Mycolic acid as antigen or analyte in tuberculosis

MONICA NUNES GOMES

Submitted in partial fulfilment of the requirements

for MSc: Biochemistry

in the Department of Biochemistry
Faculty of Natural and Agricultural Sciences
University of Pretoria

February 2007

I declare that the thesis/dissertation that I hereby submit for the degree in Biochemistry at the University of Pretoria has not previously been submitted by me for degree purposes at any other university.

SIGNATURE: …… DATE: ……

ACKNOWLEDGEMENTS

I would like to acknowledge several people and institutes who contributed to this dissertation:

- Prof. J. Verschoor for his assistance, support and encouragement throughout my studies.

- Mrs. Sandra van Wyngaardt for her continual assistance, advice, guidance and friendship.

- Yvonne for her unwavering friendship, guidance and support even from afar. Thank you!

- The TB team and the department of Biochemistry.

- My parents for all they have sacrificed in order for me to continue this far and their loving and valuable support. My siblings and friends for being there for me always, during good and bad times

- The National Research Foundation (NRF), Medical Research Council (MRC), Adcock Ingram Limited and the University of Pretoria for their financial support, which is greatly appreciated and acknowledged.

- Our Creator through whom all things are possible.

TABLE OF CONTENTS

CHAPTER 2: MYCOLIC ACIDS AS ANTIGEN IN A PROTEIN-A BASED ANTIBODY ASSAY FOR THE DIAGNOSIS OF TUBERCULOSIS

List Of Abbreviations

5-BMF	5-(bromomethyl)fluorescein
Ab	Antibody
Abs	Antibodies
Ag	Antigen
Ags	Antigens
BCG	Bacille Calmette-Guerin
c. m. c.	Critical micelle concentration
Cas	Casein
$CHCl_3$	Chloroform
Chol	Cholesterol
CIC	Circulating immune complexes
CPC	Cetylpyridiniumchloride
dddH_2O	Ultra-pure double distilled de-ionized water
DMF	Dimethylformamide
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HNTP	HIV negative TB positive
HPLC	High performance liquid chromatography
HPTP	HIV positive TB positive

IAsys	Interaction analysis system
IgG	Immunoglobulin G
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$KHCO_3$	Sodium bicarbonate
KOH	Potassium hydroxide
LAM	Lipoarabinomannan
Mϕ	Macrophage(s)
MA	Mycolic acids
MALADE	MA liposome adsorption-desorption assay
MBSA	Methylated bovine serum albumin
MRC	Medical Research Council
N_2	Nitrogen
Na_2HPO_4	Sodium hydrogen phosphate
NaCl	Sodium chloride
NaN_3	Sodium azide
NC	Negative control
NNR	Nearest-neighbour recognition
NR	No regeneration
OPD	o-phenylenediamine
PBS	Phosphate buffered saline
PBS/AE	Phosphate buffered saline-azide EDTA buffer
PC	L-α-phosphatidylcholine
PCR	Polymerase chain reaction
PPD	Purified protein derivative

RIA	Radio-immunoassay
RPLA	Reverse particle latex agglutination
Seph-prot A	Sepharose protein A
SPICA	Sepharose protein A immunoglobulin capture assay
TACO	Tryptophan aspartate-containing coat protein
TB	Tuberculosis
TDM	Trehalose-6, 6'-dimycolate
TN	TB-negative
TNF	Tumour necrosis factor
TP	TB-positive
TRIS	Tris(hydroxymethyl)-aminomethane
VL	Visceral leishmaniasis
WHO	World Health Organization

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: LITERATURE REVIEW

"There is a dread disease which so prepares its victim, as it were, for death… a dread disease, in which the struggle between soul and body is so gradual, quiet, and solemn, and the results so sure, that day by day, and grain by grain, the mortal part wastes and withers away, so that the spirit grows light… a disease in which death and life are so strangely blended that death takes the glow and hue of life, and life the gaunt and grisly form of death-a disease which medicine never cured, wealth warded off, or poverty could boast exemption from-which sometimes moves in giant strides, or sometimes at a tardy sluggish pace, but, slow or quick, is ever sure and certain."
- Charles Dickens (Nicholas Nickleby)

1.1 HISTORY OF TUBERCULOSIS

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) has infected humans for thousands of years. Fragments of the spinal column from Egyptian mummies from 2400BC have been found that show definite pathological signs of tubercular decay (Pickering, 2000).

In 460BC Hippocrates identified 'phthisis' (a Greek word meaning consumption) as the most prevalent disease of the times. He also observed that it killed nearly everyone it infected and warned other doctors not to visit patients in late stages of the disease due to the danger of catching it themselves.

In Medieval times, it was called 'The King's Evil' because the newly crowned kings of England and France were thought to have powers to heal TB with their touch (Pickering, 2000). The TB epidemic in Europe during the 18th century was referred to as the 'The White Plague'. The most familiar historical term for TB is 'consumption', due to the wearing away of the body.

In 1865, a French military doctor, Jean-Antoine Villemin (Fig 1.1a), discovered that the disease could be passed between humans and cattle, and between cattle and rabbits. This led to the understanding that the disease is caused by a specific organism and that it did not arise spontaneously in each affected organism as had been thought.

The fight against the organism truly began in 1882 when Robert Koch (Fig 1.1b) discovered a staining technique that allowed him to see the specific mycobacterium that is now known to cause TB.

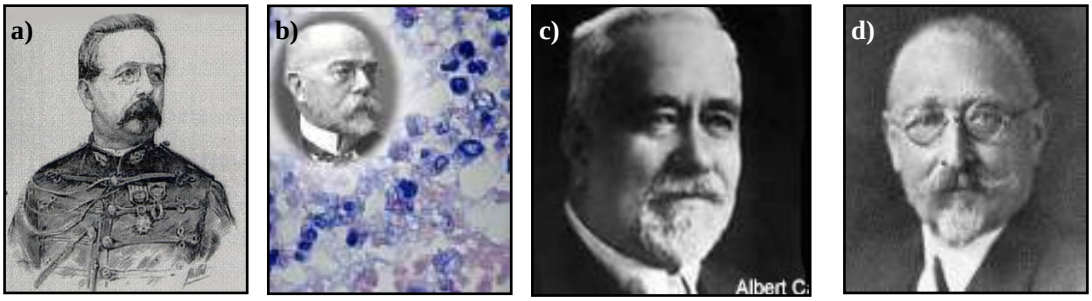


Figure 1.1: Scientists a) Jean-Antoine Villemin and b) Robert Koch who made the initial breakthroughs into the study of the microorganism, *Mycobacterium tuberculosis* (Source: (http://www.umdnj.edu/~ntbcweb/history.htm). c) Albert Calmette and d) Camille Guerin developed the first TB vaccine.

Almost 20 years later, Albert Calmette and Camille Guerin (Fig 1.1 c & d), seeking to overcome the problem of bacillary clumping associated with mycobacteria, grew bovine tubercle bacilli in dispersed culture that contained ox bile (Bloom *et al*., 1992). An avirulent variant was observed on the 39th passage and in the 231st passage; this variant was first used to immunize a child. This vaccine, BCG (bacille Calmette-Guerin), is currently the most widely used anti-TB vaccine in the world.

