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Serological diagnosis for active tuberculosis in Malaysian population: Comparison of four protein candidate

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ABSTRACT

Objective: To assess the ability of 4 types of Mtb proteins—ESAT6, SCWP, MAN and Ag85 to serve as indicator for active tuberculosis among Malaysian population. **Methods:** Sera from 90 individuals, 60 from confirmed pulmonary tuberculosis patients and 30 healthy PPD negative individuals were tested for presence of anti-IgG and anti IgA by ELISA assay. **Result:** Mean concentration of IgG and IgA were higher in patients compared to healthy. Positivity of the ELISA test were calculated, taking the cut off value at mean +2 SD of healthy sera. The sensitivity of the ELISA IgA assay for ESAT 6, SCWP, MAN and Ag85 were 81.1%, 83.3%, 11.7% and 53.3% respectively. The sensitivity of the ELISA IgG assay for ESAT 6, SCWP, MAN and Ag85 were 71.0%, 71.0%, 71.0% and 21.0% respectively. **Conclusion:** Detection of IgA against SCWP promised a good indicator for active tuberculosis infection among Malaysian.

1. Introduction

Tuberculosis (TB) is the leading cause of death in adults due to a single pathogen. Although the bacterium that cause it, *Mycobacterium tuberculosis* (MTB), was discovered way back in 1882, it has remained a major global problem.

Over the years, there has been steady increase in the number of TB notifications.

In 2006, there were an estimated 9.2 million new cases of TB, that is, an increase from 9.1 million cases in 2005, supposedly due to population growth [1]. In 2005, the 2,259,000 new cases in South-East Region constituted 34 percent of global incidence TB cases. India, China, Indonesia, South Africa and Nigeria

rank first to fifth respectively in terms of absolute number of cases (WHO, 2008). The situation is so pervasive that according to WHO, someone in the world is newly infected with TB bacillus every second.

In Malaysia, TB is the second highest notifiable infectious disease compared to dengue. In the year of 2010, there were 19337 tb cases (68 of 100000 population) reported, where from that given number, 18517 cases (96%) were new cases and 820 case (4%) were recurrence TB (Annual Report MOH, 2011).

Current diagnosis of pulmonary TB is relies on clinical presentation, supported by laboratory investigations particularly direct smear and culture method. Direct smear is very fast and cheap method but it is lack of sensitivity (40–60%), since it relies a lot on the quality of samples and requires experience technologies to identify the acid fast bacilli. Culture method is more sensitive but requires longer incubation times. Thus, the development of a simple, cheap, fast and reliable antibody or antigen detection assay might be of great benefit for early disease detection and disease control.

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Studies on the antibody response to *Mycobacterium tuberculosis* infection date back to 1898 when Arloing documented specific antibody production in tuberculosis (TB) patients. Since then, research has been directed towards identifying antigens having sero–diagnostic potential and understanding the role of antibodies in disease pathogenesis and protective immunity

Antibodies response towards tuberculosis infection varies in different stages of disease and also heterogeneity of the geographical background (Hoff, S.T., *et al.*, 2007). Among all infectious diseases, TB posed a prime example of the effects of intra and inter–population variation, because the outcome of exposure of the human host to M tuberculosis is determined by interactions ranging from the molecular to the population level (Kato–Maeda., *et al.*, 2001), with variation operating at each of these levels. For example, it is estimated that only 30% of exposed persons show evidence of infection, and of those infected, only 10% ever become ill. It is very clear that the targets and magnitude of the immune response to M tuberculosis show marked variation among individuals (Jepson, A. *et al.* 2001), with both host and pathogen factors being implicated. So far there is no study done to study the presence of specific M tuberculosis antibodies among Malaysian population.

The aim of this paper is to optimize four different protein panels, which include recombinant ESAT6, SCWP, lipoarabinomannan and Ag85 and to apply the ELISA assay for detection of the IgG and IgA antibodies among confirmed TB patients in Malaysia. The antibody titer presence in the patients were compared with healthy PPD negative individuals.

2. Materials and methods

2.1. Study population.

The study was conducted in a Hospital Universiti Sains Malaysia (HUSM). This is an 800 bedded teaching hospital in east coast region of Malaysia. The study has been carried out with approval of the human ethics committee, Universiti Sains Malaysia. Informed consent was taken from all the participants involved in this study.

