Low temperature incubation improves the performance of anti-phenolic glycolipid-I antibody detecting ELISA in leprosy patients

Leprosy, caused by *Mycobacterium leprae*, is a devastating human disease where damage to the peripheral nerves leads to deformities of the hand, foot, face and in some cases eye. A strategy to diagnose leprosy at an early stage and subsequent treatment could help in preventing both serious disability in the patient and transmission, as untreated leprosy is contagious. As of now, accurate and efficient diagnosis of leprosy is considered as a great challenge for leprosy health care. Employing the skin smear test, leprosy patients are classified as smear-positive (for acid-fast bacilli) multibacillary (MB) and smear-negative (for acid-fast bacilli) paucibacillary (PB) types (World Health Organization, 1988). Regarding serology in leprosy, phenolic glycolipid-I (PGL-I) is a highly specific antigen of *M. leprae* (Hunter & Brennan, 1981) and is known to give rise, predominantly, to IgM antibodies against its terminal trisaccharide. Using PGL-I based antibody detecting tests, well over 90 % of MB patients have high levels of antibodies whereas 15–40 % of the PB patients have detectable antibodies (Buhner *et al.*, 1998; Chanteau *et al.*, 1989, 1993; Cho *et al.*, 1983, 1991; Fujiwara *et al.*, 1984; Hussain *et al.*, 1990; Roche *et al.*, 1991; Saad *et al.*, 1990). Thus, the major limitation of the serological assays has remained the detection of a limited number of smear-negative PB patients that could be due to an undetectable weak antibody response to *M. leprae*. Hence, a strategy to improve the detection rate for such patients is clearly needed. In the present study we have investigated the effect of incubating the reactants at low temperature in order to enhance the performance for detecting antibodies to phenolic glycolipid-I antigen.

Study sera included samples from 56 leprosy patients collected at National JALMA Institute for Leprosy and Other Mycobacterial Diseases. The diagnosis was made by clinical criteria defined by World Health Organization (1998), according to which persons with one or more

characteristic symptoms, such as hypopigmented or reddened skin lesion(s) with definite loss of sensation, and/or involvement of the peripheral nerves as demonstrated by palpable thickening with loss of sensation, and skin smear positivity for acid-fast bacilli, were included as leprosy patients. The skin smears from all leprosy patients were tested for the presence of acid-fast bacilli by Ziehl-Neelsen staining. Our study included 18 smear-positive MB patients and 38 smear-negative PB patients. All of these patients either were untreated or had started therapy less than one month earlier. Additionally, serum samples from 15 clinically active pulmonary tuberculosis patients, 14 other skin diseased patients and 20 healthy individuals were included as controls. Serum samples were obtained and stored at -20°C until use. Approval for the study was obtained from our institute's ethics committee for scientific researches. Informed consent was obtained from the patients and controls.

Antibodies against PGL-I were detected by ELISA as described previously (Parkash *et al.*, 2006). Our variations to this assay were that the serum samples added in the wells were incubated: for 2 h at 37°C for the conventional assay, for 24 h at 4°C for the one-step modified assay and for 2 h at 37°C followed by 24 h at 4°C for the two-step modified assay. To score a test as positive, cut-off points were determined using receiver operator characteristic curve analysis by calculating the percentage of true positives and false positives at various absorbance values obtained by ELISA. The absorbance value at which assay performance was optimal [(true positives + true negatives)/total sample number] was used as a cut-off point. The cut-off points for the conventional assay, one-step modified assay and two-step modified assay were 0.240, 0.400 and 0.375, respectively. A sample with an absorbance value of greater than the cut-off value in the respective assays was considered as positive. The differences between groups, with

respect to the proportion of positively testing subjects, were assessed by chi-square test.

The number of positives and percentage positivity for all the assays are given in Table 1. On considering the results of all leprosy patients (MB + PB) together, the sensitivity of single-step modified approach was found to be higher than that of the conventional approach, but lower than that of two-step modified approach. However, the sensitivities of the three assays did not differ significantly ($P > 0.05$) from one another. Surprisingly, one sample from a patient with a bacterial index (a criterion to estimate the bacterial density in the leprosy lesion) of 3^{+} was negative in all the assays. The failure to detect this patient could be due to immune-complex formation of anti-PGL-I antibody with the corresponding antigen, thus making the antibody unavailable for the assay. Specificity remained appreciably high (95.9–100 %) and statistically similar ($P > 0.05$) with all the assays. However, on further analysis, it became evident from the data that in case of two-step modified approach, the sensitivity with smear-positive MB patients was similar (94.4 %) to that of the conventional or one-step modified assay; whereas, in the smear-negative PB group it was (68.4 %) significantly higher ($P < 0.05$) when compared to (36.8 %) the conventional assay but not significantly higher (68.4 vs 52.6 %; $P > 0.05$) than the one-step modified assay. Added to this, all the samples positive by the conventional assay were also scored as positive by the one-step modified assay. Though the positivity with the one-step assay was higher than the conventional assay, the results of the two assays were statistically similar ($P > 0.05$).