1.2 CURRENT TB DILEMMA

TB remains a major health problem worldwide. The rise in the number of infections is due to drug-resistant strains of *Mycobacterium tuberculosis*, and to more incidences of co-infection with HIV (Houghton *et al*., 2002). In 2003 more than 15 million people were coinfected with the human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* and of the approximately three million deaths of individuals with HIV infection, at least 600 000 died of TB (Kaufmann, 2004).

Lapses in public health programmes contribute to the increase of TB infection despite the fact that TB can be effectively treated with chemotherapy. The treatment, however, is lengthy; requiring a combination of at least three or four drugs for six months (Kaufmann, 2004).

This long drawn out process and complicated treatment schedule often affects compliance, thus resulting in development of resistant strains (Kaufmann, 2004). Therefore, early identification and proper treatment of individuals with active TB has a great impact on public health (Chan *et al*., 2000).

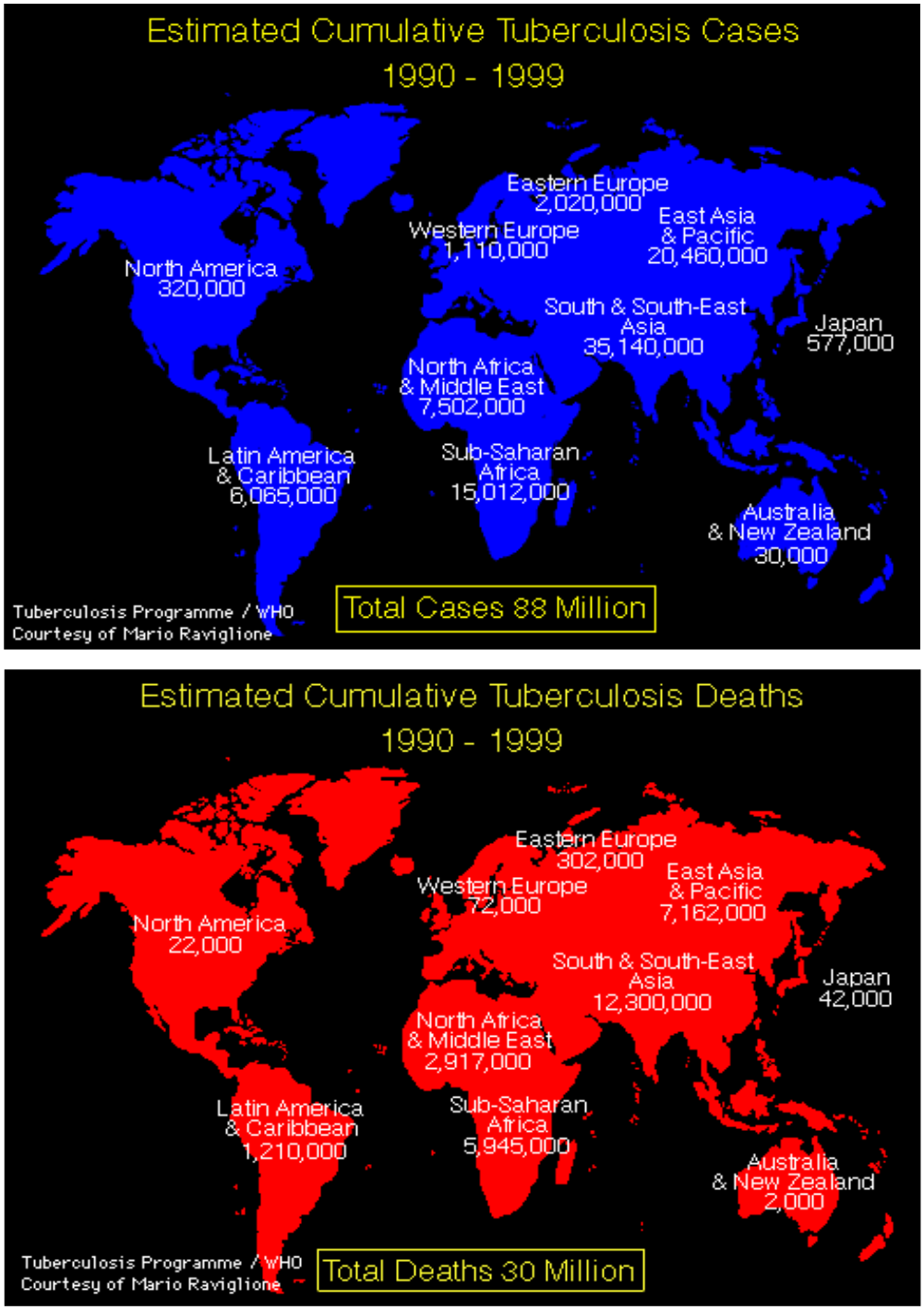


Figure 1.2: Estimated cumulative TB cases and deaths between 1990 and 1999 (Source: WHO, Mario Raviglione).

1.3 TB & HIV CO-INFECTION

TB is generally acquired via the respiratory route. Where the immune system is competent this phase is usually asymptomatic and is only manifested by the development of a positive skin response to tuberculin. Of exposed individuals, only about 10% of subjects develop TB (Fitzgerald *et al*., 1991).

The hallmark of HIV infection is progressive depletion and dysfunction of CD4 cells, coupled with defects in macrophage (Mϕ) and monocyte function. Because CD4 cells and Mϕ have a central role in anti-mycobacterial defenses, dysfunction of these cells places patients with HIV infection at high risk for primary or reactivation TB (Barnes *et al*., 1991).

Findings from several studies suggest that active TB may accelerate HIV-induced immunological deterioration. First, active TB is associated with transient CD4+ T-lymphocyte depression. Second, TB causes immune stimulation and increased production of cytokines, such as tumour necrosis factor (TNF), which increase HIV replication *in vitro*. Third, HIV-infected patients with TB appear to have a higher risk of opportunistic infections and death than do HIV-infected patients with similar CD4+ T cell counts but without TB. Finally in one study, preventative therapy with isoniazid for HIV-infected patients not only reduced the risk of active TB but also appeared to delay other opportunistic infections and death (Helbert *et al*., 1990; Pape *et al*., 1993; Shafer *et al*., 1996). There are different manifestations of TB in 'early' and 'late' HIV infection that have an influence on the final diagnosis of the patient (see Table 1.1).

Table 1.1: Manifestations of TB in 'early' and 'late' HIV infection (Source: Murray, 1990):

	Early	Late
Skin test	positive	negative
Adenopathy	unusual	common
Distribution	upper lung	lower-middle lung
Lung cavitations	present	absent
Extrapulmonary	10-15%	more than 50%

HIV has increased reactivation of old latent TB infection, increased re-infection and recurrent disease following treatment; in poor communities there has been loss of disease control, the clinical picture has changed (increase in extrapulmonary and disseminated disease), an increase in presentation of the disease in young adults and finally an increase in mortality (Murray, 1990).

