A Study on the Reproducibility of Two Serological Assays for Detection of *Mycobacterium leprae* Infection

TO THE EDITOR:

The detection of *Mycobacterium leprae* infection is one of the important tools for effective control of leprosy. It is known that phenolic glycolipid-I (PGL-I) and 35-kDa protein are the two components of *M. leprae* which are major targets for antibody response in leprosy patients. Employing PGL-I and a monoclonal antibody against 35-kDa antigen, two *M. leprae*-specific serological assays—the PGL-I-based indirect enzyme linked immunosorbent assay (PGL-ELISA) and the monoclonal antibody inhibition test (MAIT)—have been developed ([4, 8, 10, 12]). These assays are highly specific and sensitive for detection of *M. leprae* infection, mainly in lepromatous patients. Apart from the diagnosis of *M. leprae* infection, PGL-ELISA might also be useful for application in monitoring the response of the patients to chemotherapy ([2, 9]), to measure the magnitude of *M. leprae* infection ([3, 7]), to identify the emergence of relapse ([6]) and lepra reactions ([1]). On the other hand, the MAIT has been studied mainly for diagnostic applications ([13]) and for monitoring the efficacy of chemotherapy ([5, 11]). It is well understood that accuracy and reliability of any test depend upon its reproducibility. Therefore, reproducibility is considered as an essential characteristic of any ideal diagnostic assay. To our knowledge, regarding PGL-ELISA and MAIT there is no documented information about their reproducibilities. Therefore, this study was carried out to investigate intra-assay and inter-assay variability for these two assays.

Measurement of anti-PGL-I antibodies by ELISA. IgM anti PGL-I antibodies were measured by indirect ELISA following the major steps described by Sinha, *et al.* ([11]) with slight modifications where required. In brief, the wells of a round-bottom microtiter plate (Immunoplate; Nunc, Raskilde, Denmark) were coated (by incubating the wells overnight at 4°C) with 50 µl/well (containing required concentration of antigen, i.e., 2 ng/50 µl) of trisaccharide conjugated to bovine serum albumin (NT-P-BSA). Uncoated antigen was removed by dumping, followed by washing (three times) with phosphate buffered saline (PBS) containing 0.1% Tween-20 (PBST) and patting of the plates on tissue paper to make them free of washing buffer. Wells of the plate were then blocked with 2.0% bovine serum albumin (150 µl/ml) in phosphate buffered saline (BSA-PBS) for 1.5 hr. After washing the wells, respective patients' sera, diluted 1:300 with BSA-PBS, were put into wells (50 µl/well). The wells were then incubated for 2 hr at 37°C and washed. This was followed by the addition of 1:1000 diluted proxidase labelled anti-human IgM. The wells were incubated at 37°C for 1 hr. The washing step was repeated and then the wells were incubated (at room temperature for 20 min) with ortho-phenylene diamine dihydrochloride (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) supplemented with hydrogen peroxide. Finally, the reaction was stopped by adding 7.0% H_2SO_4 (50 µl/well) and the optical density (OD) values were recorded at 492 nm in a microplate ELISA reader. Serum samples with an absorbance of greater than or equal to 0.2 were considered positive.

Measurement of anti-*M. leprae* antibodies by monoclonal antibody based assay. The anti-*M. leprae* antibodies were measured by a competitive inhibition type of ELISA. The main guidelines for performing this test were obtained from the previously described method ([11]) with slight modifications. Briefly, the round-bottom wells of microtiter plates (Nunc) were coated with 50 µl/well of *M. leprae* antigen (containing 5 µg/50 µl of antigen) by overnight incubation at 4°C. After this, unbound antigen was removed by washing with PBST. The wells were then blocked with 2.0% BSA-PBS for 1.5 hr at 37°C. The wells were then emptied and washed. Next, serum samples (diluted 1:10 in BSA-PBS) were added (25 µl/well) to the wells and incubated at 37°C for 1 hr. This was then followed by the addition of 25 µl/well of diluted

TABLE 1. *Intra- and inter-assay variations for test readings of PGL-ELISA.*

Serum	Assays	Mean OD	S.D.[a]	% C.V.[b]	
			Intra-assay		
High	16	1.136	0.041	3.61%	Overall
Medium	16	0.381	0.023	6.04%	C.V.
Low	16	0.045	0.013	28.89%	12.85%
			Inter-assay		
High	10	0.910	0.194	21.32%	Overall
Medium	10	0.314	0.087	27.71%	C.V.
Low	10	0.072	0.023	31.94%	26.99%

[a] S.D. = Standard deviation.
[b] %C.V. = Percent co-efficient of variation.

