The Myanmar Health Sciences Research Journal, Vol. 21, No. 3, 2009

Application of new serological (Major Membrane Protein II) Enzyme Linked Immunosorbent Assay for leprosy patients in Myanmar

**Khin Nwe Oo, *Mi Mi Htwe, *Khine Zar Win, **Khin Thet Wai, ***Kyaw Myint & ****Kyaw Kyaw*

*Bacteriology Research Division
**Epidemiology Research Division
Department of Medical Research (Lower Myanmar)
***Department of Health
****Central Special Skin Clinic, Yangon

As serodiagnosis is the easiest way of diagnosing a disease, the utility of (Major Membrane Protein II) MMP-II antigen in the serodiagnosis of leprosy was examined and compared with NTP-BSA ELISA test. It was carried out on the blood samples of new leprosy cases and their contacts from Nyaungdon Township, and on new adult pulmonary TB cases of Yangon TB Center and childhood TB cases of Yangon Children's Hospital and North Okkalapa General Hospital between November 2006 and December 2007. The sensitivity of the tests on detecting leprosy patients who had not been treated was 58/77 (75.32%) and 54/77 (70.1%) for MB patients using MMP-II and NTP-BSA ELISA tests, respectively. It was 48/64 (75.0%) and 40/64 (62.50%) for PB patients using MMP-II and NTP-BSA ELISA tests, respectively. The sensitivity of MMP-II ELISA test was higher than that of NTP-BSA ELISA test on both leprosy patients. The sensitivity of MMP-II ELISA test was also higher than that of NTP-BSA ELISA test on TB patients in our study. Therefore, our data indicate that MMP-II ELISA could be useful as a supporting serodiagnostic tool in diagnosis of leprosy and childhood tuberculosis.

INTRODUCTION

Leprosy represents a broad spectrum disease caused by *Mycobacterium leprae* with lepromatous leprosy at one pole and tuberculoid leprosy at another pole, depending on the clinical manifestation, which is an ultimate effect of the immunity of the host [1]. In all forms of the disease, *M. leprae* induces skin lesions and a chronic progressive peripheral nerve injury, to a lesser or greater extent, which leads to systemic deformity [2, 3]. Therefore, early detection of *M. leprae* infection is the key to avoiding deformities. The diagnosis of leprosy is based on microscopic detection of acid-fast bacteria (AFB) in skin smears or biopsies along with clinical and histo-pathological evaluation. Acid-fast staining requires at least a thousand organisms per gram of tissue for reliable detection [4], resulting in an extremely low sensitivity especially for the tuberculoid form of the disease where AFB are rare or absent.

Serodiagnosis is generally accepted as the easiest way of diagnosing a disease. For leprosy, the only antigen currently used is phenolic glycolipid 1 (PGL-1), which is supposedly specific to *M. leprae* [5, 6]. Since the identification of PGL-1 in 1981 by Hunter and Brennan, a number of serological tools have been developed [7]. Simple assays such as the Serodia-Leprae method, a dip-stick assay and lateral flow tests based on PGL-1 antigen have been used to detect leprosy patients in areas

where leprosy is endemic [8, 9]. However, these tests seem to be insufficient for detection of both multibacillary (MB) and paucibacillary (PB) patients as well as for early diagnosis.

Recently, real time PCR based methods have been developed [10], but the sensitivity of the test for clinical specimens is still problematic. In developing countries where leprosy is endemic, diagnosis still relies on clinical observations and easy inexpensive tests. Serodiagnosis is the easiest and tangible way of diagnosing a disease.

In Myanmar, leprosy elimination (Prevalence Rate <1/10,000) was achieved at national level at the end of January 2003. Until 2003, NCDR (New Case Detection Rate >4/100,000) was still high in some townships of Yangon, Ayeyawady, Bago, Mandalay, Magwe, Sagaing Divisions and Shan State [11].

To date, various antigens of *M. leprae* have been studied [12], but due to the lack of either specificity or sensitivity, their use had been limited. Major membrane protein II (MMP-II, ml2038c, gene name bfrA, also known as bacterioferritin) had been identified previously from the cell membrane fraction of *M. leprae,* as an antigenic molecule capable of activating both antigen-presenting cells and T cells [13, 14]. These findings prompted examination of the role of MMP-II as the role of humoral responses of patients.

As serodiagnosis is the easiest way of diagnosing a disease, the utility of MMP-II in the serodiagnosis of leprosy was examined. The percent positivity by an ELISA for anti MMP-II antibody was 82.4% for MB leprosy and the specificity of the test was 90.1%. For PB leprosy where cell-mediated immunity predominates, 39% showed positive results. These percentage values were significantly higher than those obtained by existing PGL-1 based methods suggesting that MMP-II antibody detection would facilitate the diagnosis of leprosy [15].