A study was done in Kampala, Uganda, to determine the diagnostic utility of an enzyme-linked immunosorbent assay (ELISA) for IgG antibody (Ab) to the 30-kDa antigen (Ag) of *Mycobacterium tuberculosis* in HIV-infected persons (Daniel *et al*., 1994). The sensitivity of the ELISA assay, using the absorbance values, dropped from 0.62 in non HIV-infected tuberculous patients to 0.28 in HIV-infected patients. It was subsequently concluded that ELISA serodiagnosis of TB might have a markedly decreased usefulness in populations where HIV infection is prevalent.

1.4 TB DIAGNOSIS

The diagnosis of active TB still largely depends upon initial clinical assessment and radiographic findings, with subsequent laboratory confirmation by bacteriologic studies.

1.4.1 Tuberculin skin test

The skin reaction (Mantoux test) is frequently used to test for exposure to TB organisms and is currently the only proven method for identifying TB infection in children. The tuberculin skin test involves the intradermal injecting of 0.1ml of tuberculin solution into the volar or dorsal surface of the forearm (De Charnace & Delacourt, 2001). A pale elevation of the skin (Fig 1.3b) should be produced when the injection was administered correctly. Between 48 and 72 h after the injection, the result is read by measuring the area of induration at its largest transverse diameter in millimeters. Infection by other mycobacteria and BCG vaccination may result in false-positive results. This test has only limited diagnostic application in developing countries because it does not distinguish between latent and active TB. Especially in Sub-Saharan Africa, the majority of individuals test tuberculin-positive because of previous exposure to *Mycobacterium tuberculosis* and other mycobacterial species that cross-react immunologically to give false positive results (Daniel, 1989).

1.4.2 Direct microscopy

Microscopy for mycobacteria in sputum samples has traditionally used the Ziel-Neelsen stain; that contains the carbol-fuchsin dye, which is retained by the mycolic acid-rich cell wall of mycobacteria after washing with acid alcohol (Gray, 2004). The use of fluorescence microscopy and staining with auramine phenol has reduced the time taken for microscopic examination. However, examination of smears stained by either method is not sensitive enough and can only detect 5 x 10^3 AFBs/ml (Watterson & Drobniewski, 2000). This is a problem for paediatric microbiology laboratories because the bacterial load in childhood TB is lower than that in post-primary TB in adults. It is thought that with HIV co-infection the sensitivity of the sputum smear will be even less, because much tuberculous infection in AIDS is not retained in the lungs (Daniel, 1990). These microscopic techniques are also unable to distinguish between *Mycobacterium tuberculosis* and other *Mycobacterium* species.

1.4.3 Culture

Mycobacterium tuberculosis is a slow-growing and fastidious bacterium that requires specialized culture media. Traditional culture (Fig 1.3a) is performed on solid and liquid media such as Löwenstein-Jensen, or Kirchner and various Middlebrook formulations (Watterson & Drobniewski, 2000). The average time to detect growth on conventional culture media is 2 – 4 weeks although cultures are maintained for up to 12 weeks before being reported as negative (Gray, 2004). Radiometric detection of carbon dioxide produced by growing organisms is used in the BACTEC system but this technique is too expensive and too technological complex for widespread application in laboratories in developing countries with a high prevalence of TB (Daniel, 1989).

1.4.4 The type of mycobacterial infection by mycolic acids identification

Garza-Gonzalez *et al*. (1998) investigated the identification of mycobacteria through MA pattern recognition by high performance liquid chromatography (HPLC). The MA pattern of fluorescent derivatives obtained from pure strains and from smear-positive clinical specimens by HPLC had the same number of peaks and relative heights that had been reported previously with UV detection, but differences were found in retention times. Laval *et al*. (2001) applied MALDI-TOF mass spectrometry to accurately determine the molecular mass of MA. It was possible to obtain a fingerprint of the whole mycolate content from a given mycobacterial strain by analyzing the whole

bacterial fatty acid mixture. Watanabe *et al*. (2001) used mass spectrometry and ^{1}H-NMR to determine the chain lengths and *cis*- and *trans*-double bond and cyclopropane ring content of mycobacteria. They concluded that there is considerable variation in the mycolate composition of members of the *Mycobacterium tuberculosis* complex and related mycobacteria.

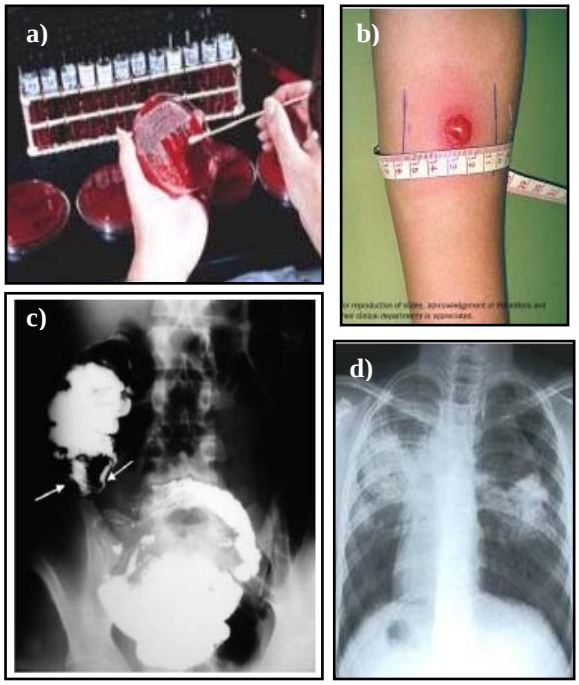


Figure 1.3: Different TB diagnostic techniques: a) sputum culture is still the reference method for the diagnosis of pulmonary TB (Source: emedicinehealth.com); b) the tuberculin skin test which should be used in combination with other diagnostic assays (Source: emedicinehealth.com); c) computed tomography (CT) of ileocecal TB which can be misdiagnosed since ileocecal TB often mimics Crohn's disease (Source: Pereira *et al*., 2005) and d) x-rays of the lungs where TB is only detected in its progressed state (Source: emedicinehealth.com).

1.4.5 Serodiagnosis

With the increase of TB/HIV co-infection, many patients are being misdiagnosed mainly because HIV infection changes the clinical, radiological and microbiological features of TB (Fätkenheuer *et al*., 1999). Diagnosing active TB using the tuberculin skin test is futile due to the unacceptably high number of both false-negative and false-positive results. Extrapulmonary TB is not detected using x-rays. Pulmonary TB needs to be in a progressed state before it can be detected via x-rays resulting in lowered probability of effecting a cure. With HIV co-infection, pulmonary TB with atypical chest x-ray appearance occurs while the incidence of extrapulmonary TB is higher.

