CHAPTER 2

Validation of the MARTI-assay on IAsys biosensor

2.1 Introduction

Active tuberculosis is diagnosed by detecting *Mycobacterium tuberculosis* complex bacilli in specimens from the respiratory tract (pulmonary TB) or in specimens from other sites of the body (extrapulmonary TB). Although many new diagnostic methods have been developed, acid fast bacilli (AFB) smear microscopy and culture are still the gold standards for diagnostic of active TB, especially in low resource countries (Palomino *et al*., 2007). Microscopic identification and culture of *Mycobacterium* species in sputum are the most common methods for diagnosis of pulmonary disease, but the detection of extrapulmonary TB is often more difficult. In the search for rapid and cost-effective diagnostic methods for TB, immunodiagnosis is considered an attractive option, because it uses the specific humoral and cellular immune responses of the host to infer the presence of infection or disease; thereby avoiding the problem of sensitivity of detection of traces of the infectious agent itself. A wide variety of serological tests for the detection of antibodies in individuals suspected to have TB have been evaluated to detect active disease (Chan *et al*., 2001; Schleicher *et al*., 2002; Pan *et al*., 1999). Serology has additional advantages in situations where the patient is unable to produce adequate sputum, where TB is extrapulmonary and where sputum smear and culture results are negative (Palomino *et al.*, 2007).

Our previous studies (Schleicher *et al*., 2002) and that of others (eg. Pan *et al*., 1999) have shown the prevalence of anti-mycolic acid antibody in TB patients with ELISA. Schleicher *et al.* investigated the diagnostic potential of an ELISA, based on the detection of antibodies to *M. tuberculosis* mycolic acids in sera of HIV seropositive and HIV seronegative tuberculosis patients, in a population with a high prevalence of HIV. Although they observed a higher signal of antibody to mycolic acids in TB positive patients than in TB negative patients, they also found quite a number of false positive and false negative results. From their studies they concluded that the ELISA has inadequate sensitivity and specificity to detect anti-mycolic acid antibody and is

therefore not suitable as a reliable serodiagnostic assay for the diagnosis of pulmonary TB.

Our previous study on the IAsys biosensor showed good potential in detecting anti-mycolic acid antibodies in seventeen active TB patient serum samples (Thanyani, 2003). A recent study done by Nagel *et al*. (2007) reported a specificity of 100% and a sensitivity of 75% for detecting antibodies as surrogate markers of active TB using immobilized 30-kDa antigen from *M. tuberculosis* and contacting it with undiluted blood serum samples when optical biosensors were utilized. However, the dilution of the samples decreased the sensitivity of the assay (Nagel *et al*., 2007). Their results are probably not universally applicable, as many studies have shown a decrease in specificity and sensitivity of immunoassay when the 30-kDa is used in an HIV burdened population (Daniel *et al*., 1994; Hendrickson *et al.,* 2000). It is known that the production of antibodies to protein antigens generally depends on the help of $CD4^+$ T cells and the infection with HIV results in depletion of $CD4^+$ T cells and inhibition of function of the remaining T cells (Price *et al.*, 2001). The reason why the antibody response to glycolipid antigens such as mycolic acid is preserved in HIV seropositive TB patients, despite declining CD4 T lymphocyte counts, has been reported to be due to the novel CD1-restricted lipid antigen presentation pathway (Moody *et al.*, 1997; Schleicher *et al*., 2002; Simonney *et al*., 2007). However, the lipid antigen presentation to B-cells in humoral immune responses has not yet been reported. It is believed that mycobacterial lipid antigen epitopes may be presented by novel mechanisms different from the classical MHC class I or class II restricted proteins antigens (Fujita *et al*., 2005a).

2.1.1 Prevalence of HIV in TB

Tuberculosis has re-emerged as a global health problem due to co-infection with HIV and the emergence of drug resistant strains of *Mycobacterium tuberculosis*. There is a need for a reliable and fast serodiagnostic assay to reduce the time required for test results from weeks to hours, in order to better control the spread of the disease. World Health Organization (2006) has reported that, in countries with the highest HIV prevalence, more than 75 % of cases of tuberculosis are HIV-associated. Individuals with HIV infection are at increased risk for TB infection and more serious disease due

to their weak immune system when compared to those without HIV infection. Strategies that are normally effective in healthy populations cannot be transferred directly to control TB in persons with HIV infection (Frothingham *et al*., 2005). It is estimated that 50% of the population of sub-Saharan Africa is latently infected with TB. Once infected with *M. tuberculosis*, progressive deterioration of cell mediated immunity caused by HIV infection increases the risk of TB disease by a hundred fold or more (Frothingham *et al*., 2005). In Africa, TB is often the first manifestation of HIV infection, and it is the leading cause of death among HIV-infected patients. Corbett *et al.* (2006) state that every opportunity should be taken to screen HIV-infected patients for active TB, just as every patient with TB should be screened for HIV. The timing of the initiation of antiretroviral therapy in patients with HIV and TB co-infection is also difficult, due to quick immune deterioration in such patients (Frothingham *et al*., 2005). WHO (2006) guidelines suggest starting antiretroviral drugs within two months of tuberculosis treatment. Since patients who start antiretroviral drugs early in their TB treatment can be predisposed to immune reconstitution syndrome, which is frequent, have symptoms overlapping with worsening TB and can be life threatening to the patients (Lawn *et al*. 2005). Only a rapid and reliable diagnostic assay can reduce the TB infection, especially to those co-infected with HIV because they are more likely to develop drug resistant TB (Frothingham *et al*., 2005). Previous studies done by Schleicher *et al.* (2002) has shown that anti-mycolic acid antibodies can be shown also in HIV infected patients. Our previous studied on the IAsys biosensor has shown a potential development of a serodiagnosis assay in TB patients co-infected with HIV (Thanyani, 2003). The CD1-restricted lipid antigen presentation pathway could probably be the reason why the antibody response to mycolic acids is preserved in HIV-seropositive patients despite a declining CD4 T-lymphocyte count (Schleicher *et al*., 2002).

2.1.2 Advantages of the IAsys Biosensor

The amount of both ligand and analyte needed to obtain informative results is low and the time required to perform an assay is very short. Another advantage is that the cuvette can be reused many times. This indeed lowers the costs with the only limitation being the repeated verification of the stability of the immobilized ligand (Bertucci and Cimitan, 2003). The IAsys biosensor ensures complete solution

homogeneity throughout the cuvette by means of a vibro-stirrer, which is of extreme importance in order to minimize mass-transport effects. The IAsys cuvettes are available with one or two cells. The two cells offer an advantage since one cell could be used as control in comparative measurements. The internal aspirators are used to remove solutions from the cells without removing the cuvette from the system, which makes the addition of the solution easy and fast.