In the present study, the serological test using MMP-II as an antigen for serodiagnosis of leprosy was carried out and the sensitivity and specificity was compared with NTP-BSA (Natural Trisaccharide Phenyl-Propionyl-Bovine Serum Albumin) ELISA test. Both ELISA tests are sandwich types of ELISA.

MATERIALS AND METHODS

This study was a community and laboratory-based cross-sectional type of study and was conducted to compare the sensitivity and specificity of MMP-II with NTP-BSA ELISA on new MB and PB cases of leprosy and their contacts and tuberculosis cases. The blood samples were collected from new leprosy cases and their contacts of Nyaungdon Township, and new adult pulmonary TB cases of Yangon TB Center, childhood TB cases from the Yangon Children's Hospital and North Okkalapa General Hospital. The samples were collected between November 2006 and October 2007.

Sample collection

The blood samples were collected from new leprosy cases and their contacts from Nyaungdon Township, Ayeyawady Division. They were also collected from smear-positive and negative pulmonary tuberculosis cases attending the Yangon TB Center and childhood TB cases of YCH and NOGH. To get the community acceptability, the cases and subjects were informed that a research study of leprosy would be done. The objectives of the study were explained. Two millilitres of blood were taken after obtaining informed consent from each subject who is above 12 years of age. If the subject is less than 12 years of age, informed consent was taken from his//her parents or guardians. The blood was withdrawn using sterile needles and syringes. The clotted blood samples were transported under cold storage to the Bacteriology Research Division, Department of Medical Research (Lower Myanmar).

NTP-BSA (Natural Trisaccharide Phenyl-Propionyl–Bovine Serum Albumin) ELISA and MMP-II (Major Membrane Protein II) ELISA tests were carried out on these sera.

Leprosy examination

Leprosy examinations were performed by trained leprosy field workers of Nyaungdon Township. Leprosy was clinically diagnosed based on detailed skin examination, including testing for anesthesia and examination for enlargement of nerves and confirmed by the Regional Officer. Classification was based on the WHO system of lesions counting (paucibacillary PB patients=1-5 lesions, multibacillary MB patients=>5 lesions) [16].

Procedure for indirect ELISA

NTP-BSA (Natural Trisaccharide Phenyl-Propionyl–Bovine Serum Albumin) and MMP-II (Major Membrane Protein II) ELISA tests, both sandwich types, were carried out on the samples. The sero-positives were identified from cut-off point of controls (apparently healthy blood donors of National Blood Bank, Yangon). The ELISA tests were carried out between December 2006 and November 2007. The ELISA microtitre plate (Fastec microplate U, Fujirebio Inc.) with 96 U-shaped wells was coated with 50µl of 1 µg/ml and 4 µg/ml concentrations of soluble antigens: NTP-BSA and MMP-II on each plate, respectively, and incubated at 37°C in moist chamber for 2 hours and then placed in 4°C for overnight. The coating antigens were diluted with carbonate-bicarbonate buffer to get final concentrations. The goat anti-human IgM (for NTP-BSA) and IgG (for MMP-II) conjugated to horse-radish-peroxidase (Dako, Denmark) were used for 2^{nd} antibody reaction and ortho-phenylene-diamine) (Dako, Denmark) was used as the substrate. The optical density (OD) was measured by ELISA reader (Stat Fax 3200, USA) at wavelength of 492 nm within 15 minutes. Pooled sera from 10 multi-bacillary patients and 10 apparently healthy donors of the National Blood Bank, Yangon were used as positive and negative controls, respectively. The cut-off values of ELISA tests were 0.015 for MMP-II and 0.07 for PGL-1 tests.

Study population

This study was carried out on 77 MB and 64 PB cases, their contacts and 80 blood donors. It was also studied on sputum smear-positive and negative adult pulmonary and childhood tuberculosis cases.

Informed consent

All eligible subjects were invited verbally for the study and procedure on taking blood samples was explained. Informed consent was requested from each adult. For children, less than 12 years of age, consents were obtained from their parents or guardians.

Study subjects

The diagnosis of leprosy was carried out according to the WHO guidelines and was performed by trained leprosy field workers from Nyaungdon Township. All suspected cases were examined by the Leprosy Regional Officer, Ayeyawady Division and were confirmed by the Central Special Skin Clinic, Yangon. All TB cases were diagnosed by specialists from Yangon TB Center and pediatricians and confirmed by radiologists.

Data analysis

The sensitivity and specificity of MMP-II ELISA test was calculated and compared with NTP-BSA ELISA test using ROC curve (Area under the curve) analysis.