Newer and more rapid diagnostic techniques have been investigated in an attempt to improve the accuracy of diagnosis of TB. Among the newly developed methods for rapid diagnosis of TB, nucleic acid amplification methods such as the polymerase chain reaction (PCR) seem most promising (Rodrigo *et al*., 1992). Unfortunately these newly developed methods are expensive, require culture and are not applicable to field use (Houghton *et al*., 2002).

Two principal components are necessary for successful serodiagnosis: (i) a technically simple and reproducible test and (ii) highly specific reagents, i.e. antigens (Ags), to detect circulating antibodies (Abs) and Abs to detect Ags (Khomenko *et al*., 1996). For a successful TB serodiagnostic test, the two principal components mentioned above need to be met and yet with all the ongoing research, a successful TB serodiagnostic test has not been developed or is financially not worth developing and/or the tests' sensitivity and specificity are so low that it is not worth marketing.

Failures of TB serodiagnosis tests have been reported due to: (i) immunodeficiencies (Barnes *et al*., 1991; Daniel *et al*., 1994), (ii) cross-reactivities with Ags from saprophytic mycobacteria (Rawlinson & Basten, 1989; Geluk *et al*., 2004; Tsao *et al*., 2005) and (iii) rheumatoid factors (Rapoport *et al*, 1990; Djavad *et al*., 1996; Jelinek *et al*., 2000). Technically simple and reproducible tests are available but the challenge lies in the selection of suitable specific Ags to detect circulating Abs. Abs to detect Ags are not always present, especially in the case of HIV co-infection where Abs directed towards specific protein Ags wane with progression of AIDS. Yet, a serodiagnostic test remains an attractive option, because it is easier to perform, produces faster results and can be readily configured in a dipstick assay. Serodiagnostic assays exist in a variety of configurations:

1.4.5.1 Radio-immunoassay (RIA)

The radio-immunoassay (RIA) combines the specificity of the immune reaction with the sensitivity of radioisotope techniques. This technique is based on the competition between unlabelled Ag and a finite amount of the corresponding radiolabelled Ag for a limited number of Ab binding sites in a fixed amount of antiserum (Wilson &Walker, 1994). RIA was used in the detection of circulating immune complexes (CIC) in pulmonary TB and it was concluded that the detection of CIC in the sera of patients was

not of much diagnostic or prognostic value but may be useful in evaluating therapeutic responsiveness and progress of disease (Ashtekar & Samuel, 1985; Samuel *et al*., 1984). RIA is precise, has high sensitivity and specificity but the costs of the equipment and reagents, the relatively short shelf-life of reagents, the radiological hazards and the highly skilled technical staff needed, make it an expensive technique that cannot be applied in developing third world countries.

1.4.5.2 Latex agglutination tests

Polystyrene latex beads are typically coated with mycobacterial Ags and agglutinated with Abs from patient sera (Cole *et al*., 1972; Ganju *et al*., 1991). When the beads are coated with monoclonal Abs that are directed towards a specific mycobacterial Ag, the Ag in the patient sera becomes the agglutination agent and this is known as reverse particle latex agglutination (RPLA) (Sada *et al*., 1992; Tasaka *et al*., 1995). In both cases the coated beads are then exposed to a certain concentration of serum and the degree of agglutination is rated.

Although latex agglutination tests are easy to perform and fairly accurate in some trials, there are reasons why it has not become the standard diagnostic tool for TB diagnosis:

- Latex agglutination tests are not necessarily able to differentiate between TB and other mycobacterial diseases due to the specific Ag chosen;
- Proteins present in serum have inhibitory effects on latex agglutination;
- The sensitivity for extrapulmonary TB is quite low or not detectable at all, and,
- The sensitivity of agglutination tests is low in HIV co-infected patients because of decreased levels of specific Abs.

1.4.5.3 Enzyme-linked immunosorbent assay (ELISA)

An important indirect serodiagnostic method is ELISA, which has been widely explored. Many antigenic materials have been employed in the ELISA method in an effort to improve both the sensitivity and specificity. These have included protein antigens from *Mycobacterium tuberculosis* (Al-Hajjaj *et al*., 1999; Amicosante *et al*., 1999), BCG sonicate, purified protein derivatives (PPD) antigen (Amicosante *et al*., 1997) and mycobacterial glycolipids (Tessema *et al*., 2002a). Theoretically, combining different antigen ELISA tests may be another way to improve diagnostic yields and yet

when tested, the effort was ineffective since the improvement was negligible (Chiang *et al*., 1997). Although the ELISA system is very practical and sensitive, the testing equipment required is not always available in areas where TB is widespread. In addition, changes in antigen conformation that may occur as a result of passive coating of the antigens to solid supports may cause technical artefacts resulting in false-positive and false-negative reactions (Attallah *et al*., 2005). Compared to protein antigens, ELISA using lipid antigens shows better stability and reproducibility and low cross-reactivity (Simonney *et al*., 1997; Julian *et al*., 2002; Schleicher *et al*., 2002). Interestingly, the serological response to mycobacterial antigens among TB patients seems to vary with geographic location and HIV co-infection status.

1.4.5.4 Biosensor

Biosensor technology has allowed the detection of molecules with low binding affinity in a biological medium. This new technology makes it possible to visualize on a computer screen the progress of binding of biomolecules as a function of time, in terms of changes in mass accumulation occurring on a sensor surface (Van Regenmortel *et al*., 1999). Biosensors have been used to study binding interactions in a number of different applications: Ag-Ab interactions, protein-protein interactions (Piehler, 2005; McGill *et al*., 2005) and lipophilic drugs-protein interactions (Cimitan *et al*., 2005). Lately, the possible application of biosensor technology as a possible diagnostic test for TB (Thanyani, 2003) has been investigated. Appropriate sensor surface modifications are important issues in the development of successful binding assays. Biosensor technology does not require that the interaction partners be labelled and has high sensitivity and specificity, but the cost of the equipment and the highly skilled technical staff needed, make it an expensive technique that cannot easily be adapted for use of TB screening in developing countries.

1.5 MYCOBACTERIAL ANTIGENS

The mycobacterial cell wall (Fig 1.4) is a complex and intriguing mixture of components, which sets *Mycobacterium tuberculosis* apart from all other known bacterial species (Lee *et al*., 1996).

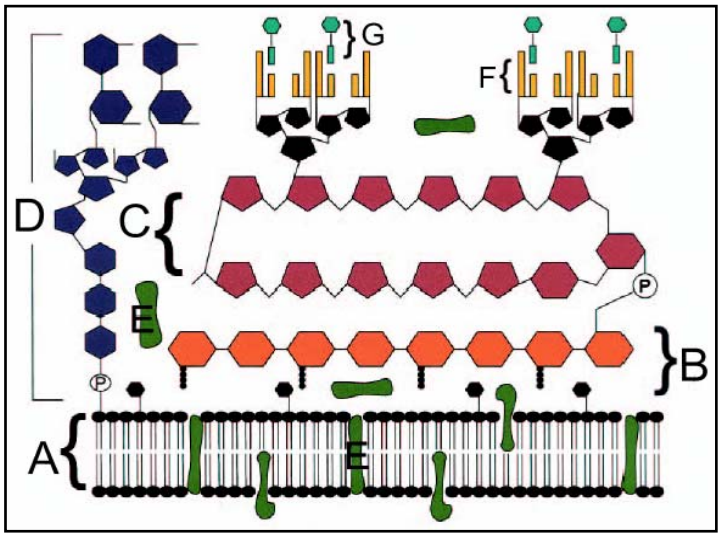


Figure 1.4: Schematic representation of the cell envelope of *M. tuberculosis*. The components include the (A) plasma membrane, (B) peptidoglycans, (C) arabinogalactan, (D) mannose-capped lipoarabinomannan, (E) plasma membrane- and cell envelope-associated proteins, (F) mycolic acids and (G) glycolipid surface molecules associated with the mycolic acids (Source: Karakousis *et al*., 2004).