The IAsys affinity biosensor requires about one tenth (5µl) of the amount of patients' sera that is required for ELISA and other standard serological tests (Siko, 2002). Since a patient's serum is a limited resource, the ability to use a minimal amount of serum could make the IAsys affinity biosensor an instrument of choice for the detection of anti-mycobacterial antibodies in patients infected with *M. tuberculosis*.

2.1.3 Immobilization of mycolic acids antigen on IAsys biosensor

The immobilization of mycolic acids was first reported by Siko (2002). This was followed up by Thanyani (2003) to give a proof of principle of the MARTI (Mycolic acid Antibody Real-Time Inhibition)-assay, whereby liposomes carrying mycolic acids could be immobilized on the non-derivatized IAsys biosensor cuvettes and then used for monitoring the binding of anti-mycolic acids antibodies for the development of a serodiagnostic method for tuberculosis. Siko (2002) initially immobilized liposomes containing both mycolic acids and cholesterol onto the surface of the hydrophobic cuvette, but found that the coated surface was not stable. Altin *et al*. (2001) also showed that immobilization of lipid membranes on IAsys hydrophobic cuvettes from a solution of lipids in organic solvents did not always produce consistent results, even when the procedure was carried out according to IAsys protocols manual. Siko (2002) firstly activated the surface of a non-derivatized cuvette with a cationic detergent, cetyl pyridinium chloride (CPC) to make the hydrophilic surface hydrophobic and could then stably coat with mycolic acids and cholesterol containing liposomes. In their studies Siko (2002) and Thanyani (2003) showed adequate binding of mycolic acid and cholesterol containing liposomes occurred after activation of the non-derivatized hydrophilic surface with the cationic detergent CPC. A neutral surfactant, saponin, was used to further stabilize the surface and also to block the non-specific binding to immobilized liposomes. Saponins are

highly soluble in water and show a typical surfactant behaviour, i.e. forming colloidal solutions that easily generate foam at low concentrations. The saponins are known as biologically highly active substances. Previous studies (Thanyani, 2003) showed that an optimum concentration needs to be determined on the mycolic acids and cholesterol surface for each batch of saponin that is obtained. The same approach was followed in this study to validate the MARTI-assay on IAsys biosensor using tuberculosis patient sera collected by Schleicher *et al.* (2002).

2.3 Aims

To validate the mycolic acid antibody real-time inhibition (MARTI)-assay on an IAsys biosensor for its application to detect anti-mycolic acid antibodies in human serum samples from patients suffering from active tuberculosis due to infection with *M. tuberculosis*.

2.4 Materials and Methods

2.4.1 Materials

2.4.1.1 General reagents

Cetyl-pyridinium chloride (1-hexadecylpyridinium chloride), L-α-phosphatidylcholine (L-α-Lecithin, 99%), batches of saponin and ethylene diamine tetra-acetic acid (EDTA) were from Sigma (St Louis, MO). Sterile double distilled water was used throughout for the preparations of aqueous solutions. Sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), and sodium hydrogen phosphate (Na_2HPO_4) were from Merck (NT laboratories, SA). Chloroform, potassium hydroxide (KOH) and ethanol (98%) were from Saarchem (SA).

2.4.1.2 Enzyme Linked Immunosorbent Assay (ELISA)

Serowell ELISA plates: flat-bottom 96-well plates; disposable pipette tips; Sterile, disposable 50 ml centrifuge tubes and disposable pipettes were from Bibby Sterilin Ltd, Stone, UK. Goat anti-human IgG (Heavy and Light chain) antibody conjugated to peroxidase was obtained from Sigma, St Louis, MO, USA. Carbohydrate- and fatty acid free casein was from Calbiochem, La Jolla, CA and hydrogen peroxide from Merck (Darmstadt, BRD). *o*-Phenylenediamine and polyethylene glycol (PEG) from Sigma, St Louis, MO, USA.

2.4.1.3 ELISA Buffers

PBS buffer: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 (anhydrous) and 1.05 g Na_2HPO_4 (anhydrous) per 1 liter distilled water, adjusted to pH 7.4.

Neutralisation buffer: K_2HPO_4 (1 M in dddH_2O) adjusted to pH 9.0 with H_2KPO_4 (1 M) if necessary.

Acidification buffer: Glycine HCl (0,2 M, pH 2.8).

Diluting buffer: 0.5% (m/v) carbohydrate- and fatty acid free casein in PBS buffer adjusted to pH 7.4 was used for diluting the sera and the immunoreagents.

2.4.1.4 Resonant mirror biosensor apparatus

The IAsys resonant mirror biosensor system and twin-cell non-derivatized cuvettes were from Affinity Sensors (Cambridge, United Kingdom).

2.4.1.5 Human sera

Serum samples were selected from 101 patients (aged between 18 and 65) collected for another study by Schleicher *et al*. (2002), who were admitted to the general medical wards of the Helen Joseph Hospital; Johannesburg, South Africa, including a number with active pulmonary tuberculosis. The study population consisted of a tuberculosis-positive (TB^+) group and a control tuberculosis-negative (TB^-) group. The TB^+ group consisted of patients with newly diagnosed smear-positive pulmonary tuberculosis of which some were HIV-seropositive. The TB^- patients that were used for control had medical conditions other than TB and were recruited from the general medical wards. None of the TB^+ patients were on anti-TB chemotherapy at the time of serum collection.

2.4.1.6 Mycolic acids

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum *et al*. (2001).

2.4.1.7 Biosensor Buffer

Phosphate buffered saline (PBS) azide EDTA buffer (PBS/AE): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.05 g Na_2HPO_4 per liter ultra-pure, distilled water with 1 mM EDTA and 0.025% (m/v) sodium azide, adjusted to pH 7.4.

