\circ}\text{C}$ , DNA contained in the supernatant fraction was precipitated by addition of 2 volumes of ice cold isopropyl alcohol. The solution was kept at  $-20^{\circ}\text{C}$  for 24 h. After a new centrifugation at  $8117 \times g$  for 5 min at  $4^{\circ}\text{C}$ , the supernatant was discarded and the tubes were left open overnight at RT to allow the alcohol to evaporate. Subsequently, 50  $\mu\text{l}$  of sterile distilled water was added to each tube and left in a water bath for 1 h at  $37^{\circ}\text{C}$ . The DNA samples were stored at  $-20^{\circ}\text{C}$  until further analysis.

DNA primers S13 (5'-CTCCACCTGGACCGCGAT-3') and S62 (5'-GACTAGCTGCCAAGTCG-3') were selected based on the nucleotide sequence of the 531-bp fragment (proline-rich region) of a specific gene that encodes the 36-kDa antigen of *M. leprae*.<sup>21</sup>

The PCR technique was performed by adding 5.2  $\mu\text{l}$  sterile water, 2  $\mu\text{l}$  Tween 20, 10  $\mu\text{l}$  Master Mix (Promega, USA), 0.4  $\mu\text{l}$  primers, and 2  $\mu\text{l}$  DNA template to each microtube. Subsequently, the samples were subjected to 38 amplification cycles after an initial denaturation step at  $94^{\circ}\text{C}$  for 5 min. An amplification cycle consisted of 1 min at  $94^{\circ}\text{C}$ , 2 min at  $56.5^{\circ}\text{C}$ , and 2 min at  $72^{\circ}\text{C}$ . A

**Table 1**Frequency of positive serum and salivary anti-PGL1 antibody titers and nasal *M. leprae* DNA among leprosy household contacts

Leprosy contacts—clinical form of the index case	Anti-PGL1 antibodies				<i>M. leprae</i> DNA
	Serum IgG, n (%)	Serum IgM, n (%)	Saliva IgA, n (%)	Saliva IgM, n (%)	Nasal, n (%)
Paucibacillary (n = 50)	8 (16%)	26 (52%)	11 (22%)	5 (10%)	3 (6%)
Multibacillary (n = 85)	6 (7.1%)	54 (63.5%)	30 (35.2%)	9 (10.5%)	9 (10.6%)
Fisher's test p-value	>0.1	>0.1	>0.1	1.0	>0.5

final elongation step of 7 min at 72 °C completed the amplification. Four microliters of the amplicon were subjected to electrophoresis in a 2% agarose gel. The DNA was visualized under ultraviolet light after staining the gel with ethidium bromide.

### 2.6. Acid-fast stain of nasal smears

Nasal smears were collected, spread on a slide, heat-fixed, and stained by Ziehl–Neelsen method.

### 2.7. Statistical analysis

The data were analyzed using non-parametric tests as the data did not follow a Gaussian distribution (Kolmogorov–Smirnov test,  $p < 0.0001$ ). The Wilcoxon and Spearman tests were used to compare the antibody isotype titers between paired groups. The Mann–Whitney test was used to compare the isotype antibody titers between unpaired groups. Fisher's test was used to calculate the probability of association between the clinical form of the leprosy disease and the frequency of positive or negative antibody/molecular results; also for comparing serum and salivary antibody parameters. The software used for the statistical analysis was Prism GraphPad Instat version 3.10. The level of statistical significance was considered to be 0.05.