2.2. Healthy Subjects

Healthy subjects were recruited among the university students age 20–24 years old. They were interviewed and signed the informed consent form. All the subjects do not have known tuberculosis case in the family, BCG vaccinated and PPD skin test negative.

2.3. Serum samples from confirmed TB patients

All the serum samples of patients clinically diagnosed as pulmonary tuberculosis were taken and kept at –20°C until used. The diagnosis of pulmonary tuberculosis was made based on the clinical history, physical examination, sputum direct smear and sputum culture onto the Ogawa egg–based solid medium. In this study the ELISA assay were only performed on the confirmed smear and culture positive. Serum was obtained

by collecting the blood in plain test tube. The samples were spin down at 8000 rpm for 3 minutes to separate the serum. The serum were kept at –20°C until used.

2.4. Antigens

The antigens used in this study were purchased from Pasteur Institute, France and some donated by Colorado State University (CSU). The detail descriptions of all the proteins used were explained as below.

ESAT–6, is the 6 kDa early secretory antigenic target of *Mycobacterium tuberculosis*, is a secretory protein and potent T cell antigen.

Soluble Cell Wall Protein (SCWP) is a component of the cell wall fraction.

ManLAM from *M. tuberculosis* H37Rv: LAM is a cell wall product possessing many biological activities including immunogenicity, induction of TNF and the release of other cytokines, and inhibition of antigen processing. The LAM were extracted by suspension of the cell in a PBS buffer containing 4% Triton X–114 and broken by French Press.

Ag85. The Ag85 complex contains three gene products of 30–31 kDa. Detail description on the methods of protein extraction is beyond the scope of this paper.

2.5. ELISA assay.

Each well of the 96–well flat–bottomed microtiter plate (NUNC Immuno™ Maxisorp, Denmark) was coated with 100 µl antigen in carbonate–bicarbonate buffer (pH 9.6). The plate was then covered with aluminum foil and incubated in a humid chamber at 4 °C, overnight. Plates were blocked with 1% BSA in phosphate–buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS–T) for 1 h at 37 °C and washed four times with PBS–T. Serum was diluted 1:500, whereas ALS was diluted in 1:100, in PBS–T containing 0.1% BSA, and 100µl of diluted samples were added to antigen–coated wells in duplicate and incubated for 2 hours at 37°C on shaker. After washing with PBS–T, plates were incubated with 100µl/well goat anti–human immunoglobulin G conjugated with horseradish peroxidase (Zymed,USA) in PBS–T plus 0.1% BSA for 30 min at 37 °C. Plates were then washed with PBS–T, and enzyme activity was assayed by incubation for 20 min at 37°C with 100 µl/well OPD substrate kit (Sigma). Reactions were stopped by adding 50µl of 1N H₂SO₄. Optical density at 492 nm (OD₄₉₂) was measured with an automated microplate reader VersamaxPLUS.

2.6. Result analysis.

Mean value of OD for patients sample and healthy control were calculated, and unpaired student t test were employed to calculate the p value of the difference. The determination of the sensitivity of the test was done by measuring the mean value of the healthy control plus two standard deviation (mean +2SD).

2.7. Determination of sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV)

The sensitivity of a test is the probability that the test is

positive when tested with a group of patients with the disease. The formula for sensitivity is:

$$Sn = [TP / (TP + FN)] \times 100$$

The specificity of a test is the probability that the test is negative among patients who do not have the disease. The formula for specificity is:

$$Sp = [TN / (TN + FP)] \times 100$$

where TP (true positive), TN (true negative), FP (false positive), FN (false negative)

2.8. Ethics

The study was approved by the Universiti Sains Malaysia human ethical committee. Informed consent was obtained from patients and healthy individuals.

3. Results

All together, 90 individuals were enrolled in this study. The serum of 60 confirmed pulmonary tuberculosis patients

and 30 healthy PPD negative individuals were tested for presence of anti-IgG and anti IgA . The direct ELISA assay for ESAT6, SCWP, MAN and Ag85 were successfully optimized. Direct ELISA assay were performed to detect anti-IgG and anti- IgA antibodies titer against 4 types of Mtb proteins mentioned. The mean OD for both IgG and IgA antibodies titer were calculated and compared to the mean OD of healthy individuals. The summary of mean OD titer of ELISA assay for anti IgA antibodies and anti IgG antibodies are as listed in the Table 1 . Unpaired student t test were applied to calculate the significance difference of the mean OD of the TB patients and the healthy individuals. There is significance difference in the mean OD of TB patients and healthy groups against all the 4 types of proteins used in this study.