RESULTS

The 2 ELISA tests were carried out on 77 multibacillary and 64 paucibacillary cases and 80 blood donors. The specificity of the tests, as calculated by using results of sera from 80 blood donors, was 69/80 (86.25%) for both MMP-II and NTP-BSA ELISA tests. The sensitivity of the tests in detecting leprosy patients who had not been treated was 58/77 (75.32%) and 54/77 (70.1%) for

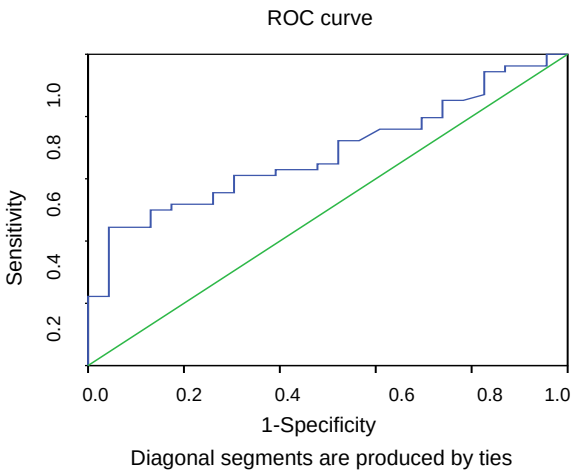
MB patients using MMP-II and NTP-BSA ELISA tests, respectively. It was 48/64 (75%) and 40/64 (62.5%) for PB patients using MMP-II and NTP-BSA ELISA tests, respectively. The sensitivity of MMP-II ELISA test was higher than that of NTP-BSA ELISA test in both leprosy patients (Table 1).

Table 1. ELISA results of leprosy and non-leprosy patients in Myanmar

No. of cases	MMP-II (%)	PGL-1 (%)
Patient (141)	106 (75.2)	94 (66.7)
Normal (80)	11 (13.8)	11 (13.8)
Contacts (472)		
Contact to MB (317)	281 (88.60)	74 (23.3)
Contact to PB (155)	123 (79.4)	36 (23.20)
Total	404 (85.6)	110 (23.3)
TB patients (196)	27 (55.10)	0 (0)
Smear + TB (49)		
Smear - TB (52)	7 (13.5)	0 (0)
Child NOGH (50)	18 (36)	4 (8.00)
Child YCH (45)	37 (82.2)	4 (8.9)
Total	89 (45.4)	8 (4.1)

Cut-off value: >0.015 for MMP-II
>0.07 for PGL-1

By using ROC curve analysis; for MMP-II serodiagnostic test, area under the ROC curve was 0.855; standard error, 0.028; 95% CI, (0.795 to 0.902); and p=0.0001.

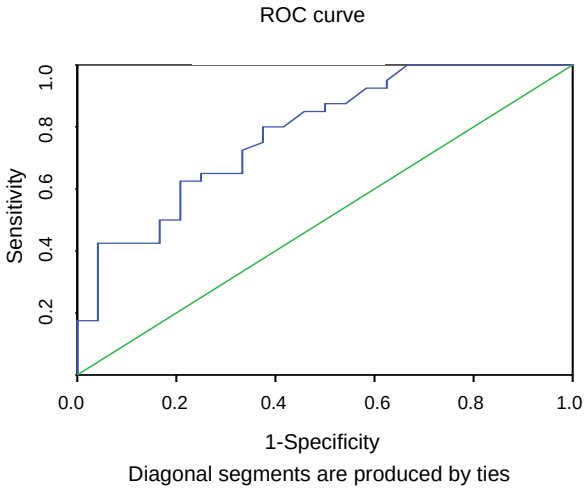


Area under the curve

Test result variable(s): mmp2 value

Area	Std. error (a)	Asymptotic sig.(b)	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
.682	.061	.012	.564	.801

Fig. 1. ROC curve of multibacillary cases



Area under the curve

Test result variable(s): mmp2 value

Area	Std. error (a)	Asymptotic sig.(b)	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
.782	.059	.000	.666	.898

Fig. 2. ROC curve of paucibacillary cases

For NTP-BSA ELISA test, area under the ROC curve was 0.872; standard error, 0.026; 95% CI,(0.815 to 0.917); and p=0.0001. The sensitivity of MMP-II ELISA test was higher than that of NTP-BSA ELISA test in TB patients of this study (Fig. 1& 2).

DISCUSSION

In the study involving Vietnamese leprosy patients, there is a significant difference between MMP-II ELISA and PGL-1 ELISA in detecting both MB and PB leprosy. The positivity rate of anti-MMP-II antibody for MB leprosy was approximately 85% and that for PB leprosy was 48%, significantly higher than the titers obtained by PGL-1 ELISA (57% and 20%, respectively) [14]. Also in our study, the sensitivity of MMP-II ELISA test was higher than that of NTP-BSA ELISA test in both leprosy patients.