2.4.2 Methods

2.4.2.1 Preparations of liposomes

Stock solution of phosphatidylcholine (100 mg/ml) (Sigma, St Louis, MO) was prepared by dissolving the weighed amounts in chloroform. Mycolic acids containing liposomes were prepared by adding 90 µl phosphatidylcholine stock to 1 mg dried mycolic acids. Empty liposomes, i.e. with no mycolic acids, were prepared by using 90 µl of phosphatidylcholine stock solution only. During pipetting, everything was kept on ice to avoid evaporation of chloroform. The liposome ingredients were dried with nitrogen gas in a heat block at 85 °C for about 10 min. Liposome formation was induced by addition of 2 ml saline (0.9% NaCl) and placing in a heat block at 85 °C for 20 min, with vortexing every 5 min. The liposomes were then sonicated for 2 min at 30% duty cycle at an output of 3% with a Model B-30 Branson sonifier (Sonifier Power Company, USA). The sonicator tip was washed with chloroform and rinsed with distilled water before and after use. The liposomes (200 µl) were aliquoted into ten tubes and kept at –20 °C overnight before freeze-drying. After freeze-drying, 2 ml of phosphate buffered saline (PBS) azide EDTA (Sigma, St Louis, MO) buffer (PBS/AE, pH = 7.4) was added to each tube containing liposomes. The tubes containing liposomes were placed in a heat block for 20 min. and sonicated as before.

2.4.2.2 ELISA of patient sera

Mycobacterial mycolic acids were isolated from a culture of *M. tuberculosis* H37Rv (American Type Culture Collection 27294) as described by Goodrum *et al*. (2001). Mycolic acids (250 µg) were dissolved in 4 ml hot phosphate-buffered saline (PBS, pH 7.4) for 20 min at 85 °C and sonicated (Virsonic 600, United Scientific, USA) at 20% duty cycle and optimal output level for 1 min. The solution was kept at 85 °C during pipetting into ELISA plates (Sero-Well®, Bibby Sterilin Ltd, UK), after which

the plates were placed in plastic bags and incubated overnight at 4 °C. The final antigen load was approximately 3 µg/well. Control wells were coated with hot PBS only. After overnight incubation, the ELISA plates were flicked out and the wells blocked with 0.5% (m/v) carbohydrate- and fatty acid-free casein in PBS for 2 h at room temperature. The solution was flicked out, filled with 50 µl serum or serum precipitate in triplicate and incubated for 1 h at room temperature, flicked out and washed three times with PBS/0.5% casein. The wells were aspirated to remove proteinaceous froth. The plates were incubated for 30 min. at room temperature with peroxidase-conjugated goat anti-human IgG (whole molecule, Sigma) diluted 1/1000 in PBS/0.5% casein, flicked out, washed three times with PBS/0.5% casein and aspirated. The presence of antibody was revealed using 50 µl/well of hydrogen peroxide (40 mg) and *o*-phenylenediamine (50 mg) in 50 ml 0.1 M citrate buffer (pH = 4.5). Measurement of the yellow colour was done after 30 min at 450 nm using a Multiskan Ascent photometer (Thermo-Labsystems, Finland). To correct for background binding in serum, the signal generated with those samples in PBS coated wells was subtracted from that generated in mycolic acid coated wells.

2.4.2.3 Detection of anti-mycolic acids antibody with IAsys affinity biosensor

The IAsys resonant mirror biosensor sensor was set for a data-sampling interval of 0.4 sec, temperature of 25 °C and stirring rate of 75% for all experiments. The cells were rinsed three times prior to use with 96% ethanol (Saarchem, SA), followed by extensive washing with PBS/AE. A 60 µl volume of PBS/AE was pipetted into each cell of the cuvette to obtain a stable baseline for 1 min. The PBS/AE was subsequently aspirated and the surface activated with 50 µl of 0.02 mg/ml CPC, which was freshly prepared every week, for 10 min. This was followed by five times washing with 60 µl PBS/AE and then substituting with 25 µl PBS/AE for a new baseline before immobilization of mycolic acids containing liposomes to the surface for 20 min. The immobilized liposomes were then finally washed five times with 60 µl PBS/AE, substituted with 50 µl of saponin and incubated for 10 min. This latter step was to avoid non-specific binding on the surface of the cuvette during the subsequent binding events. The cells were then washed five times with PBS/AE, the content of each cell substituted with 25 µl of PBS/AE and left for about 5 - 10 min to achieve a stable baseline. Inhibition studies were performed using patient's serum that

was first placed at room temperature to thaw completely. After obtaining a stable baseline, a 1/1000 dilution of serum antibodies (10 µl) in PBS/AE was added in each cell, to compare the responses of the two cells over 10 min. A pre-incubation of 1/500 dilutions of serum with solutions of liposomes containing mycolic acids and empty liposomes (phosphatidylcholine alone) were allowed for 20 min. These were then added (10 µl) for binding inhibition studies in different cells, one with mycolic acids liposomes and the other with empty liposomes as a control, and allowed to bind for 10 min. Finally, dissociation of antibodies was effected with three times PBS/AE washing and measurement of the response for 5 min.

2.4.2.4 Regeneration of non-derivatized cuvettes

Regeneration was effected by initial three times washing with 96% ethanol for 1 min, followed by seven times washing with 70 µl PBS/AE for 1 min. The surface was then finally treated with 50 µl potassium hydroxide (12.5 M) for 2 min followed by seven times washing with 70 µl PBS/AE for 1 min.

2.5 Results

2.5.1 Biosensor criteria applied for validation

In this study, 102 patients were analysed on the IAsys biosensor to identify anti-mycolic acid antibodies in human patient's sera, to determine the specificity and sensitivity of the MARTI-assay. After analyzing all the patient sera, the profiles were then analyzed for accuracy of measurement and the following criteria were applied for the acceptance of the data point: the cuvette cell calibration curves of the high dilution serum in the two cells of one cuvette had to fall within 90 – 100% identity in terms of the relative response amplitudes, calibration curve profiles had to be similar by eye, and the amplitude of binding of the calibration curves had to be at least the average of all 102 samples analyzed minus one standard deviation. This translated into 480 – 145 = 335 arc.seconds as minimum response amplitude required for the calibration curve. Figure 2.1 indicates one of the sensorgrams that was not accepted due to the difference in response of the two channels. Of the 102 sera that were analyzed, 61 met the criteria above. These were divided into 32 TB positive, 11 TB negative and 18 $HIV^{+}TB^{-}$ samples.