Figure 1 showed the whisker box displayed the data set of OD value obtained from the ELISA assay. This figure suggests that the detection of antibodies against SCWP and ESAT (for both IgG and IgA) in the serum of Tb patients of Malaysian are significantly higher among the four tested proteins.

Table 1

Summary of Mean of OD value, Standard Deviation (SD) and their respective p value of antibodies against various protein panels in serum samples of healthy and TB patients.

	Healthy		Patients		P value
	Mean	(SD)	Mean	(SD)	
rESAT6 IgG	0.228	(0.044)	0.371	(0.102)	0.0001
rESAT6 IgA	0.285	(0.058)	0.540	(0.165)	0.0001
SCWP IgG	0.195	(0.038)	0.325	(0.117)	0.0001
SCWP IgA	0.133	(0.032)	0.284	(0.130)	0.0001
Mannan IgG	0.266	(0.059)	0.446	(0.193)	0.0010
Mannan IgA	0.132	(0.035)	0.184	(0.082)	0.0013
Ag 85 IgG	0.115	(0.058)	0.221	(0.121)	0.0001
Ag 85 IgA	0.0791	(0.044)	0.191	(0.121)	0.0001

Table 2

Sensivity and specificity of the anti-IgG ELISA assay using various types of Mtb proteins.

	Sensitivity		Specificity	
	True Positive (n=60)	%	True Negative (n=30)	%
ESAT6	49/60	81.7%	29/30	96.6%
SCWP	50/60	83.3%	28/30	93.3%
LAM	7/60	11.7%	30/30	100%
Ag85	32/60	53.0%	29/30	96.6%

Table 3

Sensitivity and specificity of the anti-IgG ELISA assay using various types of Mtb protein.

	Sensitivity		Specificity	
	True Positive (n=60)	%	True Negative (n=30)	%
ESAT6	43/60	71.0%	28/30	93.3%
SCWP	42/60	71.0%	29/30	96.6%
LAM	43/60	71.0%	29/30	96.6%
Ag85	13/60	21.7%	30/30	100%

Serum level of IgG and IgA were quantified using ELISA assay. The cut off value were calculated at the mean OD plus 2 OD of the healthy individuals. Every ELISA reading above the OD were considered as positive.

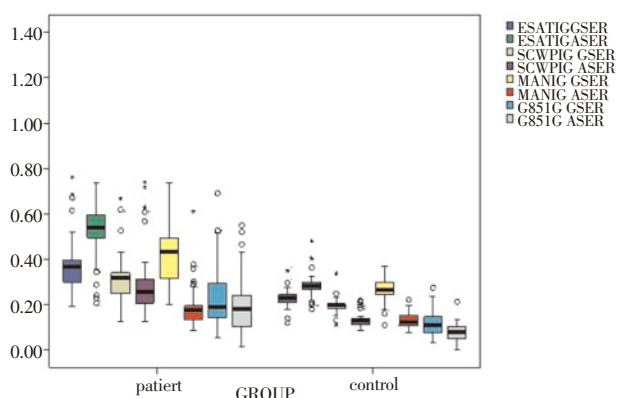


Figure 1. Box and Whisker plot demonstrating the anti-IgG and anti IgA antibodies in the sera of TB patients and healthy individuals (control). Serum level of IgG and IgA were quantified using ELISA assay. TB patients have markedly higher mean OD titer as compared to healthy individuals.