As a result, cell wall Ags and several other mycobacterial Ags have been identified and widely used in attempts to diagnose TB since the end of the 19th century (Table 1.2).

Table 1.2: Antigens used in serological diagnosis of TB (Source: Chan *et al*., 2000).

Antigens used in the serological diagnosis of TB:
Mycobacterial sonicates
Extracted glycolipids
Tuberculin purified protein derivatives (PPD)
Antigen 5 (38-kDa antigen)
A60 antigen
45/47-kDa antigen complex
Antigen Kp90
30 kDa antigen
P32 antigen
Cord factor (trehalose dimycolate, TDM)
Lipoarabinomannan (LAM)

1.5.1 Tuberculin purified protein derivatives

The tuberculin PPD contain a complex mixture of proteins present in culture filtrate material from *Mycobacterium tuberculosis*. Some of these proteins are largely specific but others share a variety of antigenic determinants common to many species of *Mycobacteria*. This antigenic cross-reactivity leads to false-positive results in intradermal skin tests and serological assays and is a major problem for disease diagnosis (Fifis *et al*., 1991).

1.5.2 Lipoarabinomannan

Lipoarabinomannan (LAM) (Fig 1.5) is a phosphatidylinositol-anchored lipoglycan composed of a mannan core with oligoarabinosyl-containing side-chains with diverse biological activities (Karakousis *et al*., 2004). LAM is a prominent component of the mycobacterial cell envelope, accounting for up to 5mg.g^{-1} bacterial weight (Hunter *et al*., 1986). LAM is known to cause abrogation of T cell activation, inhibition of γ-interferon-mediated activation of murine Mϕ, inhibition of protein kinase C activity and evocation of a large number of cytokines associated with Mϕ such as TNF (Lee *et al*., 1996).

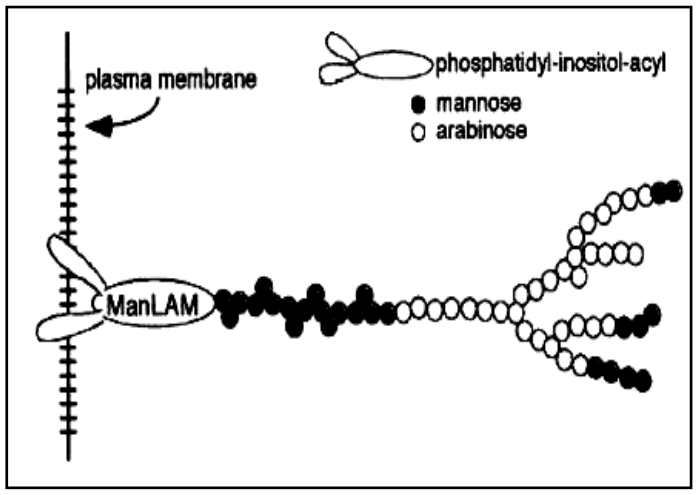


Figure 1.5: Diagramme of mannose-capped lipoarabinomannan. The fatty acid chains (acyl groups) are attached to the plasma membrane (Source: Chan *et al*., 2000).

Various techniques have been explored where LAM was used as antigen for the possible diagnosis of TB in HIV-infected and non-infected patients. LAM can occur in urine and experiments have been carried out to determine whether urinary LAM can be used in a possible TB diagnostic test (Tessema *et al*., 2001; Hamasur *et al*., 2001;

Tessema *et al*., 2002a). The sensitivities and specificities of these assays (using TB patients not co-infected with HIV) varied from 65.4–81% and 82.9–87% respectively.

Sada *et al*. (1990) evaluated LAM for the serological diagnosis of TB in a population of patients with TB from the Republic of Mexico and found that most of the patients had Abs against LAM. A specificity of 91% and sensitivity of 72% was obtained. Julián and co-workers (1997) evaluated the MycoDot test (Genelabs, Switzerland), which detects specific immunoglobulin G (IgG) Abs against LAM, in patients with newly acquired TB, and patients with relapse TB. They found that all non-HIV infected patients with relapse TB were MycoDot positive; as opposed to only 11.1% of those with new TB. Of patients with relapse TB who were also co-infected with HIV, 26.6% were MycoDot positive and of patients with new TB who were also co-infected with HIV 7.7% were MycoDot positive.

A coagglutination technique was established for the detection of LAM in human serum samples and evaluated for its utility in the diagnosis of TB (Sada *et al*., 1992). The test had a sensitivity of 88% in patients with positive sputum-smears and active pulmonary TB and 67% sensitivity in patients with active pulmonary TB negative for acid-fast bacilli in sputum. The sensitivity decreased to 57% when patients with TB and HIV were tested. Another disadvantage of the LAM Ag is that the serological response to it among TB patients varies with geographic location (Tessema *et al*., 2002b).

1.5.3 Antigen 5 (38-kDa antigen)

The immunodominant 38-kDa protein (also known as Antigen 5 or Pab) is an extracellular lipoprotein and a phosphate-binding protein (Chang *et al*., 1994). The 38-kDa protein possesses species-specific epitopes as defined by various monoclonal Abs. Andersen *et al*. (1989) observed two reactivity patterns when monoclonal Abs were used: one group of monoclonal Abs were dependent on the presence of the ultimate 91 amino acids of the protein, whereas another group of antibodies recognized an antigenic domain located in the middle portion of the molecule. This Ag has been used in the early development of commercial assays for TB detection. While highly specific for TB, it lacks sensitivity, particularly in the detection of disease in smear-negative but TB-infected individuals, as well as those patients with HIV-TB co-infections (Houghton *et al*., 2002).

1.5.4 Carbohydrates

Carbohydrates have been reported to be associated with antigenic proteins of pathogenic *Mycobacteria* (Espitia *et al*., 1989; Fifis *et al*., 1991). The importance of carbohydrates attached to proteins in immune recognition has been demonstrated by the decreased capacity of the *Mycobacterium tuberculosis* 45/47-kDa recombinant protein to stimulate T-cell lymphocyte responses when its mannosylation pattern is changed (Lara *et al*., 2004).

