

## Serological heterogeneity against various *Mycobacterium leprae* antigens and its use in serodiagnosis of leprosy patients

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae* primarily affecting the peripheral nerves and skin. It still continues to be a health problem mainly in endemic countries such as India, Brazil, Myanmar, Madagascar, Nepal, Mozambique, Democratic Republic of Congo, Tanzania, Angola and the Central African Republic (World Health Organization, 2005). Worldwide, more than 400 000 new cases of leprosy are reported each year and most (more than 85.0%) originate from the endemic countries mentioned above. Routinely, the disease is diagnosed clinically by specialists. However, due to integration of leprosy control programmes into general health services, the continued availability of sufficiently experienced health workers to diagnose leprosy is being threatened. Hence support tools are needed to ensure correct and timely diagnosis as well as treatment of leprosy patients even in the situation of non-availability of a specialist's diagnosis. Assays based on the detection of specific antibodies in serum are considered attractive due to their technical simplicity, speed, low cost and easy implementation under the conditions commonly encountered in developing countries. Today, the most extensively studied serological tests to detect *M. leprae* infection are IgM antibody detecting assays based on the *M. leprae*-specific phenolic glycolipid-I or its analogue (Oskam *et al.*, 2003). However, these tests are limited by the fact that about 60–85% of the leprosy patients with low bacterial load (paucibacillary or PB patients) lack detectable serum antibody levels. We have therefore investigated the potential of other *M. leprae*-specific antigens, such as the *M. leprae* 45 kDa, ESAT-6 and CFP-10 antigens (Parkash *et al.*, 2006a, b, 2007). However, even though the 45 kDa antigen performed better than PGL-I in similar assays, our studies have indicated that these antigens in general are not recognized widely enough by serum

samples from leprosy patients, particularly PB patients (sensitivities ranged from 39 to 64%), for the development of tests with sufficiently increased sensitivity to help diagnose all PB leprosy patients. In the present study, we have analysed the serological heterogeneity among assays based on PGL-I, 45 kDa antigen, ESAT-6 and CFP-10, and explored the performance of a combinatorial approach employing these four *M. leprae* antigens.

Approval for the study was obtained from our institute's (National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra) ethics committee for scientific research. After informed consent was obtained, blood samples from the patients and controls were collected. The diagnosis was based upon clinical criteria defined by the 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 patients were classified (World Health Organization, 1988) as multibacillary (MB) or PB according to the presence or absence, respectively, of acid-fast bacilli (detected by Ziehl–Neelsen staining) in the skin smears. As controls, serum samples from clinically active pulmonary tuberculosis patients, patients with other skin diseases and healthy individuals were collected. In all, serum samples from 97 subjects were examined. The subjects included 17 smear-positive MB leprosy patients and 33 smear-negative PB leprosy patients. All of these patients were either untreated or had started therapy less than 1 month earlier. Serum samples from 13 clinically active pulmonary tuberculosis patients, 14 patients with other skin diseases and 20 healthy individuals were included as controls. After collecting,

serum samples were stored at  $-20^{\circ}\text{C}$  until use.

Antibodies against PGL-I, the 45 kDa antigen, ESAT-6 and CFP-10 were detected by ELISAs as described elsewhere (Parkash *et al.*, 2006a, b, 2007). To score tests as positive, cut-off points for assays with various antigens were determined by constructing receiver operator characteristic curves, by calculating the per cent true-positives and false-positives at various absorbance values obtained by ELISAs. The absorbance value at which assay performance was optimal (true positives + true negatives/total sample number) was used as a cut-off point. A sample with an absorbance value of greater than the cut-off value was considered positive. The differences between groups with respect to the proportion of positively testing subjects were tested by the chi-square test.

The positivity patterns against various antigens among MB and PB patients are shown in Fig. 1(a) and Fig. 1(b), respectively. The positivity with all the antigens (PGL-I + 45 kDa + ESAT-6 + CFP-10) was higher (12/17) in the MB group and lower (1/33) in the PB group. When considering all leprosy patients (MB + PB), only 13 samples were positive by all the assays (PGL-I + 45 kDa + ESAT-6 + CFP-10). However, only 6, 2, 2 and 2 samples were exclusively positive by the assays based on the PGL-I, 45 kDa, ESAT-6 and CFP-10 antigens, respectively, whereas 2, 2, 0, 1, 7, 1, 0, 1, 2 and 0 samples were positive jointly by the assays based on the PGL-I + 45 kDa + ESAT-6, PGL-I + 45 kDa + CFP-10, PGL-I + ESAT-6 + CFP-10, 45 kDa + ESAT-6 + CFP-10, PGL-I + 45 kDa, PGL-I + ESAT-6, PGL-I + CFP-10, 45 kDa + ESAT-6, 45 kDa + CFP-10 and ESAT-6 + CFP-10 antigens, respectively. Thus the heterogeneity in MB patients was 29.4% (5/17), which was lower than that (69.7%; 23/33) among the PB group of patients. Concerning heterogeneity in



sensitivity (17/17; 100.0%) for detection of MB patients did not differ ( $P > 0.05$ ) from that of the assays based on PGL-I (16/17; 94.1%), ESAT-6 (14/17; 82.5%) or CFP-10 (14/17; 82.5%). However, for PB leprosy patients, the combined (PGL-I + 45 kDa + ESAT-6 + CFP-10) approach yielded a sensitivity of 72.7% (24/33), resulting in an increase of 40.4, 21.2, 54.5 and 54.5% over that of PGL-I ( $P < 0.01$ ), the 45 kDa antigen ( $P > 0.05$ ), ESAT-6 ( $P < 0.01$ ) and CFP-10 ( $P < 0.01$ ) as single antigens, respectively. When all leprosy patients (MB + PB) were taken together, the combined sensitivity with all the antigens was 82.0%, implying a 28.0, 14.0, 42.0 and 42.0% increase in sensitivity over that of the anti-PGL-I ( $P < 0.01$ ), 45 kDa ( $P > 0.05$ ), ESAT-6 ( $P < 0.001$ ) and CFP-10 ( $P < 0.001$ ) antibody-detecting assays, respectively. Although the increase in the sensitivity of 45 kDa antigen was comparable ( $P > 0.05$ ) to that of PGL-I, it was significantly different from that of ESAT-6 ( $P < 0.05$ ) or CFP-10 ( $P < 0.05$ ). Interestingly, the specificity invariably remained more than about 94.0% in all the assays.

Thus the combinatorial approach, considering various antigen-based assays, appears to be the most sensitive one. Adopting this approach could lead to detection of 21.2–54.5% more of the smear-negative PB leprosy patients who otherwise would have been missed with any of the above-mentioned single-antigen-based assays. Hence, we consider this combined approach to be promising for application for serodiagnosis of leprosy.

Nevertheless, for better diagnostic applicability, the combined sensitivity needs to be increased further, while maintaining overall specificity. In this context, the availability of the complete gene sequence of *M. leprae* (Cole *et al.*, 2001) may help to identify additional *M. leprae* antigens with serodiagnostic potential. Recently, several groups have reported a number of new *M. leprae*-specific antigens and peptides capable of stimulating B and T cells (Geluk *et al.*, 2005; Spencer *et al.*, 2005; Araoz *et al.*, 2006; Reece *et al.*, 2006; Groathouse *et al.*, 2006) which could form the basis for

a new immunological tool(s) for diagnosis of leprosy patients. The results of the present study provide a new outlook for the development of improved methods by exploring new antigens in combinatorial multiplex studies to improve the serodiagnosis of leprosy.

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