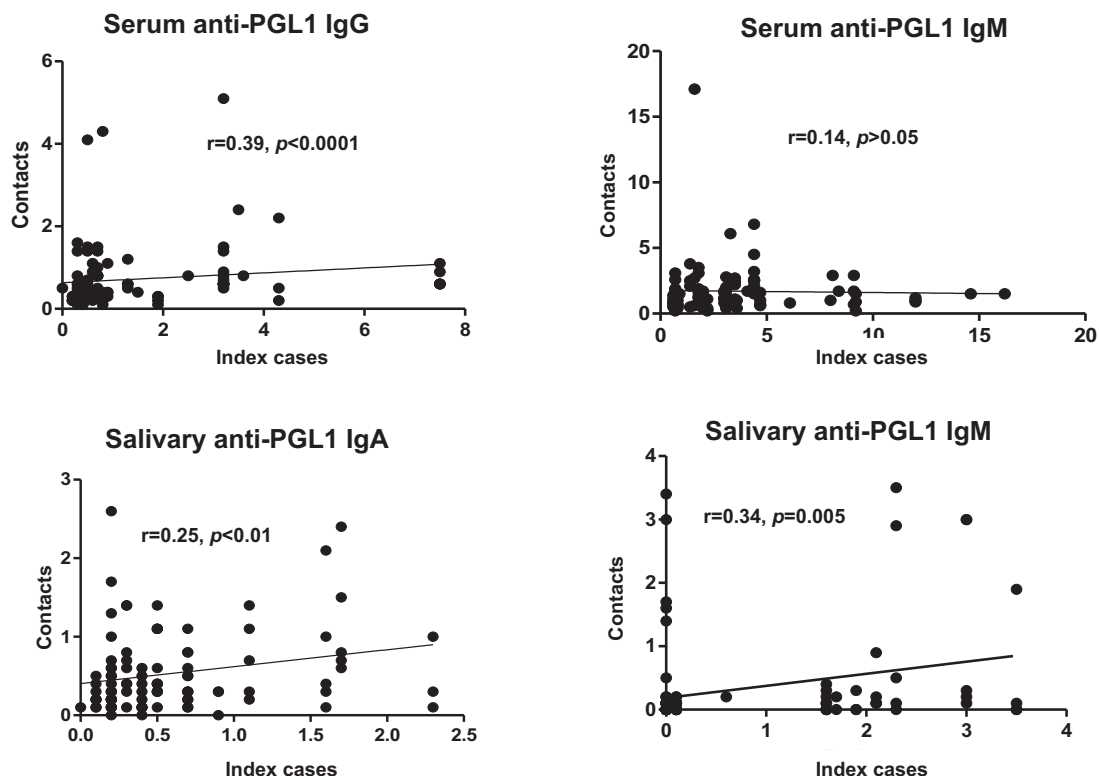
## 3. Results

### 3.1. Serological and molecular data in household leprosy (HHL) contacts

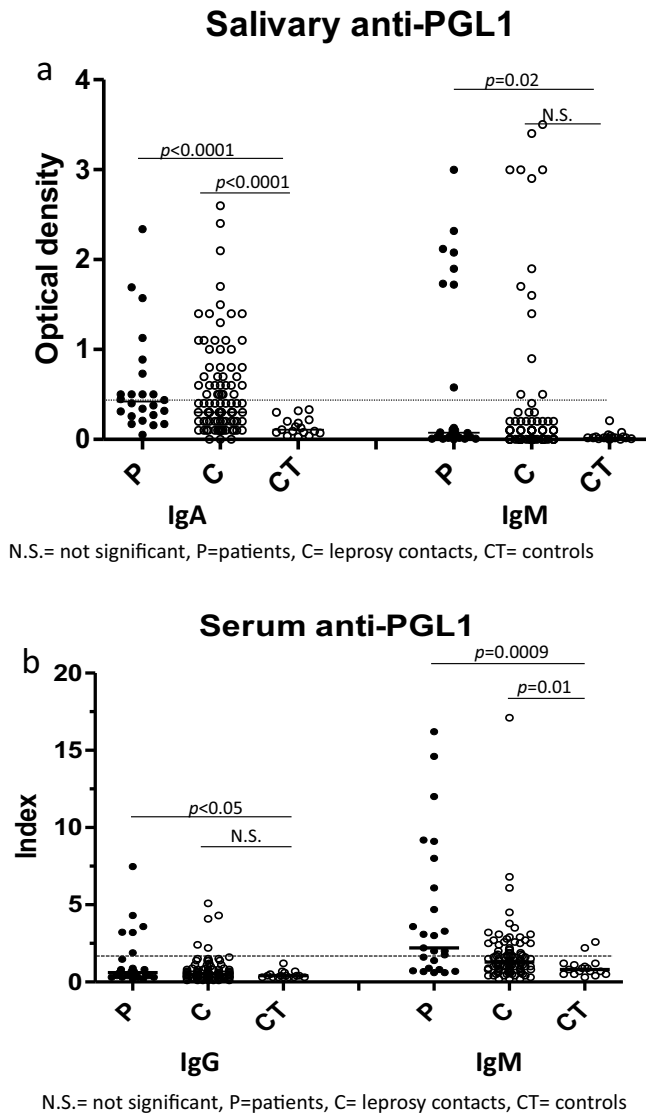
Table 1 presents the positivity of serological and molecular parameters in HHL contacts. Serum anti-PGL1 IgG and IgM were positive in 14 (10.4%) and 80 (59.3%) out of 135 samples, respectively. Salivary anti-PGL1 IgA and IgM titers were positive in 41 (30.3%) and 14 (10.4%) out of 135 samples, respectively. Twelve out of 135 samples (26.7%) were positive for *M. leprae* DNA. No statistical significance was found when the frequencies of positive serum and salivary antibodies or the *M. leprae* DNA were associated with the PB and MB HHL contacts.

