Utility of serodiagnostic tests for leprosy: a study in an endemic population in South India

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Summary In order to evaluate the usefulness of natural disaccharide (PGL1) and 35 kDa antigens based serology in diagnosis of leprosy and in detecting high risk groups for leprosy, this study was conducted in an endemic population in South India. Out of 3346 cases and their households and neighbouring household contacts, serum samples from 2994 and 2875 individuals were screened for antibodies against PGL1 and 35 kDa antigens respectively. While the overall positivity for contacts and leprosy cases was 3.3% for PGL1 antibody, the positivity for 35 kDa antibody was 6.3%. The positivity for contact population was 2.7% and 5.4% for PGL1 and 35 kDa antibodies, respectively. Lepromatous and borderline lepromatous patients showed positivity of 35.1% for PGL1 antibody and 45.7% for 35 kDa antibody. Follow-up of contacts showed that the majority (>90%) remained seronegative for both the antibodies and most of the new cases emerged from the seronegative group. The study clearly indicates that specific serological assays are not sensitive enough for application, both for diagnosis and for identifying any individual at risk for leprosy in the south Indian endemic population.

Introduction

With the establishment of antibody based serological assays using antigens of Mycobacterium leprae, efforts were directed towards their use in the diagnosis of leprosy and their potential use for determining subclinical infection with special emphasis on the detection of future multibacillary cases of leprosy.

Several serological tests have been developed for the diagnosis of leprosy.1–5 Two serological tests, one based on antibody response to phenolic glycolipid-1 (PGL1)/natural

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disaccharide (PGL1) BSA conjugated (PGL1-O-BSA) antigen\textsuperscript{3,4} and the other based on antibody response to 35 kDa antigen,\textsuperscript{5} were reported as specific and reproducible in several laboratories in the world.\textsuperscript{6–11}

Several studies have been carried out using these assays to find out their utility in identifying leprosy cases.\textsuperscript{3–11} In the recent past, PGL1/PGL1 based enzyme linked immunosorbent assay (ELISA) has been used by several workers in field conditions to find out its suitability as a screening test for leprosy infection in patients and contacts with equivocal results.\textsuperscript{12–18} The present study was carried out in an endemic population for evaluation of both PGL1-based and 35 kDa-based serological assays and in the diagnosis of cases of leprosy and in identifying contacts at risk for getting leprosy. The study was conducted as part of several pre-vaccine trial studies in the late 1980s. The results are being presented in view of their continuing relevance to understand leprosy epidemiology.

**Materials and methods**

The baseline survey for immuno-epidemiological studies in leprosy was completed between August 1987 and February 1988. The first follow-up at the end of 6 months was between May and June 1988, and the 1-year follow-up was between February and April 1989.

Three hyperendemic villages for leprosy with a total population of approximately 15,000 were selected. Initial survey was undertaken to detect all the patients of leprosy. All the members of the patients’ household and all the members of the immediate neighbouring household were included in the study as contacts. In the baseline survey 3346 individuals were examined and blood samples could be collected from 3287 individuals. Healthy contacts were followed up every 6 months for 1 year for detecting incidence cases of leprosy. All the clinical examinations were performed by trained and standardized field workers and cases or suspects were confirmed by a medical officer. All the examiners and medical officer did not know previous clinical or serological status of the study subjects.

**CLASSIFICATION OF PATIENTS**

Leprosy patients were classified clinically broadly based on number of lesions following the earlier Indian Association of Leprologists (IAL) classification.\textsuperscript{19} In addition, neuritic patients and doubtful cases (not satisfying all the cardinal signs of leprosy) were also included in the study. The patients and contacts were classified into different categories from 0 to 9 based on clinical examination and skin smear examination for acid fast bacilli [0 = no leprosy, 1–3 = doubtful cases, 4 = neuritic (N), 5 = indeterminate (I), 6 = tuberculoid (TT), 7 = borderline tuberculoid (BT), 8 = borderline lepromatous (BL), and 9 = lepromatous leprosy (LL)].