(1:1000) peroxidase labelled MLO4. Incubation was further carried out for 2 hr at 37°C. All the wells were then washed with PBST and blotted dry before the addition of substrate (orthophenylene diamine dihydrochloride) for 20 min. Finally, the enzymatic reaction was stopped with H_2SO_4 and readings were recorded by an ELISA reader. The results were expressed as percent inhibition, by anti-*M. leprae* antibodies in patients' sera, of binding of labelled monoclonal antibody MLO4 to *M. leprae* antigen coated on the wells. The serum causing ≥50% inhibition was taken as positive.

The reproducibilities of the assays were determined by testing three serum specimens for each assay, having varying quantities of anti-*M. leprae* antibodies. Serum samples with high levels of antibody were strongly positive. Samples with medium levels of antibodies were close to the cut-off point. Low antibody level sera had antibodies below the cut-off value. As described above, the PGL-

TABLE 2. *Intra- and inter-assay variations for test readings of MAIT.*[a]

Serum	Assays	Mean of % inhibition	S.D.[b]	% C.V.[c]	
			Intra-assay		
High	24	66.83	4.49	6.72%	Overall
Medium	24	44.60	5.30	11.88%	C.V.
Low	24	10.21	4.10	40.16%	19.59%
			Inter-assay		
High	8	77.16	17.73	22.50%	Overall
Medium	9	41.92	9.06	21.61%	C.V.
Low	9	12.69	3.94	31.05%	25.05%

[a] MAIT = Monoclonal antibody inhibition test.
[b] S.D. = Standard deviation.
[c] %C.V. = Percent co-efficient of variation.

TABLE 3. *Intra- and inter-assay reproducibilities for scoring the results as positive or negative by PGL-ELISA.*

Serum	Assays	Positive	Negative	Result	% Reproducibility	
			Intra-assay			
High	16	16	0	+	100%	Overall reproducibility 100%
Medium	16	16	0	+	100%	
Low	16	0	16	–	100%	
			Inter-assay			
High	10	10	0	+	100%	Overall reproducibility 96.7
Medium	10	9	1	+	90%	
Low	10	0	10	–	100%	

ELISA readings were recorded as OD values; whereas for MAIT the results were expressed as percent inhibition of binding of peroxidase labelled anti-*M. leprae* monoclonal antibody (MLO4) to coated *M. leprae* antigen. The variability in test readings was expressed as a percent co-efficient of variation (%CV). Data obtained by PGL-ELISA are shown in Table 1 and MAIT data are given in Table 2. The results indicate that intra-assay variations of both the assays were smaller than 12% for sera having high and medium levels of antibodies and >28% for sera containing low levels of antibodies. The overall intra-assay variation (12.9%) for PGL-ELISA was slightly lower than that for MAIT (19.6%). However, inter-assay variations with all the sera were moderately high (ranging from 21.3% to 31.9%) for both the assays. Another interesting observation was that with sera having high levels of antibodies variation was less. Contrary to this, low antibody containing serum showed the highest variation. Thus, a

TABLE 4. *Intra- and inter-assay reproducibilities for scoring the results as positive or negative by MAIT.*

Serum	Assays	Positive	Negative	Result	% Reproducibility	
			Intra-assay			
High	24	24	0	+	100%	Overall reproducibility 93.1%
Medium	24	5	19	–	79%	
Low	24	0	24	–	100%	
			Inter-assay			
High	8	8	0	+	100%	Overall reproducibility 92.6%
Medium	9	2	7	–	78%	
Low	9	0	9	–	100%	

kind of gradation, depending upon the antibody levels, in variation was seen.

Having observed these variations in readings of the assays, it became tempting to investigate the reproducibilities (intra-assay as well as inter-assay) of the approach to score the test results as positive or negative. According to our findings, the reproducibitity rates were better with the PGL-ELISA (Table 3) than with the MAIT (Table 4). All sera having various levels of anti-*M. leprae* antibody showed promising reproducibility (90%–100%) with the PGL-ELISA. On the other hand, with the MAIT, sera having high and low levels of anti-*M. leprae* antibodies showed 100% reproducibility; whereas sera having medium levels of antibody showed comparatively lower reproducibility (i.e., 79% for intra-assay and 78% for inter-assay). Nevertheless, the overall reproducibilities of both the assays were remarkably high (almost above 92%).