The difference in sensitivity between PGL-1 ELISA and MMP-II ELISA may be due to differences in the biochemical features of the antigens. PGL-1 is a glycolipid component, and as such, it might be retained in some infected cells for a longtime after

the initial exposure [17]. Therefore, the usefulness of PGL-1 based tests for early diagnosis is limited. MMP-II is a protein antigen and is considered to be one of the immunodominant antigens of *M. leprae* [14]. Therefore, in individuals who had been exposed to *M. leprae* but did not have leprosy, antigen presenting cells expressing MMP-II might feasibly be eliminated from the body by immune cells such as cytotoxic T lymphocytes and thus lack the ability to produce anti MMP-II antibodies through antigen presenting cell dependent mechanisms.

Since the MMP-II protein is conserved in response to exposure to other mycobacteria, such as *M. tuberculosis* and *M. avium,* the sensitivity of MMP-II ELISA test was slightly higher than that of NTP-BSA ELISA test on TB patients in our study. Therefore, our data indicate that MMP-II ELISA could be useful as a supporting serodiagnostic tool for leprosy and childhood tuberculosis.

ACKNOWLEDGEMENT

This study was granted by the International Medical Center of Japan. The coating antigen of MMP-II ELISA test was a gift from Professor M Kai, National Institute of Infectious Diseases, Tokyo, Japan.

REFERENCES

1. Ridley DS & Jopling WH. Classification of leprosy according to immunity: A five group system. *International Journal of Leprosy and Other Mycobacterial Diseases* 1996; 34: 255-273.
2. Stoner GL. Importance of the neural predilection of *M. leprae* in leprosy. *Lancet* 1979; 2: 994-996.
3. Job CK. Nerve damage in leprosy. *International Journal of Leprosy and Other Mycobacterial Diseases* 1989; 57: 532-539.
4. Shepard CC & McRae DH. A method for counting acid fast bacteria. *International Journal of Leprosy and Other Mycobacterial Diseases* 1968; 36: 78-82.
5. Schuring RP, Moet FJ, Pahan D, Richardus JH, & Oskam L. Association between anti PGL-1 IgM and clinical and demographic parameters in leprosy. *Leprosy Review* 2006; 77: 343-355.
6. Sekar B, Sharma RN, Leelaba G, Ananda D *et al*. Serological response of leprosy patients to *Mycobacterium leprae* specific and myco-bacteria specific antigens; possibility of using these assays in combination. *Leprosy Review* 1993; 64:15-24.
7. Hunter SW & Brennan PJ. A novel PGL from *M. leprae* possibly involved in immunogenecity and pathogenicity. *Journal of Bacteriology* 1981; 147: 728-735.
8. Izumi S, Fujiwara T, Ikeda M, Nishimura M, Sukiyama K & Kawatsu Y. Novel gelatin particle agglutination test for serodiagnosis of leprosy in the field. *Journal of Clinical Microbiology* 1990*;* 28: 525-529.
9. Kai M, Maeda Y, Maeda S, Fukutomi Y, *et al*. Active surveillance of leprosy contacts in country with low prevalence rate. *International Journal of Leprosy and Other Mycobacterial Diseases* 2004 ; 72: 50-53.
10. Martinez AN, Britto CFPC, Nerry JAC, Sampaio EP, Jardim MR, Sarno EM & Moraes MO. Evaluation of real time and conventional PCR targeting complex 85 genes for detection of *M. leprae* DNA in skin biopsy samples from patients diagnosed with leprosy. *Journal of Clinical Microbiology* 2006; 44: 3154-3159.
11. Ministry of Health, Yangon, Myanmar. In: *Leprosy Control Program Report, Yangon*. 2004.
12. Hunter SW, Revoire B, Mera V, Bloom BR & Brennan PJ. The major native proteins of the leprosy bacillus. *Journal of Biological Chemistry* 1990; 265: 14065-14068.
13. Pessolani MC, Smith DR, Revoire B, Mc Cormick J, Hefte SA, Cole ST & Brennan PJ. Purification, characterization, gene sequence and significance of a bacterioferritin from *M. leprae*. *Journal of Experimental Medicine* 2004; 180: 319-327.
14. Maeda Y, Mukai T, Spencer J & Makino M. Identification of an immunomodulating agent from *M. leprae. Infection and Immunity* 2005; 73: 2744-2750.
15. Maeda Y, Mukai T, Kai M, Fukutomi Y *et al*. Evaluation of major membrane protein II as a tool for serodiagnosis of leprosy. *FEMS Microbiology Letter* 2007; 272: 202-205.
16. Fine PEM. Leprosy: the epidemiology of a slow bacterium. *Leprosy Review* 1982; 4: 161-188.
17. Gelber RH, Li F, Cho SN, Bryd S, Rajagopalan K & Brennan PJ. Serum antibodies to defined carbohydrate antigens during the course of treated leprosy. *International Journal of Leprosy and Other Mycobacterial Diseases* 1989; 57: 744- 751.