**MICROSCOPIC EXAMINATION OF SKIN SMEARS**

Skin smears were taken from all suspects and leprosy cases to identify the presence of acid-fast bacilli (AFB). The smears were taken using a standard procedure and were stained by acid-fast staining before subjecting them to oil immersion light microscopy and were classified bacteriologically on Ridley’s scale.
COLLECTION OF BLOOD SAMPLES

The method of Burgess et al.\textsuperscript{20} was followed for collection of capillary blood obtained by finger prick. Four drops of blood were collected from each donor on pieces of Whatman no.3 filter paper (Whatman International Limited, Maidstone, UK) and air dried. For children below 5 years of age, 0.5 ml of intravenous blood was collected. The dried samples were allotted coded numbers and were sealed in polythene envelopes and sent to the laboratory at CJII, Agra by mail for analysis. Information on clinical symptoms or diagnosis of leprosy of these individuals was not provided to the laboratory. After receiving the samples the polythene envelopes containing the samples were preserved at 4°C until use.

SEROLOGICAL ASSAYS

Filter paper blood samples were processed for antibody assays within 1–6 months from the day of collection in the field.

All the laboratory investigations were performed by trained laboratory staff without knowledge of the clinical status of the study subjects.

ELISA for determination of antibodies against \textit{M. leprae} specific epitopes on the 35 kDa protein and PGL1 was performed after elution of samples from blood spots collected on filter paper. A metallic punch of 16 mm diameter was used to cut out the central portion of blood blot from each filter paper and each punched out filter paper disc (corresponding to approximately 50 μl of whole blood) was placed in wells of a 24-well tissue culture plate (Laxbro, Pune, India). Phosphate buffered saline 250 μl (0.1 mol/l, pH 7.4) was added in each well and the plates were kept in a rocking platform overnight at 4°C in a humid box for elution. The eluted samples which corresponded to approximately 1:10 serum dilution were used for ELISA.\textsuperscript{21}

35 kDa ANTIBODY ASSAY

The assay was performed by competition ELISA as reported earlier.\textsuperscript{22} ELISA plates (NUNC, Denmark) were coated with soluble antigen (2.5 μg/50 μl/well at +4°C overnight) of \textit{M. leprae} (armadillo derived, procured from IMMLEP, WHO through Dr R. J. W. Rees, National Institute for Medical Research, London, UK). After removing the antigen solution, plates were blocked (2 h at room temperature) with 1% skimmed milk powder (Anik Spray; Lipton India Limited) in Tris buffered saline (0.01 mol/l Tris, 0.15 mol/l saline, pH 7.4) containing 0.05% Tween 20 (TBST). Antigen coated wells in duplicate were incubated (60 min, 37°C) with serial 10-fold dilution of each sample (25 μl/well). The incubation was continued for a further 120 min at 37°C after addition (25 μl/well of 1:1000 dilution in 1% milk) of peroxidase conjugated monoclonal antibody, MLO4 (kindly provided by Professor J. Ivanyi, MRC Laboratory, Hammersmith Hospital, London, UK). The plates were washed with TBST and colour was developed with O-phenylene diamine (Sigma, USA) substrate solution (50 μl/well, 20 min, 37°C). The reaction was stopped by adding 50 μl/well 2.5 NH\textsubscript{2}SO\textsubscript{4} and optical densities (ODs) were read at 492 nm using an ELISA reader (Titertek, Multiskan, Flow Laboratories, Sweden). Relative percent bindings were calculated (using 100% as the OD value for binding of MLO4 alone to the antigen well) and plotted against corresponding serum dilution. The dilution that would cause 50% inhibition of MLO4 binding is referred to as ID\textsubscript{50} titre. On the basis of results obtained with non-leprosy controls,
a serum with ID$_{50}$ titre of 1:10 or more was considered positive. We have earlier reported a good correlation between the results of ELISA obtained using sera and corresponding blood blot samples.\textsuperscript{21}