In light of the above findings, I wish to suggest that sera showing values near the cut-off point must be repeated at least three times, particularly when using the MAIT in order to get accurate results. Contrarily, testing for samples having high and low levels of antibodies need not be repeated.

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REFERENCES

1. Andreoli, A., Brett, S. J., Draper, P., Payne, S. N. and Rook, G. A. Changes in circulating antibody levels to the major phenolic glycolipid during erythema nodosum leprosum in leprosy patients. Int. J. Lepr. **53** (1985) 211–217.
2. Bach, M.-A., Wallach, D., Flageul, B., Hoffenbach, A. and Cottenot, F. Antibodies to phenolic glycolipid-I and to whole *Mycobacterium leprae* in leprosy patients: evolution during therapy. Int. J. Lepr. **54** (1986) 256–267.
3. Baumgart, K. W., Britton, W. J., Mullins, R. J., Basten, A. and Barnetson, R. St. C. Subclinical infection with *Mycobacterium leprae*—a problem for leprosy control strategies. Trans. R. Soc. Trop. Med. Hyg. **87** (1993) 412–415.
4. Brett, S. J., Payne, S. N., Gigg, J., Burgess, P. and Gigg, R. Use of synthetic glycoconjugates containing the *Mycobacterium leprae*-specific immunodominant epitope of phenolic glycolipid-I in the serology of leprosy. Clin. Exp. Immunol. **64** (1986) 476–483.
5. Chaturvedi, V., Sinha, S., Girdhar, B. K. and Sengupta, U. On the value of sequential serology with a *Mycobacterium leprae*-specific antibody competition ELISA in monitoring leprosy chemotherapy. Int. J. Lepr. **59** (1991) 32–40.
6. Chin-A-Lien, R. A., Faber, W. R., van Rens, M. M., Leiker, D. L., Naafs, B. and Klatser, P. R. Follow-up of multibacillary leprosy patients after discontinuation of treatment using PGL-I ELISA. Are increasing ELISA values indicative of relapse? Lepr. Rev. **63** (1992) 21–27.
7. Cho, S.-N., Kim, S. H., Cellona, R. V., Chan, G. P., Fajardo, T. T., Walsh, G. P. and Kim, J. D. Prevalence of IgM antibodies to phenolic glycolipid-I among household contacts and controls in Korea and The Philippines. Lepr. Rev. **63** (1992) 12–20.
8. Cho, S.-N., Yanagihara, D., Hunter, S. W., Gelber, R. H. and Brennan, P. J. Serological specificity of phenolic glycolipid-I from *Mycobacterium leprae* and its use in serodiagnosis of leprosy. Infect. Immun. **41** (1983) 1077–1083.
9. Klatser, P. R., de Wit, M. Y. L., Fajardo, T. T., Cellona, R. V., Madarang, M. G., Abalos, R. M. and Steenbergen, G. J. Evaluation of *Mycobacterium leprae* antigens in the monitoring of dapsone based chemotherapy of previously untreated lepromatous leprosy patients in Cebu, Philippines. Lepr. Rev. **60** (1989) 178–186.
10. Mwatha, J., Moreno, C., Sengupta, U., Sinha, S. and Ivanyi, J. A comparative evaluation of serological assays for lepromatous leprosy. Lepr. Rev. **59** (1988) 195–199.
11. Sinha, S., McEntegart, A., Girdhar, B. K., Bhatia, A. S. and Sengupta, U. Appraisal of two *Mycobacterium leprae*-specific serological assays for monitoring chemotherapy in lepromatous (LL/BL) leprosy patients. Int. J. Lepr. **57** (1989) 24–32.
12. Sinha, S., Sengupta, U., Ramu, G. and Ivanyi, J. A serological test for leprosy based on competitive inhibition of monoclonal antibody binding to the MY2a determinant of *Mycobacterium leprae.* Trans. R. Soc. Trop. Med. Hyg. **77** (1983) 869–871.
13. Sinha, S., Sengupta, U., Ramu, G. and Ivanyi, J. Serological survey of leprosy and control subjects by a monoclonal antibody-based immunoassay. Int. J. Lepr. **53** (1985) 33–38.