Detection of serum antibodies to *M. leprae* Major Membrane Protein-II in leprosy patients from Indonesia


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Summary

**Background** Sero-diagnostic methods are the easiest way of diagnosing an infectious disease in developing countries. In leprosy, phenolic glycolipid-1 (PGL-I) based methods for the detection of leprosy are currently available, but the use of these methods has been hindered due to the inherent problems of sensitivity. We previously showed that antibodies to Major Membrane Protein-II (MMP-II) derived from *Mycobacterium leprae* could be used to diagnose leprosy in Japan.

**Methods** Sera from patients and healthy individuals were collected with informed consent and the anti-MMP-II antibody levels of the sera were measured by enzyme-linked immunosorbent assay. The study was conducted at South Sulawesi and Bali, in Indonesia. The study population included 40 each of multibacillary leprosy and paucibacillary leprosy patients, 30 tuberculosis and 16 patients with typhoid.

**Results** We evaluated the anti-MMP-II antibody levels in Indonesian individuals. The cut-off value was determined from receiver operator characteristic curve as 0·124 using the O.D. titers for patients with multibacillary leprosy, so that the sensitivity of the test was 97·5% and the specificity taking healthy individuals as controls was 98·4%. Using the determined cut-off values, 98% of multibacillary (MB) leprosy and
48% of paucibacillary (PB) leprosy patients had positive levels of anti-MMP-II antibodies, 13% of patients with typhoid and 22% of the household contacts of MB leprosy had positive levels of anti-MMP-II antibodies.

Conclusions Our results suggest that measuring anti-MMP-II antibody levels could facilitate the detection of leprosy in endemic countries.

Introduction

*Mycobacterium leprae*, the causative agent of leprosy, induces skin lesions and peripheral nerve injuries which may lead to physical deformities.\(^1\)\(^,\)\(^2\) Despite efforts by the World Health Organization to reduce the global leprosy burden, 254,525 new patients were detected during 2007, compared with 265,661 during 2006, which indicates that the progress towards complete elimination of leprosy is slow.\(^2\) Timely detection of new patients and prompt treatment is the key to reducing the burden of leprosy. The most widely used antigen for serodiagnosis of leprosy is phenolic glycolipid-I (PGL-I).\(^3\) Simple lateral flow test and dipstick assays, based on detecting the antibodies to the PGL-I antigen, have been developed to classify leprosy patients and identify contacts with an increased risk of developing leprosy.\(^4\)\(^,\)\(^5\) A particle agglutination test has also been developed to detect anti-PGL-I antibodies qualitatively and quantitatively.\(^6\)\(^,\)\(^7\) However, there are limitations in using these kits especially in terms of sensitivity. Numerous antigens of *M. leprae* have been tested for diagnostic feasibility,\(^8\) but their use has been limited due to their poor performance. Duthie et al. performed a world-wide serological study using two *M. leprae* antigens namely ML0405 and ML2331, and found that these two proteins could be recognised by patients’ sera from diverse regions.\(^9\) We have previously identified Major Membrane Protein-II (MMP-II) from the cell membrane fraction of *M. leprae* as one of the antigenic molecules capable of activating both antigen-presenting cells and T cells.\(^10\) When we evaluated the anti-MMP-II IgG levels in Japanese leprosy patients, 82.4% of MB leprosy and 39.0% of PB leprosy patients had positive results.\(^11\) Here, we evaluated the use of MMP-II as a tool for serodiagnosis of leprosy in Indonesia, which is an endemic countries with foci of high leprosy rates.

Material and Methods

Sera were obtained under informed consent from healthy individuals, leprosy patients, household contacts of leprosy patients, tuberculosis patients and typhoid patients from different parts of Indonesia. Sera were frozen at \(-30^\circ\)C until use. All patients who were clinically diagnosed were selected, so that there was no bias in the system, and double-blind test for ELISA was conducted. The population studied included multibacillary (MB) and paucibacillary (PB) leprosy patients, tuberculosis patients and typhoid patients. All patients diagnosed with tuberculosis had either acid fast bacilli in sputum smears stained with Ziel Neelsen stain and/or *M. tuberculosis* grown on culture. Patients diagnosed with typhoid had *S. typhimurium* grown on culture. All patients were newly diagnosed. We defined household contacts of leprosy patients (n = 50), as people living under the same roof and sharing food with the patient for at least 6 months. These contacts were collected either by home visits or in clinics from those family members who accompanied the patients.
Prior to the study, we received ethical clearance from the Ethical Research Committee of the Hasanuddin University and informed consent for participation in the study was obtained from all participants or their parents/guardians. We also received approval from the ethics committee of the National Institute of Infectious Diseases, Tokyo. Classification of leprosy was carried out according to Ridley-Jopling’s classification and WHO recommendations (http://www.who.int/lep/classification/en/index.html). Briefly, Ridley-Jopling’s five group classification system is based on clinical, bacteriological and cytopathological findings, and WHO classification is based on clinical and skin smear test findings. Sera from *M. bovis* BCG-vaccinated healthy volunteers and blood donors (n = 62), who were local ethnic people from Makassar which is an endemic region in Indonesia, were collected from the Makassar blood transfusion unit and used as negative controls in ELISA tests to determine the cut-off.