**PGL1 Antibody Assay**

The ELISA for anti-PGL antibodies was performed following the reported protocols.\textsuperscript{22,23} Briefly, wells of ELISA plates were coated with either the synthetic antigen PGL1-O-BSA (kindly provided by Dr Delphi Chatterjee, Colorado State University, Fort Collins, USA) (1 μg carbohydrate/50 μl/well, overnight at 4°C) or with coating buffer. After blocking with 1% milk-TBST, a dilution of each sample equivalent to 1:300 serum was incubated (50 μl/well, 37°C, 60 min) in four wells (a pair of each of antigen coated and buffer coated). The plates were washed with TBST and incubated with 1:2000 dilution of peroxidase conjugated affinity purified anti-human IgM antibody (Sigma, USA; 50 μl/well, 37°C, 60 min). The remaining steps (washing, colour development, reading) were same as for 35 kDa ELISA. On the basis of results obtained with non-leprosy control sera (mean SD), a serum sample (1:300) was regarded positive if it showed an OD of >0.2.\textsuperscript{23}

**Results**

**PGL1 Antibody**

Out of a total of the 3287 cases and contacts, samples from 2994 of PGL1-antibody tested individuals were analysed (Table 1). The remaining samples from 293 (about 9%) individuals could not be analysed due to wastage of samples during storing, processing and testing.

The overall positivity for antibody was 3.3% (100 out of 2994). Of all the confirmed cases of leprosy (406), 32 cases were positive (7.9%). Further, a total of 68 out of 2555 (2.7%) contacts showed positivity. However, maximum positivity of 35.1% (13 out of 37) was noted in BL/LL cases.

**Table 1.** Distribution of contacts and patients by readings to PGL1-O-BSA and 35kDa antibodies at the baseline survey

<table>
<thead>
<tr>
<th></th>
<th>Number of contacts and leprosy cases</th>
<th>Leprosy category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1–3</td>
</tr>
<tr>
<td>PGL1-O-BSA, test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive*</td>
<td>68 (2555)</td>
<td>0 (33)</td>
</tr>
<tr>
<td>% positive</td>
<td>2.7</td>
<td>0</td>
</tr>
<tr>
<td>35 kDa, test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive**</td>
<td>133 (2318)</td>
<td>3 (32)</td>
</tr>
<tr>
<td>% positive</td>
<td>5.4</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate total samples tested.

* Any OD value of ≥ 0.2 was considered positive.

** Any titre ≥ 1:10 was considered positive.

Leprosy category 0 = no leprosy, 1–3 = doubtful cases, 4 = neuritic, 5 = indeterminate, 6 = tuberculoid,
35 kDa Antibody

Out of a total of 3287 cases and contacts, samples from 2875 could be analysed (Table 1). The remaining samples from 412 individuals (about 13%) could not be analysed due to the similar reasons as stated above under PGL1 antibody.

The overall positivity for the antibody in the study population of cases and contacts was 6.3% (182 out of 2875). It could be observed that 11.7% (46 out of 392) of leprosy cases were positive for the antibody. A total of 133 contacts out of 2451 (5.4%) were positive (not statistically significant difference at 5% level). Amongst the leprosy groups the highest positivity was noted in BL/LL group (16 out of 35; 45.7%).

PGL1 and 35 kDa Antibody in Smear Positive Patients

Out of the 16 smear positive individuals examined by both the serological tests, eight were seropositive for both. An additional single individual was seropositive for each of the tests individually.

Age and Sex Distribution

The age and sex distribution for all the individuals in the baseline survey with results of both the serological tests were considered. It was observed that 3.67% of the females were PGL1 antibody positive and 2.97% males were positive (no statistically significant difference at 5% level). Positivity for 35 kDa antibody was 5.92% for females and 6.79% for males (difference not statistically significant at 5% level). Amongst the contacts, positivity for antibody was similar in adults (15+ years) and children. For PGL1-O-BSA, 3.0% adults and 2.1% children were positive. For 35 kDa, 5.3% adults and 5.7% children were positive. In both the cases the difference was not significant at 5% level.

Follow-up Study

To find out how many of the healthy contacts (neighbourhood/household) who were found positive (after initial screening by employing the above mentioned serological assays)

| Table 2. Distribution of normal contacts at baseline by PGL1-O-BSA and 35 kDa tests during resurvey |
|--------------------------------------------------|----------------------------------|-----------------|-----------------|-----------------|
| Test/results | Number of persons examined | Leprosy status at II or III rounds |
|               |                               | Normal | 1–3 | 4–9 |
| PGL1-O-BSA   |                               |       |     |     |
| Negatives    | 1351                          | 1272  | 21  | 58  |
| Positives    | 26                            | 25    | –   | 1   |
| 35 kDa       |                               |       |     |     |
| Negatives    | 1249                          | 1173  | 20  | 56  |
| Positives    | 81                            | 75    | 2   | 4   |