Tiwari *et al*. (2005) examined the serological responses of mycobacterial glycolipid (glycolipids are carbohydrate-attached lipids) antigens by a liposome agglutination assay and found that the assay had an overall sensitivity and specificity of 98.5% and 85.5%, respectively. Very low anti-glycolipid Ab concentrations in the patients' sera could be detected by the agglutination assay. The specificity and sensitivity of this assay was not determined in TB patients co-infected with HIV.

1.5.5 A60 complex

Antigen complex A60, a thermostable complex present in the cytoplasm of exponentially growing *Mycobacteria*, accumulates within the cell walls of stationary cells and is released during the declining phase (Cocito *et al*., 1988). Antigen A60 triggers both humoral and cellular immune response but is not specific for Mycobacteria because it is also present in *Nocardia* and *Corynebacterium* species.

1.5.6 Antigen 85 complex

A complex of three 30 - 32-kDa proteins (designated as 85A, 85B & 85C) has been identified as the major secreted proteins of *Mycobacterium tuberculosis*. The antigen 85 complex is also present on the surface of *Mycobacteria* and in the phagosomal space of *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Clemens, 1997). The sensitivity decreases from 78% in a HIV negative population to 11% (McDonough *et al*., 1992) when using the 30-kDa protein as an Ag in an ELISA-based serological assay of TB patients co-infected with HIV.

1.5.7 Mycolic acids

Mycolic acids (MA) are high-molecular-weight α-alkyl, β-hydroxy fatty acids (Fig 1.6) found in the genera *Mycobacterium*, *Nocardia*, *Corynebacterium* and *Rhodococcus* (Brennan *et al*., 1995). MA are covalently attached to arabinogalactan and together with other lipids form the outer leaflet. The long meromycolate chain of MA usually contains two functional groups, which vary in the type, stereochemistry and spacing affecting the packing of the long hydrocarbon mycolate chains influencing the intrinsic physiological functions of the cell envelope (Watanabe *et al*., 2001).

The MA-structure is species specific (Clemens, 1997) and is the most abundant cell wall Ag by far in the mycobacteria. Goodrum *et al*. (2001) describes a protocol that allows large-scale purification of MA antigen by counter current distribution.

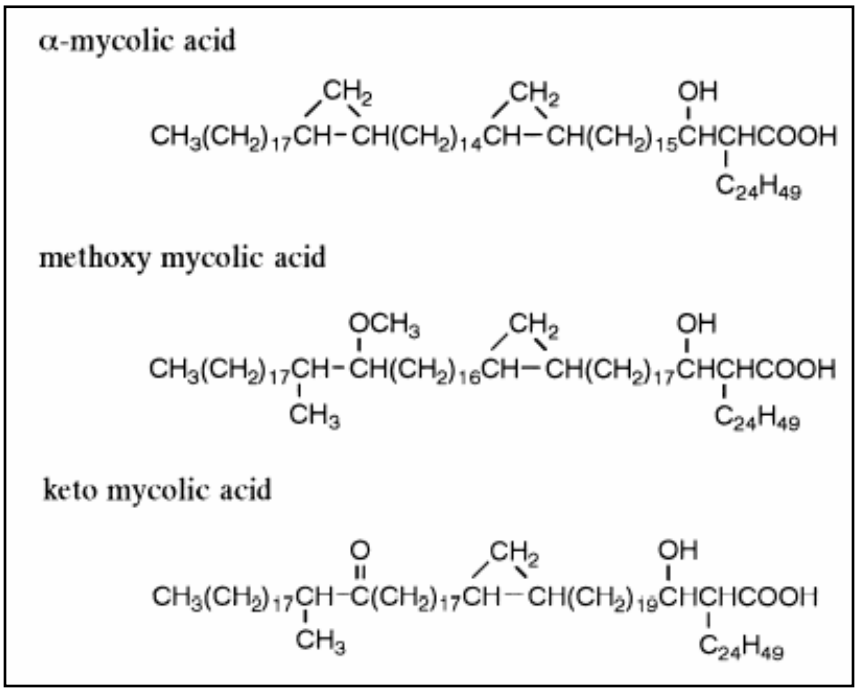


Figure 1.6: Chemical structures of *M. tuberculosis* mycolic acid subclasses (Source: Fujiwara *et al*., 1999).

The MA-containing glycolipid, trehalose-6, 6'-dimycolate (TDM) is also known as cord factor as it is thought that TDM causes virulent strains of *Mycobacterium tuberculosis* to grow in serpentine cords (Bloch, 1950; Goren, 1972). TDM is immunogenic, granulomagenic and adjuvant-active (Brennan, 2003). Kato (1972) demonstrated that Ab to cord factor is produced in sera of animals vaccinated with a complex of cord factor and cord factor-methylated bovine serum albumin (MBSA) and that the Ab

precipitates cord factor and neutralizes its toxicity either *in vivo* or *in vitro*. In 1994, Beckman *et al*. showed that αβTCR^{+}T cells (CD4^{-}CD8^{-}) recognize a broader range of Ags than previously appreciated and that at least one member of the CD1 family has evolved the ability to present lipid Ags to this subpopulation of T cells. MA (a purified CD1b-restricted Ag) was found to be presented to αβTCR^{+}T cells. Pan *et al*. (1999) reported the detection of anti-MA Abs in human serum in an ELISA-based assay and concluded that this may form a basis for a serodiagnostic test for TB. In 2002, Schleicher *et al*. investigated the diagnostic potential of an ELISA-based assay on detecting Abs to *Mycobacterium tuberculosis* MA in TB patients' sera co-infected with HIV. They found that although many HIV-positive patients in the TB group had advanced immunosupression, the Ab response to MA remained well preserved. Unfortunately, the ELISA to detect anti-MA Abs had poor sensitivity (37%) and specificity (85%). It would therefore appear that MA is a good Ag to investigate for TB diagnosis in HIV-burdened populations if one could improve sensitivity and specificity in some assay configuration.

1.6 THE IDEAL TB DIAGNOSTIC DEVICE

Dipstick assays have wide applications – from agriculture through to human diseases detection. Dipstick assays are rapid, suitable for onsite testing by technically competent personnel and can be executed in an unsophisticated laboratory or in a non-laboratory environment (Wang *et al*., 2005). Numerous dipstick assays have been developed and tested for different diseases. Boelaert *et al*. (2004) validated the rK39 dipstick test as a diagnostic criterion for visceral leishmaniasis (VL) in remote areas in Nepal where communities, often affected by VL, have poor access to health services. It was concluded that the rK39 dipstick test could replace parasitology as the basis of a decision to treat VL in Nepalese peripheral health services.

Klewitz *et al*. (2005) described a highly sensitive combination of a membrane dipstick assay and a flatbed scanner for determination of botulinum toxin type D. The assay was configured in a sandwich format using two primary Abs of distinct specificities and one secondary Ab (Fig 1.7).