The MMP-II protein used as antigen was purified as a fusion protein in *E. coli* using pMAL-c2X expression vector (New England BioLabs) as described earlier. The ELISA for the detection of anti-MMP-II IgG antibodies was performed as described as follows: 96 well plates (Imunosorb, Nunc) were coated overnight with MMP-II at a concentration of 2 μg/ml in 0.1 M carbonate buffer (pH 9.5). After blocking with 2% skim milk in PBS (pH 7) (blocking solution), the plates were washed with phosphate buffered saline containing 0.1% Tween 20 (PBST) and human sera (normal, patients or household contacts) diluted 100 times in blocking solution, were added and incubated at 37°C for 2 hours. After washing with PBST, biotinylated anti-human IgG (Vector Laboratories) in blocking solution was added at a dilution of 1:1500 and incubated for 1 hour. The plates were incubated with reagents from the ABC Kit (Vector Lab) in PBST for 30 min. After washing with PBST, substrate solution consisting of 0.2 mg/ml of OPD (o-phenylene diamine) and 0.02% H2O2 in 0.1 M citrate buffer (pH 5.5) was added until colour developed, after which the reaction was stopped with 0.5 M sulfuric acid. Optical density (O.D.) was measured using a Vmax spectrophotometer (Molecular Devices). Plate to plate variations in O.D. readings were controlled by using a common standard serum, and if this reading varied more than 0.05 O.D., then the test was repeated. For detecting anti-PGL-I antibodies, NTP-BSA was coated at a concentration of 0.5 mg/ml, and the same procedure used to detect anti-MMP-II antibodies was followed, except that the secondary antibodies used was biotinylated anti-human IgM (Vector Laboratories) at a dilution of 1:750.

For statistical analysis, Receiver Operator Characteristics (ROC) curves were drawn to describe the relation between sensitivity and specificity at various cut-off levels using the MedCalc software.

### Results

The population studied included multibacillary (MB) (n = 40) and paucibacillary (PB) (n = 40) leprosy patients from several primary health care centers in South Sulawesi and Bali, tuberculosis patients (n = 30) from Tuberculosis Hospital, Makassar and typhoid patients (n = 16) from several primary health care centers in South Sulawesi. Sera from *M. bovis* BCG-vaccinated healthy volunteers and blood donors (n = 62) were used to determine the cut-off value for the assay. The anti-MMP-II IgG antibody levels (Mean O.D. ± S.D.) for MB leprosy, PB leprosy, tuberculosis, and healthy individuals were 0.488 ± 0.152, 0.139 ± 0.096, 0.038 ± 0.023 and 0.074 ± 0.023 respectively. The cut-off value of O.D. 0.124 was defined using a ROC curve, where the specificity of the test was 98.4%
and the sensitivity was 97.5%. 39 out of 40 (98%) MB patients had positive antibody levels, and
19 out of 40 (48%) PB patients were positive for anti-MMP-II antibodies (Table 1).

None of the tuberculosis patients and two of the typhoid patients had antibodies against
MMP-II. One of the typhoid patients had a very high titer of O.D. 0.543, which is
comparable to those of patients with MB leprosy, and therefore this particular patient should
be closely monitored for any clinical signs of leprosy. Only three of the 62 (5%) healthy
individuals exhibited positive anti-MMP-II antibody levels. 

P values between the various
groups in Table 1 were calculated by the comparison of means (student’s t-test) as follows:
$p$ value was less than 0.0001 between MB and PB leprosy, PB and Healthy, MB and
Healthy. When the anti-PGL-I IgM antibody levels were examined using the same leprosy
patients, it was found that only 75% of MB patients and 28% of PB patients had positive
values (Table 2).