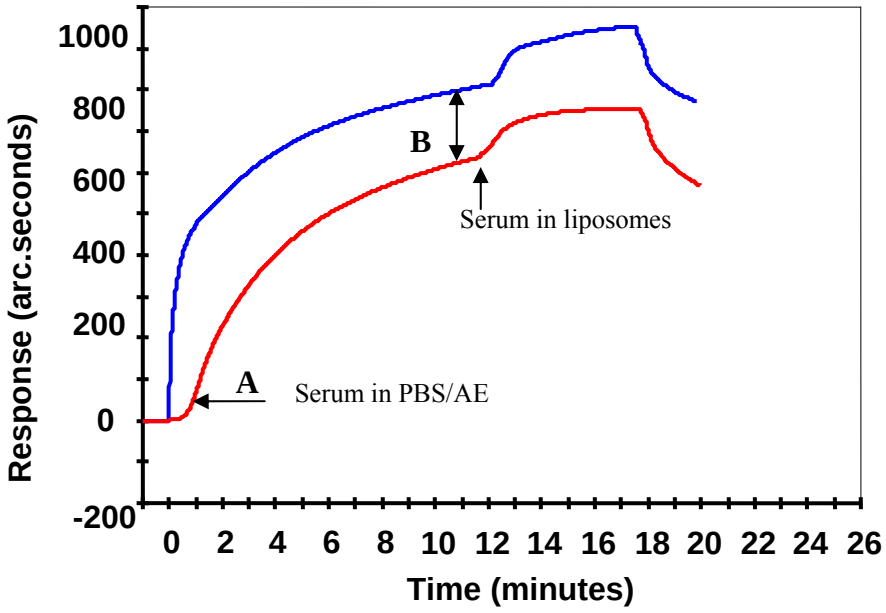

Figure 2.1: A typical inhibition binding profile on the IAsys biosensor that was not accepted due to channel differences in binding response. A and B show two manifestations of retardation in the initial binding response when the same volumes of serum in PBS/AE were added in the two cells.

2.5.2 Detection of anti-mycolic acids antibodies in human sera

The six main stages involved to measure the binding of specific antibodies to lipid antigens in liposomes in real time on the biosensor are: (A) the activation of the non-derivatised cuvette surface with CPC, (B) immobilization of the liposomes containing mycolic acids to the surface, (C) blocking with saponin to prevent non-specific protein binding, (D) binding (association) of antibodies from a high dilution of serum to calibrate the signal of the two cells of the cuvette, (E) the binding and dissociation of inhibited patient sera at higher concentration, and finally (F) surface regeneration (Fig. 2.2). The dilutions of sera used were estimated from a dilution range of one positive and one negative serum sample and are not necessarily optimal for all sera. The cuvette cell calibration curves of the high dilution serum in the two cells of one cuvette had to fall within 90 – 100% identity in terms of the relative response amplitudes in order to be accepted. A limitation of the IAsys system was found to be the difference in quality from one cuvette to another when using liposomes as antigen

coat. In rare cases, new cuvettes were not usable at all. Usually, new cuvettes were found to be reliable only after a succession of regenerations, while in other rare cases, new cuvettes could be reliably applied after a single regeneration cycle. The results (those in the rectangle, Fig. 2.2) were aligned using the Fastplot programme from IAsys.

Patient sera selected from the collection of Schleicher *et al*. (2002) were used to detect antibodies against mycolic acids on the optical IAsys biosensor. The ELISA experiments were performed as described in Schleicher *et al*. (2002). Out of the 61 patient sera that were analyzed on the IAsys biosensor, 17 were re-analyzed on ELISA to confirm that the original antibody activity as reported by Schleicher *et al.* (2002) was still intact and to compare them with the results found on the IAsys biosensor during the same period of assessment. The inhibition studies on the IAsys were determined by pre-incubating test serum with mycolic acids-containing liposomes and applying these on biosensor cuvettes coated with mycolic acids. In the control experiments, sera were pre-incubated with empty liposomes. The pre-incubation of a sputum positive TB patient serum with mycolic acids liposomes resulted in inhibition of antibody binding to mycolic acids when compared to the signal generated by the same serum pre-incubated with empty liposomes (Fig. 2.3A). This confirmed the specificity of binding of antibodies to mycolic acids in sputum positive TB patients' sera.

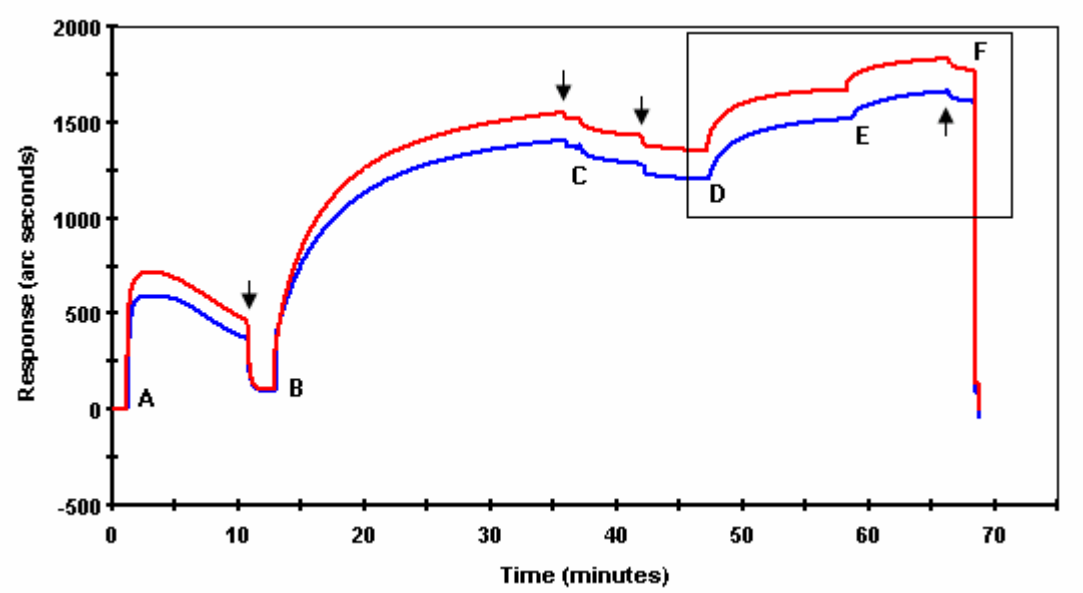

Figure 2.2: A typical graph summarizing the process of measuring antibody binding or inhibition of binding by mycolic acid and empty (phosphatidylcholine only) liposomes, in the two cells of an IAsys biosensor cuvette surface coated with mycolic acid liposomes. The surface was activated with cetyl-pyridinium chloride (A), coated with mycolic acids liposomes (B), blocked with saponin (C), calibrated with a high dilution of serum (D), applied to measure the binding and dissociation of inhibited sera at lesser dilution (E), and regenerated with potassium hydroxide (12.5 M) and 96% ethanol (F). The arrows indicate washing with PBS/AE and the response from the two cells are differentiated by red lines (channel 1, upper curve) and blue lines (channel 2, lower curve).