### 3.2. Correlation between HHL contacts and index cases

Figure 1 demonstrates the correlation of serum and salivary antibody titers between HHL contacts and index cases ( $n = 97$ ). Salivary anti-PGL1 IgA titers showed good correlation comparing HHL contacts and index cases ( $r = 0.25$ ,  $p < 0.01$ ); a similar observation was made for salivary anti-PGL1 IgM ( $r = 0.34$ ,  $p = 0.005$ ) and serum anti-PGL1 IgG levels ( $r = 0.39$ ,  $p < 0.0001$ ). The same was not observed for serum anti-PGL1 IgM ( $r = 0.14$ ,  $p > 0.05$ ).



**Figure 1.** Correlation of serum IgG/IgM and salivary IgA/IgM against PGL1 between 97 leprosy household contacts and their corresponding index cases.



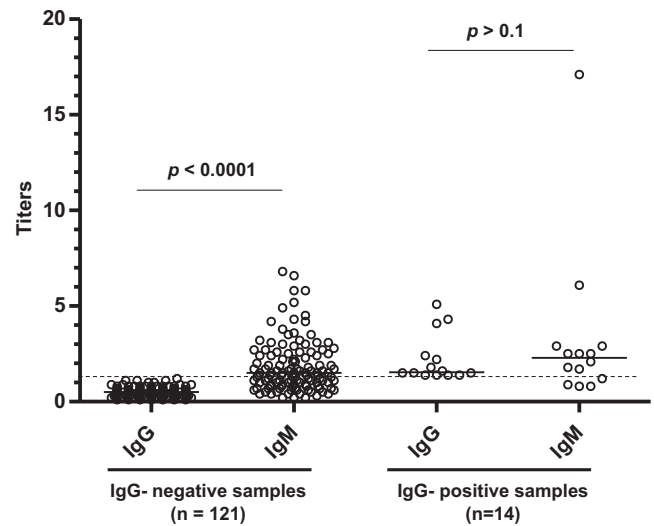
**Figure 2.** (a) Salivary and (b) serum antibody titers in leprosy patients (P,  $n = 30$ ), in leprosy household contacts (C,  $n = 135$ ), and in the control group (CT,  $n = 17$ ). The dashed lines indicate the cut-off values.

### 3.3. Controls (CT group)

Salivary IgA/IgM and serum IgG antibody titers from the CT group were below the cut-off values (Figure 2a and 2b, respectively). Two serum samples showed IgM antibody titers above 2.0 (Figure 2b). Levels of salivary anti-PGL1 IgA were much lower than those of the other groups ( $p < 0.0001$ ). In respect to salivary IgM, titers were lower than those of the HHL contact group; however, a statistical difference was only found when their levels were compared to those of the patients ( $p = 0.02$ ). In regard to serum antibodies, levels of the IgG isotype did not differ from those of the contacts, and were much lower than those of the patients ( $p < 0.05$ ). Serum anti-PGL1 IgM levels were much lower than those of the HHL contacts ( $p = 0.01$ ) and the patients ( $p = 0.0009$ ).