The cut-off value of O.D. 0.214 was determined using a ROC curve, where the specificity
of the test was 87.1% and the sensitivity was 75%. PGL-I IgM antibody levels in healthy
individuals seem to be comparatively high, which increases the cut-off value. The antibody
levels were significantly higher in MB patients than the healthy controls ($p < 0.0001$ for
PGL-I). A comparison of antibody levels were in patients with PB leprosy and healthy
controls showed that a MMP-II antibody levels were significantly higher $p < 0.0001$ than
PGL-1 antibody levels ($p = 0.018$ (PGL-I)). These results may indicate that MMP-II based
ELISA is superior to PGL-I ELISA.

In a serological survey of household contacts of 17 leprosy patients we found that 11 of 50
(22%) household contacts had positive anti-MMP-II antibody levels that were significantly

### Table 1. Evaluation of MMP-II-ELISA for serodiagnosis of leprosy in Indonesian individuals

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No.</th>
<th>No. of Positives</th>
<th>No. of Negatives</th>
<th>Percent Positivity</th>
<th>Mean O.D. values ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB leprosy</td>
<td>40</td>
<td>39 (98%)</td>
<td>1</td>
<td>98</td>
<td>0.488 ± 0.152</td>
</tr>
<tr>
<td>PB leprosy</td>
<td>40</td>
<td>19 (48%)</td>
<td>21</td>
<td>48</td>
<td>0.139 ± 0.096</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>30</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0.038 ± 0.023</td>
</tr>
<tr>
<td>Typhoid</td>
<td>16</td>
<td>2</td>
<td>14</td>
<td>13</td>
<td>0.076 ± 0.132</td>
</tr>
<tr>
<td>Household Contacts</td>
<td>50</td>
<td>11</td>
<td>39</td>
<td>22</td>
<td>0.109 ± 0.079</td>
</tr>
<tr>
<td>Healthy</td>
<td>62</td>
<td>3</td>
<td>59</td>
<td>5</td>
<td>0.074 ± 0.023</td>
</tr>
</tbody>
</table>

Cut-off value of O.D. 0.124 was defined by using the ROC curve.

### Table 2. Comparison of MMP-II and PGL-I based ELISA in Indonesian individuals

<table>
<thead>
<tr>
<th>Group</th>
<th>Total No.</th>
<th>MMP-II (IgG)</th>
<th>PGL-I (IgM)</th>
<th>Mean O.D. ± SD (PGL-I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB leprosy</td>
<td>40</td>
<td>39 (98%)</td>
<td>30 (75%)</td>
<td>0.345 ± 0.174</td>
</tr>
<tr>
<td>PB leprosy</td>
<td>40</td>
<td>19 (48%)</td>
<td>11 (28%)</td>
<td>0.158 ± 0.108</td>
</tr>
<tr>
<td>Healthy</td>
<td>62</td>
<td>3 (5%)</td>
<td>9 (15%)</td>
<td>0.114 ± 0.077</td>
</tr>
</tbody>
</table>

Cut-off value of O.D. 0.214 was defined by using the ROC curve.
<table>
<thead>
<tr>
<th>Patient No.</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
<th>#6</th>
<th>#7</th>
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<th>#12</th>
<th>#13</th>
<th>#14</th>
<th>#15</th>
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<th>#17</th>
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<tbody>
<tr>
<td>Patient Type</td>
<td>LL</td>
<td>LL</td>
<td>LL</td>
<td>BL</td>
<td>BL</td>
<td>BL</td>
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<tr>
<td>B.I. value</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
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<td>2</td>
<td>2</td>
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<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>O.D. value</td>
<td>0.588</td>
<td>0.514</td>
<td>0.616</td>
<td>0.551</td>
<td>0.521</td>
<td>0.530</td>
<td>0.482</td>
<td>0.427</td>
<td>0.607</td>
<td>0.600</td>
<td>0.596</td>
<td>0.590</td>
<td>0.198</td>
<td>0.574</td>
<td>0.386</td>
<td>0.106</td>
<td></td>
</tr>
</tbody>
</table>

Household contacts of each patient

| No.1 | 0.078 | 0.103 | 0.233 | 0.102 | 0.121 | 0.140 | 0.117 | 0.362 | 0.096 | 0.138 | 0.036 | 0.170 | 0.057 | 0.126 | 0.045 | 0.123 | 0.171 |
| No.2 | 0.099 | 0.134 | 0.063 | 0.115 | 0.084 | 0.082 | 0.036 | 0.171 | 0.094 | 0.208 | 0.080 | 0.115 |
| No.3 | 0.062 | 0.127 | 0.055 | 0.082 | 0.069 | 0.036 | 0.077 | 0.081 | 0.045 | 0.123 | 0.049 |
| No.4 | 0.061 | 0.118 | 0.078 | 0.044 | 0.074 | 0.062 | 0.053 | 0.049 | 0.053 | 0.049 |
| No.5 | 0.037 | 0.112 | 0.074 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 |
| No.6 | 0.106 | 0.062 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 |
| No.7 | 0.094 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 |
| No.8 | 0.092 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 |
| No.9 | 0.084 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 | 0.049 | 0.053 |