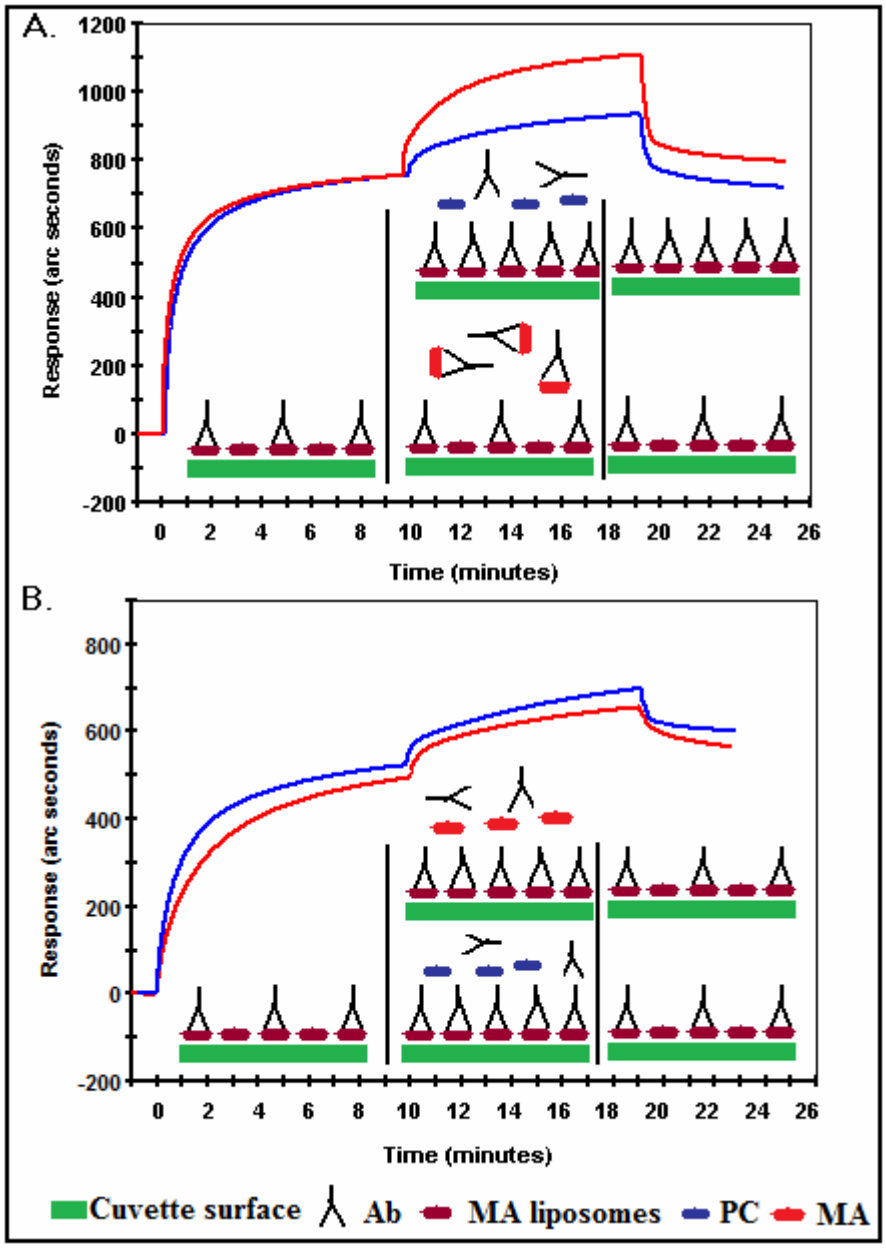

Figure 2.3: Inhibition of human TB^+ (A) and TB^- (B) patient serum antibody binding with mycolic acids or empty liposomes on an IAsys cuvette surface coated with immobilized mycolic acids liposomes. For the first 10 min, a 1/1000 dilution of serum in PBS/AE was incubated in both cells. For inhibition studies, the pre-incubated serum in a dilution of 1/500 was then added with the blue channels (A = lower curve and B = upper curve) representing the binding response of serum in mycolic acids and the red channels (A = upper curve and B = lower curve) representing that of serum in empty liposomes as control.

There was no inhibition of binding observed when a sputum negative control serum (TB^-HIV^-) was pre-incubated with liposomes containing mycolic acids and tested on the biosensor to determine binding of antibodies to mycolic acids (Fig. 2.3B). This shows that specific anti-mycolic acids antibodies can be demonstrated in TB^+ patients, after pre-incubation of serum with mycolic acids. TB negative sera from patients infected with HIV tested negative on the IAsys biosensor, with inhibition values of less than 20% (Fig. 2.4).

From 23 TB^+HIV^+ patient sera selected, two sera samples tested false negative on the biosensor (Fig. 2.4). Thirteen TB^-HIV^+ patients' sera tested "false" positive, showing an inhibition of greater than 20% on the biosensor (Fig. 2.4). It is noteworthy that these patients were HIV positive. Some patient sera that were false negative (eg. Fig. 2.5A) and false positive (eg. Fig. 2.5B) on the ELISA tested positive and negative respectively on the biosensor. The normalized signals on ELISA that were above 2 were regarded as positive and below two as negative. The TB^+ and TB^- patients that showed truly positive and negative responses of antibodies to mycolic acids on ELISA also tested truly positive and negative on IAsys biosensor respectively (Fig. 2.6). Our previous studies have also addressed the problems of detecting *M. tuberculosis*-specific antibodies to mycolic acid in TB patients co-infected with HIV on ELISA (Schleicher *et al*., 2002). Three of the patient sera tested from the thirteen HIV^-TB^- tested false positive on the biosensor, and only two serum samples tested false negative in TB^+ HIV^- population (Table 2.1). An apparently lower specificity (27.8%) was observed in TB^-HIV^+ subgroups. However, all these patients were hospitalized with diseases other than TB with the prevailing diagnostic methods. The low specificity obtained amongst the HIV^+ population could reflect true positive results, since it is known that the sputum culture assay is not sensitive enough to detect TB in HIV positive patients (Frieden *et al*., 2003). This may reflect the better ability of the serum test to detect TB in HIV^+ patients. The IAsys affinity biosensor was found to be more sensitive (91.3%) in detecting TB amongst the TB positive patients co-infected with HIV. The overall specificity and sensitivity of the assay after analyzing 61 patient sera was 48.4% (15/31) and 86.7% (26/30) respectively.