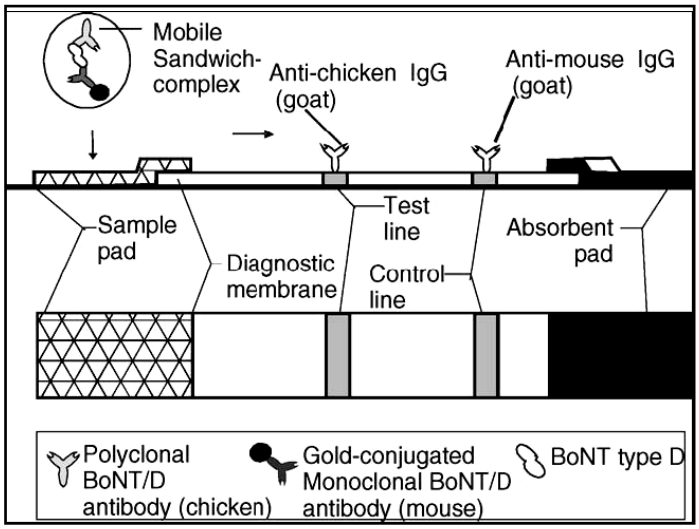


Figure 1.7: Schematic top view, cross section and reagents of a described rapid flow immunochromatographic test device for the detection of botulinum type D (Source: Klewitz *et al*., 2005).

One of the primary Abs was conjugated with colloidal gold (detector reagent); the secondary Ab (capture reagent) was immobilized within a defined zone (test line) on a diagnostic cellulose nitrate membrane. In combination with an effective sample pre-treatment, the gold conjugated Ab and the second distinct Ab formed a mobile sandwich complex with the toxin. Within the test line the mobile sandwich complex was immobilized and therefore concentrated by the secondary Ab resulting in a distinct red test line. The intensity of colour of the red test line (signal intensity) was assessed visually and by computer image analysis using a three-determination analysis. A drawback of this assay was the appearance of false positive results, which could be balanced by the implementation of the described computer image analysis and the use of a negative reference sample. The ideal TB diagnostic device would ultimately therefore be a dipstick assay, which would be blood-based and specific, i.e. requiring a specific Ag-Ab combination, of which the Ab prevalence is not affected by non-TB health aspects of the patients.

1.7 HYPOTHESIS

Mycolic acids, either as antigen or molecular probe, can be applied in a fast and affordable screening assay for TB infection.

1.8 AIMS

To develop a fast and affordable screening assay for TB infection by making use of fluorescently-labelled MA in either:

- An antibody-dependent assay using suspended Sepharose protein-A and/or
- An antibody-independent assay using immobilized MA containing liposomes.

CHAPTER 2:
MYCOLIC ACIDS AS ANTIGEN IN A PROTEIN-A BASED ANTIBODY ASSAY FOR THE DIAGNOSIS OF TUBERCULOSIS

2.1 INTRODUCTION

The serodiagnosis of TB based on the humoral immune response has long been a subject of investigation because serological testing is simple, economical, fast and non-invasive. Because it is a blood assay, serodiagnosis is not limited to cases of pulmonary TB (Fujita *et al*., 2005). Despite this, not a single serodiagnostic TB test has ever found widespread clinical utility (Arias-Bouda *et al*., 2003). To develop a clinically reliable diagnostic test, sensitivity and specificity are necessary. The World Health Organization (WHO) recommended that TB diagnostic tools for wide use should have sensitivities of over 80% (less than 20% false negatives) and specificities of more than 95% (less than 5% false positives).

2.1.1 Protein A based assays

Protein A is a bacterial cell wall protein isolated from the cell wall of the *Staphylococcus aureus* where it is covalently linked to the peptidoglycan part of the cell wall (Sjöquist *et al*., 1972). Protein A has proven useful for the study of Ags and receptors on the surface of intact cells and for the detection of Ab-secreting cells (Goding, 1978), because it binds to a domain in the Fc region of antibodies. Different approaches have been used in the study of cell surface antigens using Protein A. They include fluorescein-conjugated protein A, radiolabelled protein A (Kaplan & Quimby, 1983) and intact staphylococci and rosette formation (Ghetie *et al*., 1974). Protein A has also been used in a screening assay for the detection of antibodies to HIV (Otsyula *et al*., 1996; Benítez *et al*., 1998). Because protein A has such wide uses and is fairly cheap, it was decided to test the possibility of using protein A in a TB serodiagnostic assay with the possibility of a dipstick assay in the future. A protocol was therefore designed where MA could be used as an Ag in an Ab-dependent assay.

2.2 AIM

To investigate the feasibility of detecting Abs to MA in patient sera as surrogate marker of active TB in a configuration where the patient Abs are trapped with protein-A and where specificity is probed with liposomes containing fluorescently labelled MA*.

2.3 MATERIALS

2.3.1 Mycolic acids

Non-fluorescently labelled MA, isolated from *Mycobacterium tuberculosis* H37Rv originating from human lung tissue (ATCC 27294; Maryland, USA) was obtained from S van Wyngaardt (Department of Biochemistry, University of Pretoria). The TB culture had been maintained at the National Tuberculosis Institute of the Medical Research Council (MRC) of South Africa, Pretoria. The MA had been purified from the mycobacterial cell wall as described by Goodrum *et al.* (2001).

2.3.2 Apparatus used

The Fluoscan Ascent Fluorimeter FL Type 374 (Thermo Labsystems, Oy) was used to read the fluorescence in Nunc^{TM} dark plates (obtained from Nalge Nunc International, Denmark). The Eppendorf microfuge 5414S (Eppendorf, Germany) was used for quick centrifugation of the Seph-A suspensions at 15000 rpm at room temperature. The Retsch Vortex Mixer (Outolabor LTD, Randburg – SA) was used to mix the samples thoroughly. The ELISA plate washer, Well Wash 4, was purchased from Labsystems (Finland) and was used for all the wash steps of the ELISA. The Multiskan Ascent Spectrophotometer (Thermo Labsystems, Oy) was used to determine the absorbance values in the ELISA. The Model B-30 Branson sonifier (Sonifier Power Company, USA) was used to sonify the liposomes

2.3.3 Reagents

Ultra-pure double distilled, de-ionised water (dddH_2O) was used throughout for the preparations of aqueous solutions. The following reagents, all of analytical grade, were obtained from Merck (NT laboratories, SA): glycine, sodium chloride (NaCl), potassium bicarbonate ($KHCO_3$), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), potassium hydroxide (KOH), tris(hydroxymethyl)-aminomethane (TRIS), sodium hydrogen phosphate (Na_2HPO_4), dehydrated tri-sodium citrate and citric acid. Chemically pure chloroform ($CHCl_3$), dimethylformamide (DMF), methanol and hydrochloric acid (HCl) were purchased from Saarchem (SA). Cholesterol (5-cholesten-3β-ol) (chol), L-α-phosphatidylcholine (L-α-Lecithin) (PC), 18-crown-6-ether, and sodium azide (NaN_3) were obtained from Sigma (St Louis, MO). 5-(Bromomethyl)fluorescein (5-BMF) was purchased from Molecular Probes (Leiden, Netherlands) and Protein A immobilized on Sepharose 6MB for affinity

chromatography was obtained from Fluka (Steinham, Switzerland). Casein (Cas) [casein, bovine milk, carbohydrate & fatty acid free – fatty acids: ≤0.1%; free acid: ≤0.1%; glucose: ≤0.05%; lactose: ≤0.05%] was obtained from Calbiochem (Merck – Johannesburg, SA). Goat anti-human IgG peroxidase was bought from Cappel (Separations – Johannesburg, SA). Hydrogen peroxide (H_2O_2) from Merck (NT laboratories, SA) and o-phenylenediamine (OPD) were obtained from Sigma (St Louis, MO, USA).