O.D. values in bold represents those testing positive for anti-MMP-II antibodies.
higher \( p = 0.0012 \) than antibody levels in healthy controls. Table 3 shows the anti-MMP-II antibody titers of the patients arranged in descending order of the BI value and their household contacts.

Among the 17 leprosy patients included in our survey, eight had one or more household contacts with detectable anti-MMP-II antibodies. Patient #3, classified as LL (Bacterial Index = 4) had an O.D. titer of 0.510. Three of his nine household contacts, had detectable anti-MMP-II antibodies, although they had no clinical signs of leprosy.

Discussion

Elimination of leprosy necessitates not only treatment of leprosy by multi-drug therapy (MDT), but also early detection of patients. However, leprosy still remains a disease whose mode of transmission is unclear. Contact with MB patients is widely accepted to be responsible for the transmission of the bacilli, and the detection rate of new leprosy patients has not diminished. Thus, a survey for new cases especially in endemic regions is necessary to control the spread of the disease. For this purpose, the most easily performable method is the serological testing by a simple kit, but which antigen(s) are best suited for such a test is still not clear. A combinatorial approach using anti-phenolic glycolipid- I (PGL-I) and anti-45 kDa antibodies for detection of \( M. leprae \) infection seems to provide a method with higher specificity. Also prevalence of antibodies to culture filtrate protein-10 is observed in 83.3% of BI-positive leprosy patients. A survey of antibodies to these antigens in different leprosy endemic countries would provide further information for the diagnostic utility of these antigens in diagnosing leprosy. We previously identified MMP-II as one of the \( M. leprae \) components capable of stimulating CD4\(^+\) and CD8\(^+\) T cells, and the protein was originally recognised to be identical to mycobacterial bacterioferritin. Deshpande et al. reported that sera from leprosy patients have higher IgG titer to MMP-II, regardless of the clinical type of leprosy. Recently, we performed a comparative study among Japanese leprosy patients and discovered that MMP-II is superior to PGL-I because it was able to detect 82% of multibacillary and 39% of paucibacillary leprosy.

The present study included a population from a country geographically distinct from Japan, namely, Indonesia, in which leprosy is still endemic. There were 17 682 new patients detected in 2006, which places Indonesia among the top three countries having more than 10 000 new patients per year. We evaluated the anti-MMP-II antibody titers in leprosy patients as well as non-leprosy patients, such as tuberculosis and typhoid patients, taking the healthy blood donors as controls, and found that 98% of MB patients and 48% of PB patients had detectable anti-MMP-II antibodies. The reason for the higher positive rate observed in Indonesian leprosy patients, may be partly due to the highly active disease state of the patients and because all patients were newly diagnosed, so that all sera were collected before the start of chemotherapy. When we evaluated the household contacts of patients, 22% of these household contacts were positive for the anti-MMP-II antibodies. One of the patients examined had no detectable anti-MMP-II antibodies, but one of his household contacts had a positive titer for anti-MMP-II antibodies. Again, these contacts should be carefully monitored serologically as well as for any other clinical signs of leprosy in order to promptly initiate chemotherapy. Also, attention should be given to patients who are HIV positive or immuno-compromised individuals, since their immune system may not produce sufficient antibodies for detection. In those grounds, ELISA tests are generally sensitive and specific, if a suitable
immunogenic antigen is available. But in leprosy endemic countries we need to develop more simple tests like the visibly detectable dip-stick test, but dip-stick tests are still not as sensitive, and therefore when the level of antibodies is low, the detection by a simple system is still not feasible.

This report indicates that MMP-II could contribute to the sero-detection of MB as well as PB leprosy patients in leprosy endemic countries, where other environmental mycobacterial infections may be more prevalent, such as in Indonesia. During the course of this study, we observed that in Vietnam 85% of MB patients and 48% of PB patients were positive for anti-MMP-II antibodies. The development of sensitive, user-friendly tools for the detection of anti-MMP-II antibodies, may further contribute to the prompt detection of leprosy.

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