It is known that the gold standard of sputum growth of mycobacteria does not measure accurately in the TB^-HIV^+ cohort (Table 2.1). As the serum collection was

actually made for an earlier study, follow-up data were not available to determine the true TB status of the TB^-HIV^+ cohort tested here. When the 18 TB^-HIV^+ sera were omitted in the calculation of the performance parameters of the test based on the 61 data points, accuracy of the assay was found to be 83.7% (36/43). The sensitivity (86.7%, 26/30) remained the same after exclusion of the TB^-HIV^+ population, and the specificity was 76.9% (10/13). The assay showed a high sensitivity (91.3%) in sera from patients who were TB positive and co-infected with HIV. It is known that HIV-positive patients generally have lower levels of *Mycobacterium tuberculosis*-specific antibodies to protein and certain lipid antigens than HIV-negative patients. This shows that the IAsys biosensor can detect anti-mycolic acids antibodies in an HIV endemic population.

Table 2.1: Specificity and sensitivity of the IAsys affinity biosensor assay for detecting anti-mycolic antibody in pulmonary TB and negative control patient sera.

Patient group	*n*	False +	False -	Specificity	Sensitivity
TB^+HIV^+	23	-	2	-	91.3% (21/23)
TB^+HIV^-	7	-	2	-	71.4% (5/7)
TB^-HIV^+	18	13	-	27.8% (5/18)	-
TB^-HIV^-	13	3	-	76.9% (10/13)	-
TOTAL	**61**	**16**	**4**	**48.4% (15/31)***	**86.7% (26/30)***

(+ = positive, - = negative, and *n* = number of patients)

***Accuracy = 81.8%** (the data for specificity of the TB^-HIV^+ group is omitted because of its known underestimation of TB positiveness by standard culture growth assays)

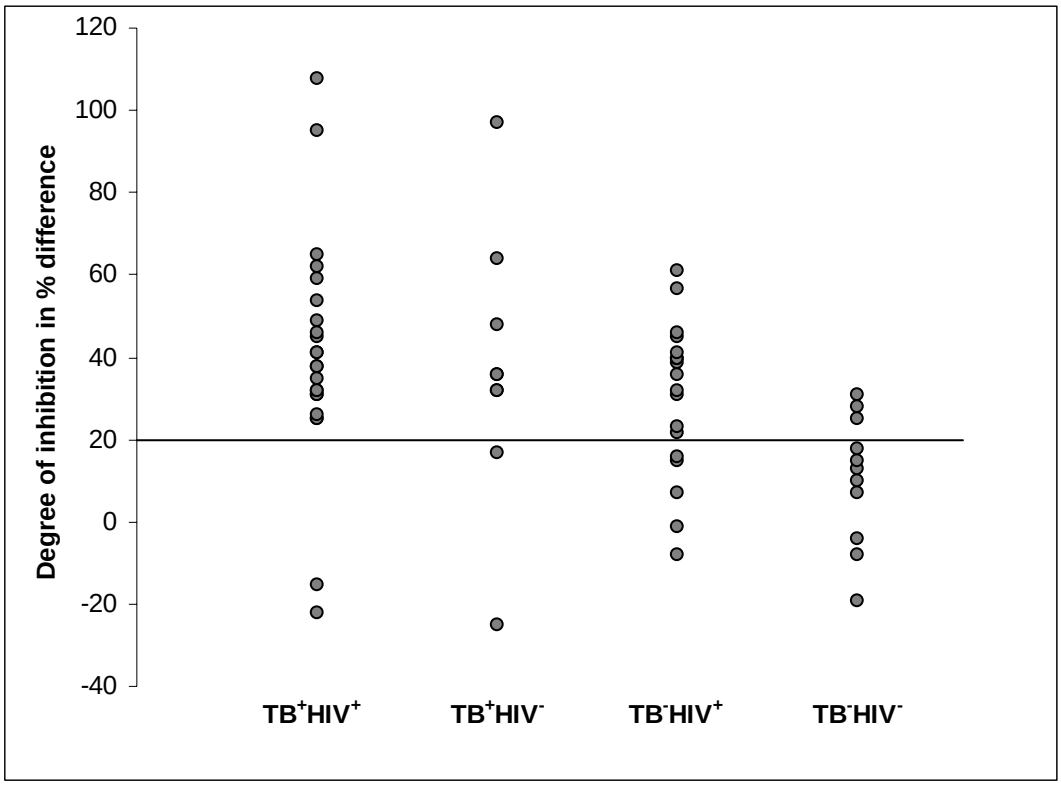

Figure 2.4: The percentage of inhibition of binding of biosensor signal for the 61 patient sera of TB^+ and TB^- controls after pre-incubation of sera with mycolic acids and empty liposomes before testing on mycolic acids coated cuvettes.