### 3.4. Serum antibody isotypes in HHL contacts

A statistically significant correlation was found between serum IgM and IgG isotypes for both the MB ( $r = 0.39$ ,  $p < 0.0001$ ) and the PB ( $r = 0.39$ ,  $p < 0.005$ ) HHL contacts.



**Figure 3.** Serum anti-PGL1 IgM and IgG titers in IgG-positive and IgG-negative samples. The dashed line indicates the cut-off value.

However, the serum IgM and IgG levels were very different from one another ( $p < 0.0001$ ). Considering serum IgG-negative and IgG-positive samples separately (Figure 3), a high frequency of anti-PGL1 IgM positivity (71 out of 121 samples; 58.6%) was found in IgG-negative samples ( $p < 0.0001$ ). For IgG-positive samples, IgM antibodies were also positive in most of the samples (10 out of 14 samples; 71.4%) and their levels did not differ significantly from the IgG titers ( $p > 0.1$ ).

### 3.5. Salivary antibody isotypes in HHL contacts

A statistically significant positive correlation between salivary IgA and IgM was found in both PB ( $r = 0.54$ ,  $p < 0.0001$ ) and MB ( $r = 0.61$ ;  $p < 0.0001$ ) HHL contacts. Twenty-four out of 45 HHL contacts who demonstrated positivity for salivary antibodies (53.3%) were negative for serum antibodies. Conversely, 64 out of 90 HHL contacts who demonstrated negativity for salivary antibodies (71.1%) were positive for serum antibodies ( $p < 0.01$ ).

### 3.6. Direct and molecular detection

No HHL contacts presented direct nasal smear detection. *M. leprae* DNA was found in the nasal swabs of nine out of the 85 MB HHL contacts (10.6%) and in three out of the 50 PB HHL contacts (6.0%). Among the 12 contacts who were positive for *M. leprae* DNA (Table 3), five were positive for serum anti-PGL1 IgM, seven were positive for salivary anti-PGL1 antibodies, and one was positive for all of the isotypes, except for salivary anti-PGL1 IgM. This individual was the only one who did not have a BCG scar.

**Table 2**

Frequency of positive and negative serum and salivary anti-PGL1 antibody titers in leprosy household contacts

Salivary IgA and/or antibodies	Serum IgG and/or IgM antibodies		
	Positive, n (%)	Negative, n (%)	Total, n (%)
Positive	21 (46.7%)	24 (53.3%)	45 (100%)
Negative	64 (71.1%)	26 (28.9%)	90 (100%)
Fisher's test	$p < 0.01$		

**Table 3**

Serum and salivary anti-PGL1 titers in multibacillary (MB) and paucibacillary (PB) leprosy household contacts whose nasal swabs were PCR-positive for *M. leprae* DNA. The presence or absence of the BCG scar was also observed. Cut-off values were 1.3 and 0.5 for serum and salivary antibodies, respectively.

Leprosy household contact	Clinical form of index case	Age (years)	BCG scar	Serum		Saliva	
				IgG	IgM	IgA	IgM
C49.3	MB	19	Yes	0.7	<b>3.1</b>	<b>0.64</b>	0.01
C13.1	MB	73	Yes	0.1	0.4	0.14	0.05
C13.2	MB	55	Yes	0.3	0.7	<b>0.58</b>	0.02
C32.1	MB	26	Yes	0.6	<b>2.2</b>	0.18	0.03
C1.3	MB	27	Yes	0.3	0.4	<b>0.50</b>	0.02
C1.6	MB	52	Yes	0.3	0.8	<b>1.70</b>	<b>3.00</b>
C9.4	MB	30	Yes	1.1	1.1	<b>1.10</b>	0.03
C8.4	MB	29	Yes	0.3	0.6	<b>0.60</b>	0.04
C53.1	MB	34	Yes	0.8	<b>1.7</b>	0.08	0.00
C46.2	PB	13	Yes	0.9	<b>3.1</b>	0.10	0.02
C12.4	PB	20	Yes	0.3	1.0	0.13	0.05
C6.1	PB	39	No	1.4	2.1	1.10	0.14

BCG, bacille Calmette–Guérin.