0–9 = same as Table 1.
became clinical cases in future, the population was re-examined and followed up with two rounds in the field at intervals of 6 months each for 1 year. A total of 1377 individuals of 2487 could be clinically re-examined. It was noted that 58 out of 1351 (4.3%) became clinical cases from PGL1 antibody negative group and 1 (3.8%) became a case from the 26 positive individuals (Table 2). Similarly, 1330 of 2318 could be clinically re-examined for the 35 kDa antibody group. While 56 out of 1249 (4.5%) manifested clinical signs of leprosy in the negative group, only four out of 81 (4.9%) from the 35 kDa positive group developed into cases of leprosy (Table 2).

All the 33 clinical suspects, examined in the resurveys, were PGL1-O-BSA negative at baseline survey. From this group 17 cases were detected.

Of the 32 clinical suspects 35 kDa tested at baseline, 29 were seronegative and three were seropositive at baseline survey. Sixteen cases were detected from the negatives and one case from the positives during the resurveys.

Discussion

After the finding of PGL1 and 35 kDa specific antigens, the antibody levels to these antigens have been shown to be associated with severity of disease or bacterial load in the patient.24–27 The present study was conducted to evaluate the utility of both PGL1 and 35 kDa antibody based serological tests in the diagnosis of leprosy and to predict future cases of leprosy in household/neighbouring contacts in a field area in south India. Because of the committed preparatory activities for a large vaccine trial, we could not examine large proportion of individuals for resurveys. However, there was no selective coverage for any groups for examination purposes. All the clinical examinations were done by our field staff without prior knowledge of leprosy or serological status of the study subjects. The criterion for selection of a seropositive individual for PGL1 antibody with OD value of >0.2 was based on our previous study after screening a large number of normal contacts.22 The same criterion of selection for a positive case was also applied by Cartel et al.17 in their field study with Polynesian population. In an endemic situation, the background cut off of control samples remains very high leading to lower levels of seropositivity. This high level of cut-off point is also likely to lead to misclassification and thus artificial inflation of seronegativity. The finding of similar and lower level of seropositivity in adults and children highlights the non-specificity of the two serological tests. We did examine different levels of cut offs, but none was found to be sufficiently sensitive and specific.

The study indicated that the overall positivity rates for both the serological tests in the contact population was low. Even for overt leprosy cases, the antibody positivity was only 6.3% and 3.3% for 35 kDa and PGL1 antigens, respectively. In the contact population, the positivity was noted to be 5.4% and 2.7% for 35 kDa and PGL1 antigens, respectively. A similar finding of a very low PGL1 antibody positivity was reported by others.16,17,27 It was further noted that in this field area, although the clinical leprosy prevalence rate was high, about 135 per 1000 in the selected group of cases and their contacts, the seropositivity rates in the healthy contact population was quite low (Table 1). Fine and Cartel earlier.16,17 also obtained similar findings in Malawi and Pacific islands respectively. Further, Fine et al.,16 from their study in Malawi, concluded that the PGL1 based assay is not sensitive enough for discrimination between contacts and non-contacts under field conditions.

Klatser28 indicated the importance of PGL1 based serological test (M. leprae dip stick
ELISA) for survey of household contact population. However, in the present study when seropositive contacts were followed up using specific PGL1 and 35 kDa based serology only one out of 26 and four out of 81 developed leprosy, respectively, within 6–12 months. In contrast, the follow-up of large number of seronegative contacts revealed 58 cases out of 1351 and 56 cases out of 1249 for PGL1 and 35 kDa groups, respectively. The majority of cases appeared from a large number of seronegative groups. In view of the small numbers of cases and possible different distributions for age, sex and other factors, we have not attempted any statistical analysis to check for significance of this finding. However, the incidence rates were very similar in both the seropositive and seronegative individuals. Thus these two serological tests do not have any added advantage for application in field situations for selective surveillance for seropositive individuals for detecting cases or for prophylactic interventions.

References


Consensus Classification of leprosy approved by IAL. *Lepr Ind*, 1982; **54**: 17–35.