ESATIGGSER–Represent the serum IgG antibodies against ESAT6

ESATIGASER– Represent the IgA antibodies against ESAT6

SCWPIGGSER– Represent the IgG antibodies against SCWP

SCWPIGASER– Represent the IgA antibodies against SCWP

MANIGGSER– Represent the IgG antibodies against MAN

MANIGASER– Represent the IgA antibodies against MAN

G85IGGSER– Represent the IgG antibodies against Ag85

G85IGASER– Represent the IgA antibodies against Ag85

The calculation of sensitivity and the specificity of the test were performed based on the standard formula mentioned in the methodology section. Positivity of the ELISA tests was calculated, taking the cut off value at mean +2 SD of healthy sera. Every ELISA reading above the OD will be considered as positive. As simplified in the table 2 and 3, the sensitivity of the ELISA IgA assay for ESAT 6, SCWP, MAN and Ag85 were 81.1%, 83.3%, 11.7% and 53.3% respectively. The sensitivity of the ELISA IgG assay for ESAT 6, SCWP, MAN and Ag85 were 71.0%, 71.0%, 71.0% and 21.0% respectively. Serum level of IgG and IgA were quantified using ELISA assay.

4. Discussion

Various serology based diagnostic assay for detection of active tuberculosis has been developed. The results of the assays vary from region to region, and also posed different sensitivities and specificities. However, despite extensive work on the diagnosis of TB, none of the antigens achieve sufficient sensitivity to replace sputum smear microscopy (Steingart, K.R. et al, 2009). In the present study, by using a direct ELISA method, we optimized 4 different types of Mtb proteins and then screen for the presence of anti-IgG and IgA antibodies in the confirmed TB patients and compare the ELISA OD titer in the healthy individuals among Malaysian populations. The data demonstrate that the mean OD of TB patients and healthy individuals has a significance difference for all 4 types of antigen. Box plot drawing offers us a better understanding of the distribution of the antibodies level.

Among Malaysian population, obviously detection of anti ESAT6 anti and SCWP antibodies promise an alternative for tuberculosis diagnosis, whereas Anti LAM and Anti-Ag85 were both give poor positivity yield. Though several previous studies have reported that Ag85 complex is the immunodominant antigen, (Iman, (2009), Sanchez-Rodriguez C, 2002). Where the IgG detection were claimed to range from 42% to 72%, Seroconversion against these two antigens among Malaysian population seems to be much less than that.

Although our study provided important information, there is a limitation that merit discussion. The ELISA assay performed was not blinded, however the ELISA protocol were adhered strictly by the operator.

Among Malaysian population, detection antibodies against ESAT6 and SCWP has a potential value for further evaluation and offer a promising diagnostic potential.

Conflict of interest statement

We declare that we have no conflict of interest.

References

- [1] Hoff ST, Abebe M, Ravn P, Range N, Malenganisho W, Rodrigues DS, et al. Evaluation of *Mycobacterium tuberculosis* specific antibody responses in populations with different levels of exposure from Tanzania, Ethiopia, Brazil and Denmark. *Clin Infect Dis* 2007; **45**(5): 575–582.
- [2] Imaz MS, Schmelling MF, Kaempfer S, Spallek R, Singh M. Serodiagnosis of tuberculosis: specific detection of free and complex-dissociated antibodies anti-mycobacterium tuberculosis recombinant antigens. *Braz J Infect Dis* 2008; **12**(3): 234–244.
- [3] Jepson, A. et al. Genetic regulation of acquired immune responses to antigens of *Mycobacterium tuberculosis*: a study of twins in West Africa. *Infect Immun* 2001; **69**(6): 3989–3994.
- [4] Kato-Maeda M, Bifani PJ, Kreiswirth BN, Small PM. The nature and consequences of genetic variability within *Mycobacterium tuberculosis*. *J Clin Invest* 2001; **107**(5): 533–537.
- [5] Malaysia, Ministry of Health annual report, 2011.
- [6] Sanchez-Rodriguez C, Estrada-Chavez C, Garcia Vigil J, Laredo-Sanchez F, Halabe-Cherem J, Pareira-Suarez A, et al. AN IgG antibody response to the antigen 85 complex is associated with good outcome in Mexican Totonaca Indians with pulmonary tuberculosis. *Int J Tuberc Dis* 2002; **6**: 706–712.
- [7] Steingart KR, Dendukuri N, Henry M, Schiller I, Nahid P, Hopewell PC, et al. Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a Meta-analysis. *Clin Vaccine Immunol* 2009; **16**(2): 260–276.
- [8] WHO. *Global tuberculosis control: surveillance, planning, financing*. Geneva: WHO; 2008. [Online] Available from http://www.stoptb.org/resource_centre/assets/documents/WHO_2008_global_TB_report.pdf.