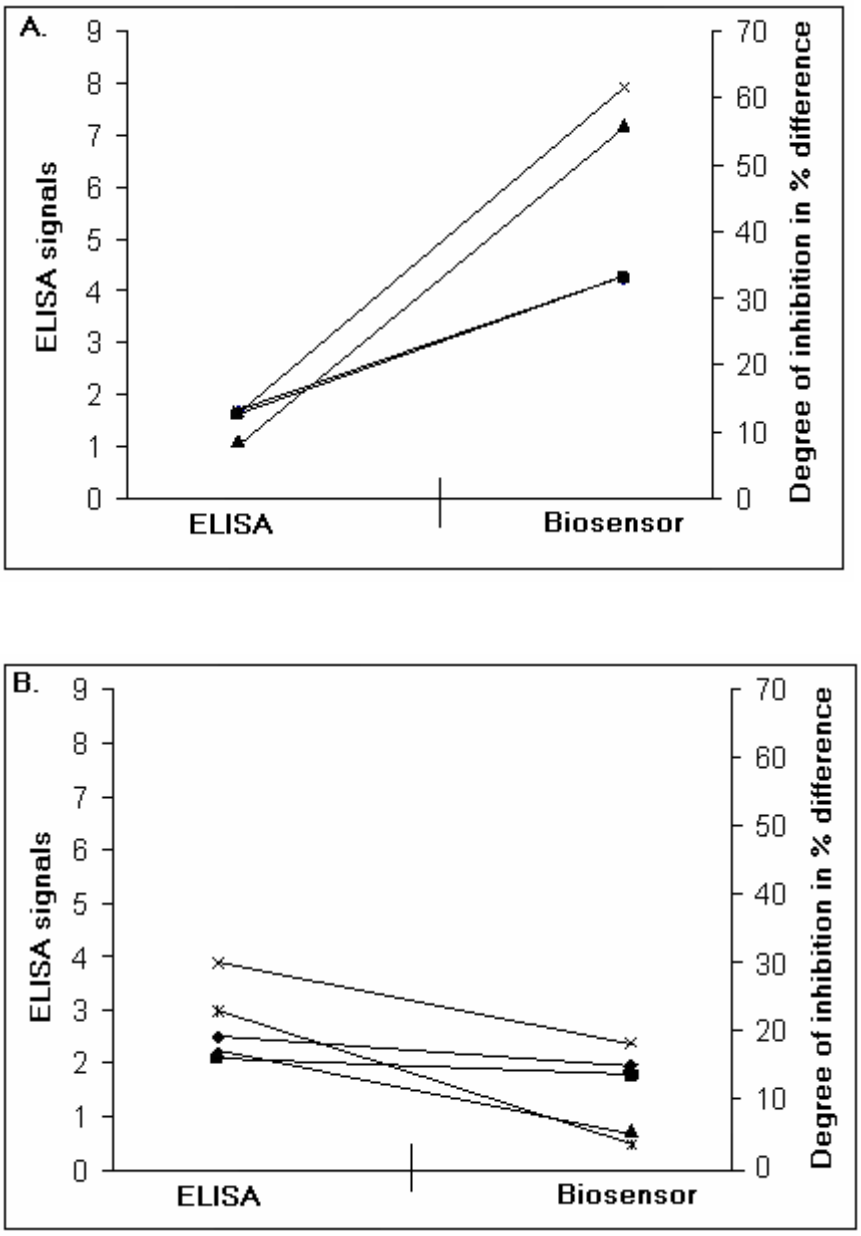

Figure 2.5: Normalized ELISA signals and the percentage of inhibition of binding of biosensor signal of false negative (A) and false positive (B) patients on ELISA who tested correctly on the biosensor.

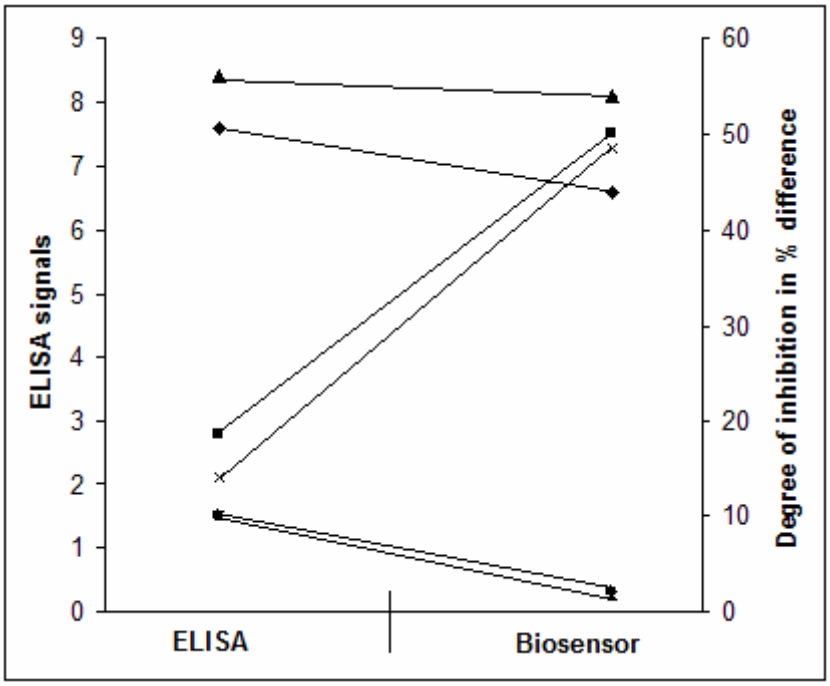

Figure 2.6: Normalized ELISA signals and the percentage of inhibition of binding of biosensor signal of true negative and true positive patients who tested correctly on ELISA and biosensor.

2.6 Discussion

In Africa, TB is often the first manifestation of HIV infection: it is the leading cause of death among HIV-infected patients. Corbett *et al.* (2006) stated that every opportunity should be taken to screen HIV-infected patients for active TB in order to prevent rapid death when both diseases manifest themselves in an individual, and to safely provide antiretroviral (ARV) treatment. The shorter the time from sampling to the diagnostic result, the more lives will be saved. Serodiagnosis with mycolic acids as antigen provides such an opportunity (Verschoor and Onyebujoh, 1999).

Pan *et al*. (1999) indicated that the anti-cord factor antibodies (IgG) in TB patients specifically recognized mycolic acid structure, especially methoxy mycolic acid methyl esters. Mycolic acid is presented by antigen-presenting cells (APC) through a mechanism that does not involve major histocompatibility complex (MHC)-class I or MHC-class II molecules (Moody *et al*., 1999). The anti-mycolic acid immune response could therefore be independent from the participation of $CD8^+$- or $CD4^+$-T cells that respond to antigen that is respectively presented on MHC I and MHC II surface proteins. Other than the MHC-presented protein antigens, mycolic acid is presented on CD1, with the ability to induce proliferation of T-cell lines, with or without the CD4 or CD8 molecules (Beckman *et al*., 1994, Goodrum *et al*., 2001). The production of antibodies to protein antigens generally depends on the help of $CD4^+$ T cells. It is known that infection with HIV results in depletion of $CD4^+$ T cells and inhibition of function of the remaining T cells (Price *et al.*, 2001). Thus, Hendrickson *et al.* (2000) showed a decreased antibody specificity and sensitivity to a mycobacterial 30-kDa protein antigen with ELISA when screening patients in a population that had a high prevalence of HIV. Ratanasuwan *et al*. (1997) showed that when a lipoarabinomannan (LAM) was used in serological tests on HIV-negative and TB positive patients, it showed sensitivities varying from 21% to 89%, but only 7% to 40% in the HIV-positive patients. Antunes *et al*. (2002) described the MycoDot serological assay for tuberculosis that is based on the detection of specific IgG antibodies against the LAM antigen, fixed onto a solid support consisting of a plastic comb designed to fit into the wells of a microtiter plate. The sensitivity values observed were definitely lower in cases of TB associated with HIV, which refuted the usefulness of the test in regions where HIV is highly endemic. They concluded that LAM as an antigen is only satisfactory in the serodiagnosis of TB as long as HIV is