#### 4. Discussion

Early detection of the disease is a strategy to interrupt *M. leprae* transmission and to prevent the occurrence of physical disabilities, a severe consequence of late diagnosis.<sup>1</sup> In endemic areas, sensitive and specific serological tests associated with molecular parameters could be of great use for following contacts at high risk of developing the disease.<sup>11</sup>

When a clinical examination of a leprosy contact is performed, suspected cases can be registered. Bazan-Furini et al.<sup>9</sup> detected new leprosy cases in 3.3% of patient contacts (co-prevalent cases). Similar results have been found by others,<sup>4,28</sup> who observed a rate of co-prevalent cases of 5.2%. In our study, we did not find any co-prevalent case among the contacts.

Many authors refer to anti-PGL1 IgM as the main serological parameter in leprosy.<sup>10,29–31</sup> It is known that the rheumatoid factor is one of the main causes of false-positive values in IgM detection. The false-positivity occurs in toxoplasmosis<sup>32</sup> and other diseases.<sup>33,34</sup> Serum anti-PGL1 IgG could be an important parameter for evaluation because only a few contacts present it. In the present study, 58% of the IgG-negative samples showed positive IgM. On the other hand, 71.4% of the IgG-positive samples were also positive for IgM. Moreover, among 17 volunteers living in a low endemic city, who reported no contact with leprosy patients, none presented seropositivity for IgG; however, two of them showed anti-PGL1 IgM titers above 2.0. This means that both isotypes should always be measured. We believe that those individuals that are positive for both of the isotypes should be followed every year. Additionally, those who present with anti-PGL1 IgM levels with titers above 3.0 (regardless of IgG) should be followed as well. We are doubtful about the usefulness of immunochromatography tests for this purpose, since they are not able to quantify antibodies in samples.

The percentage of leprosy contacts with positive titers of salivary anti-PGL1 IgA antibodies was 32.5%, much lower than the frequency reported by Smith et al. (68%).<sup>13</sup>

We found that 24 out of 45 HHL contacts (53.3%) who were positive for salivary IgA and/or IgM antibodies did not present serum antibodies. Assuming a diagnostic specificity of approximately 91% for the salivary anti-PGL1 antibody test,<sup>17</sup> we can hypothesize that 48% of the individuals were infected but did not develop serum antibodies. This probably means that they are infected with *M. leprae* but that they will not necessarily acquire the disease. Conversely, 64 out of 90 HHL contacts (71.1%) who were negative for salivary anti-PGL1 antibodies presented serum IgG and/or IgM antibodies. It is already known that the maintenance of long-term immunological memory in the mucosal surfaces is a great challenge.<sup>35</sup> After mucosal colonization, the

bacteria can translocate into the systemic circulation, reducing the bacterial load in the nasal surfaces. In this case, it is reasonable to assume that secretory antibodies will decrease, meanwhile serum antibody levels will increase.

*M. leprae* DNA was found in 8.9% of the nasal samples. Other authors have found percentages of positivity varying from 1.6% to 19.35%.<sup>10,12,13,36,37</sup>

One person showed both positive molecular and serological parameters, except for salivary anti-PGL1 IgM, and did not show a BCG scar. According to Lobato et al.,<sup>11</sup> individuals without a BCG scar are at a higher risk of developing leprosy. We believe that this person should be followed up.

We strongly suggest that serum anti-PGL1 IgG/IgM and salivary anti-PGL1 IgA/IgM measurements should be used to follow leprosy household contacts. The detection of nasal *M. leprae* DNA is not essential to follow leprosy contacts because of the high percentage of negative results in the contacts, even those with positive serology.

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*Conflict of interest:* The authors report that there are no disclosures relevant to this publication.

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