Importantly, some heterogeneity among the results of the various assays was observed. Six samples negative by the conventional assay were found to be positive by the one-step assay. Among the 3

Table 1. Results of ELISA for detection of anti-PGL-I antibodies by conventional and modified assays

Group	No. of patients	Conventional		One-step modified		Two-step modified	
		No. positive	Positivity (%)	No. positive	Positivity (%)	No. positive	Positivity (%)
Leprosy							
MB	18	17	94.4	17	94.4	17	94.4
PB	38	14	36.8	20	52.6	26	68.4*
Total	56	31	55.4	37	66.1	43	76.8
Control							
Tuberculosis	15	0	0	0	0	1	6.7
Other skin disease	14	1	7.1	0	0	0	0
Healthy	20	1	5.0	0	0	0	0
Total	49	2	4.1	0	0	1	2.0

*Significantly higher when compared with the conventional assay for the detection of PB patients.

assays, the sensitivity of the two-step assay was highest, where 14 samples negative by the conventional assay were found to be positive by the two-step assay, whereas 2 samples negative by the two-step assay were positive by the conventional assay. Further, 11 samples negative by the one-step assay were found to be positive by the two-step assay, and 5 samples negative by the two-step assay were positive by the one-step assay.

Next, we analysed the results in a joint fashion (Table 2). When all leprosy patients (MB and PB) were considered together, the combined sensitivity (85.7 %) of the conventional + one-step + two-step assays was found to be similar to that (85.7 %) of the one-step + two-step assays in

combination implying a 30.3 % increase ($P < 0.001$) in sensitivity over that of the conventional anti-PGL-I antibody detecting assay. However, the specificity (98.0 %) of the one-step + two-step assays was slightly better than that (93.9 %) of the conventional + one-step + two-step assays in combination. In terms of efficiency [(true positives + true negatives)/total], the efficiency (91.4 %) of the one-step + two-step assays in combination was found to be marginally better than that (90.5 %) of the conventional + one-step + two-step assays in combination. However, considering the MB group separately, the joint and individual sensitivities (94.4 % in each) of the one-step and two-step assays remained equivalent to that (94.4 %) of the conventional assay. On the other hand, with

PB leprosy patients, a joint sensitivity of 81.6 % (31/38) was observed, giving an increase to the tune of 44.8 ($P < 0.001$), 29.0 ($P < 0.001$) and 13.2 % ($P > 0.05$) over that of the conventional, one-step and two-step assays, respectively. Thus, the combinatorial approach of the one-step and two-step assays appears to be the most sensitive one though statistically not significantly different ($P > 0.05$) from the combined approach of the conventional and two-step assays.

In conclusion, a joint approach using the one-step and two-step assay is worth recommending. In this approach, all samples might be screened first by the one-step modified assay and then all the negatives could be repeated using the

Table 2. Results of ELISA for detection of anti-PGL-I antibodies by various combinations of conventional and modified assays

Group	No. of patients	Conventional + one-step modified		Conventional + two-step modified		One-step modified + two-step modified		Conventional + one-step modified + two-step modified	
		No. positive	Positivity (%)	No. positive	Positivity (%)	No. positive	Positivity (%)	No. positive	Positivity (%)
Leprosy									
MB	18	17	94.4	17	94.4	17	94.4	17	94.4
PB	38	20	52.6	28	73.7	31	81.6*	31	81.6*
Total	56	37	66.1	45	80.4	48	85.7†	48	85.7†
Control									
Tuberculosis	15	0	0	1	6.7	1	6.7	1	6.7
Other skin disease	14	1	7.1	1	7.1	0	0	1	7.1
Healthy	20	1	5.0	1	5.0	0	0	1	5.0
Total	49	2	4.1	3	6.1	1	2.0	3	6.1

*Significantly higher when compared to the conventional + one-step modified assays for the detection of PB patients.

†Significantly higher when compared to the conventional + one-step modified assays for the detection of all leprosy patients.

two-step modified assay. Adopting this algorithm would lead to the detection of about 44.8 % more smear-negative PB leprosy patients, who otherwise would have been missed using the conventional assay. However, the disadvantage of the described procedure is that it is time consuming as it takes 24 h more than the conventional assay and hence, the results cannot be obtained on the same day as blood sampling. Since leprosy is a chronic disease obtaining a better diagnosis should make a 24 h delay acceptable.

Acknowledgements

We are grateful to Dr P. J. Brennan and Dr J. S. Spencer from Colorado State University, USA, and the NIH/NIAID contract no. 01 AI 25469 for providing the ND-O-HSA antigen used for ELISA and to LEPRO, UK, for financial support. Thanks to Ms Astha Nigam, Department of Biochemistry, School of Life Sciences, Dr B. R. Ambedkar University, Agra, and thanks to Dr B. K. Girdhar, National JALMA Institute for Leprosy and Other Mycobacterial Diseases for providing samples and other relevant details of the patients. Thanks to Dr A. Geluk from LUMC, The Netherlands, for critical evaluation of the manuscript and to Avnish Kumar for his co-operation.

**Om Parkash,¹ Ajay Kumar,²
Amit Kumar³ and Mohan L. Arora⁴**

¹National JALMA Institute for Leprosy and Other Mycobacterial Diseases, TajGanj, Agra-1, India

²Department of Biomedical Sciences,

Bundelkhand University, Jhansi, U.P., India

³Department of Biochemistry, A.P.S. University, Rewa, M.P., India

⁴Department of Tuberculosis and Chest Diseases, Sarojini Naidu Medical College, Agra, India

Correspondence: Om Parkash
(om1234@gmail.com)

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