not highly prevalent in the population. Daniel *et al*. (1994) performed a field test in Mexico, and showed that the ELISA based on the mycobacterial 30-kDa protein antigen had a sensitivity of 70% in patients with culture-positive or smear-positive pulmonary TB and a specificity of 100% in 125 control donors. The same test was evaluated with HIV-positive and negative patients in Uganda. Although the sensitivity and specificity in HIV–negative donors were similar to the results of the Mexico test, the ELISA gave a sensitivity of 28% of 128 sera from HIV-positive donors. However, the immune response to mycolic acid could in principle proceed independently of the $CD4^+$/$CD8^+$ T cells. The human CD1 protein is known to mediate T-cell responses by presenting at least the three classes of mycobacterial lipids, i.e. free mycolates, glycosylated mycolates and diacylglycerol based glyco-phospholipids. The alkyl chains of the mycolic acid antigen have been proposed to bind directly within the hydrophobic groove of CD1 resulting in presentation of the hydrophilic caps to the T-cell's antigen receptor (Porcelli *et al*., 1996; Moody *et al*., 1999). The CD1-restricted lipid antigen presentation pathway could provide a possible explanation why the antibody response to mycolic acids is maintained in HIV-seropositive patients, despite a declining CD4 T-lymphocyte count (Schleicher *et al*., 2002). Simonney *et al.* (2007) also suggested that the CD1-restricted lipid antigen presentation pathway is the likely mechanism accounting for the perseverance of high circulating antibody responses to PGL-Tb1 antigen in HIV infected patients with TB. Simmonney *et al*. (2007) showed that about half of HIV-positive individuals produce specific anti-glycolipid antibody several months before a diagnosis of TB disease can be made.

Alving and Wassef (1999) measured the anti-cholesterol antibodies in healthy individuals and described that almost every healthy individual has various amounts of IgM and IgG anti-cholesterol antibodies partly present in complexed form with LDL and VLDL (Dijkstra *et al*., 1996; Horvath *et al*., 2001). Siko (2002) previously reported a discovery of a cross-reactivity of binding of TB patient sera antibodies between mycolic acids and cholesterol on the IAsys affinity biosensor. This work was followed up by Benadie *et al*. (2008), see Appendix B for detail. Horvath and Biro (2003) showed cholesterol concentration to be higher in HIV patients than in HIV-seronegative controls. This could also explain the false positive results in HIV patients, without TB, obtained in this study. The level of anti-cholesterol antibodies may also be high in these patients. These antibodies could then be inhibited with

mycolic acid due to the presumed molecular mimicry between mycolic acid and cholesterol. However our previous study on IAsys biosensor shows that even though the anti-mycolic acid IgG antibodies in human serum that may recognize both cholesterol and mycolic acid, the antibodies are more specific to mycolic acid and can be distinguished with the biosensor from non-specific binding (Thanyani, 2003).

Here, a significant increase in sensitivity and specificity was shown for the anti-mycolic acid antibody detection in patient sera with the inhibition assay on biosensor, as compared to that reported in our previous study using an ELISA (Schleicher *et al*., 2002). The false positive results observed amongst the $TB^{-}HIV^{+}$ population could show that the patients were true positive on the IAsys biosensor, since a sputum culture was used as gold standard method for confirming their TB status. However, it is known that sputum culture of HIV-infected patients need more incubation time than that of patients without HIV infection, which is consistent with the lower bacillary load seen in the sputum of HIV infected patients (Brindle *et al*., 1993). The culture requires 10 – 100 viable *Mycobacterium tuberculosis* per millilitre of sputum to give positive results (Colebunders and Bastian,, 2000). It has also been shown that 15 – 20% of adults with pulmonary TB whose diagnosis has been based on clinical, radiographic, and histopathological findings and response to anti-TB treatment have negative sputum cultures (Frieden *et al*., 2003).

The IAsys affinity biosensor was able to detect low affinity antibody binding to mycolic acids, in addition to high affinity antibody, which the conventional methods cannot generally achieve. In an ELISA these antibodies would have been washed away before the final step and the patient would have tested false negative. The advantage of the biosensor lies in its real-time detection of antibody binding, without the need for prior washing away of the unbound antibody excess. In addition, the inhibition of binding as an endpoint eliminates much non-specific binding interference, which adds to the increased specificity of the biosensor assay. A disadvantage of the biosensor is that it is blind to the identity of the binding ligand from the serum sample. The binding of IgG to mycolic acids was confirmed by showing that its binding inhibition could be reproduced with purified IgG from the same serum sample. The IgG experiments were co-ordinated with one the co-authors (Vanessa Roberts) of the recently published work (Appendix A). The inhibition

signals of isolated IgG from patient serum and the IgG negative control were measured on different channels of the IAsys biosensor. A significant difference in the ability of IgG positive and IgG negative to be inhibited by mycolic acid liposomes was shown. These results correlate with data obtained using whole serum and confirm that it is the IgG fraction of serum that is inhibited from binding by pre-incubation with the mycolic acid antigen (Appendix A).

The few false negative results that still remain with the biosensor analysis are probably due to the inhibition of antibody activity by circulating mycolic acid antigen in the circulation. Should this be the case, one can envisage that a duplicate test be run that is spiked with a stable source of anti-mycolic acid antibodies, such as monoclonal antibodies. A true negative will then return the spike signal, whereas a false negative will consume the signal. False positive results pose a more daunting technical challenge, but may be due to the cross-reactivity of antibodies to mycolic acids of non-tuberculous pathogenic mycobacteria, eg *M. avium*, which do occur at low frequency in especially HIV positive patients. More work is required to manage the specificity of the assay by, for instance, screening sera from patients that are TB negative, but test positive for *M. avium* disease. This work is currently underway.

Many serological assays have been developed for specific antibody detection to lipid cell wall antigens in tuberculosis patients (Lyashchenko *et al*., 1998; Pan *et al*., 1999; Pottunarthy *et al*., 2000; Julian *et al.*, 2002; Schleicher *et al*., 2002; Lopez-Marin *et al*., 2003; Fujita *et al*., 2005a), but generally they do not meet the requirements on specificity and sensitivity (Attallah *et al*., 2005). The biosensor approach may improve that by means of its unique benefits reported here. However, the technique is technically quite difficult to perform in the laboratory and the technology is not yet amenable to large scale screening of patients.

Since only 61 patients were analyzed with the biosensor in this study, more patient and control sera will have to be analyzed to properly validate it as a reliable technique to determine anti-mycolic acids antibodies as surrogate markers for active tuberculosis. However, the detection of anti-mycolic acids antibodies with the IAsys affinity biosensor appears to be technically feasible, quick and may also be made affordable by further optimisation and innovation of the biosensor hardware.

Moreover, the biosensor assay may even prove to be more sensitive than the microbiological sputum growth assay, as was suggested here with some serum samples from HIV^+ patients that tested positive with the biosensor, but negative with the sputum assay.