2.3.4 Buffers

Phosphate buffered saline (PBS) buffer (20x PBS): 160 g NaCl, 4 g KCl, 4 g KH_2PO_4, and 21 g $NaHPO_4$ per litre dddH_2O, adjusted to pH 7.4.

TRIS buffer: TRIS (10 mM, 100 mM and 1 M) was prepared by dissolving the required amounts of TRIS in dddH_2O and adjusted to pH 8.0 by using 1 N HCl.

Glycine buffer: Glycine (50 mM) was prepared by dissolving glycine in dddH_2O and adjusting to pH 3.2 by adding 1 N HCl drop for drop and checking the pH after each addition before final volume adjustment.

Citrate buffer (pH 4.5): Tri-Sodium citrate (0.1 M, 450 ml) and citric acid (0.1 M, 450 ml) solutions were prepared by weighing out 14.71 g and 10.5 g respectively and dissolving each in 450 ml ddd water. The citric acid solution was then added to the trisodium citrate solution until a pH of 4.5 was obtained. The solution was then brought to a final volume of 1000 ml with the addition of dddH_2O.

Buffers were autoclaved and aliquoted before storage at 4^{o}C.

Cas/PBS (pH 7.4): One litre 0,5% Cas/PBS buffer was prepared by weighing out 5 g Cas and mixing it into a paste with 50 ml 20x PBS. This paste was then diluted with 900 ml dddH_2O and incubated in a 37°C waterbath for 2 hours to dissolve completely. The solution was left overnight at 4°C. The pH was then determined and adjusted to pH 7.4 using 1 M NaOH. A final volume of 1000 ml was obtained with the addition of dddH_2O. The Cas/PBS was stored at 4^{o}C and used within +/- 2 hours.

2.3.5 Human sera

Serum samples were randomly selected from a group of sera that were collected for another study by Schleicher *et al*. (2002). These patients were admitted to the general medical wards of the Helen Joseph Hospital, Johannesburg (South Africa) for various conditions including active pulmonary TB. The sera were collected from the hospital between August and December 2000. Informed consent was obtained from all patients before enrolment. The Institutional Ethics Committee approved the study (University of Witwatersrand).

The TB-positive (TP) group consisted of patients with recently diagnosed smear-positive pulmonary TB of which some were HIV-seropositive. The TB-negative (TN) groups used for control had medical conditions other than TB and were recruited from the general medical wards. They had no clinical, radiological or microbiological evidence of active infection with *Mycobacterium tuberculosis*. Patients with different TB/HIV status were used: TNHN (TB negative, HIV negative), TNHP (TB negative, HIV positive), TPHN (TB positive, HIV negative) and TPHP (TB positive, HIV positive). Healthy control sera denoted MD and LP were obtained from healthy staff and students and used as negative controls. A summary of the patients used, their TB/HIV status (recorded by the Helen Joseph Hospital) and results of Ab-activity against immobilized MA (done by Y Vermaak, 2002) is found in Table 2.2.

Table 2.2: Summary of patient and control sera tested and their TB/HIV status:

Patients tested	**TB/HIV status (HNTN, HNTP, HPTN & HPTP)**	***Normalised ELISA results (Y Vermaak, 2002)**
LP	HNTN	0.894
MD	HNTN	1.000
P44	HNTP	0.138
P113	HPTN	0.405
P64	HNTP	0.630
P116	HPTN	0.686
P92	HNTN	1.306
P62	HPTP	1.619
P132	HPTP	1.720
P127	HNTP	1.866
P136	HPTP	1.935
P117	HPTN	2.289
P41	HPTN	2.465
P95	HNTP	3.053
P108	HNTP	3.601
P50	HPTP	7.396
P110	HPTN	7.405

* ≡ Normalisation of ELISA results were done as follows: patient results obtained on PBS-coated ELISA plates were subtracted from the patient results obtained on the MA-coated plates and then divided by the results obtained from the negative control on the same plate.

2.4 METHODS

2.4.1 ELISA with MA-coated plates

PBS (4 ml) was added to a vial containing 250 µg MA and one containing no MA. Both vials were placed in a heat block for 20 minutes at 85°C. The vials were then vortexed for 1 minute and the contents pulse sonified at maximum output 20 and 2% duty cycle for 1 minute. From the MA-containing vial, 50 µl of the hot solution was pipetted per well in one-half of the ELISA plate and the other half was coated with hot PBS at 50 µl per well from the vial containing PBS only. The plate was cooled down to room temperature, placed at 4°C and left overnight.

The following day, the MA and PBS solutions were flicked out and the plate was blocked with 400 µl Cas/PBS for 2 hours at room temperature. The Cas/PBS was then flicked out, the wells washed once with Cas/PBS and aspirated.

Sera were thawed at 37°C for 5 minutes. From each serum 25 µl was diluted in Cas/PBS (475 µl) and pipetted at 50 µl per well into the coated ELISA plate. It was left to incubate for 1 hour at room temperature before the serum dilutions were removed, the wells washed thrice with Cas/PBS and aspirated.

Goat anti-human IgG peroxidase conjugate (10 µl) was diluted in 10 ml Cas/PBS and 50 µl thereof was pipetted per well. The ELISA plate containing the conjugate solution was then incubated for 30 minutes. The excess conjugate solution was subsequently flicked out, the wells washed thrice with Cas/PBS and aspirated.

Substrate solution (50 µl; 10 mg OPD + 8 mg H_2O_2 in 10 ml citrate buffer pH 4.5) was added in the wells. The colour development was monitored and after 30 minutes, the ELISA plate was read using a Multiskan Ascent spectrophotometer at 450nm.

2.4.2 Labelling of MA with 5-(bromomethyl)fluorescein

2.4.2.1 Resaponification of MA

Before the MA could be fluorescently labelled, it needed to be resaponified. During resaponification, the fatty acids are converted to potassium salts of fatty acids (R-COOK). The addition of acid (HCl) to this solution forms KCl and frees the carboxyl group (R-COOH). This allows extraction into $CHCl_3$, but is converted back to the potassium salt with $KHCl_3$ before the binding of 5-BMF. To each vial containing 3 mg MA, 3 ml Reagent A [25% KOH in methanol-water (1:1)] was added and heated at 90^{o}C for 60 minutes on a heat block. The samples were cooled to room temperature and then transferred into test tubes. To each test tube, 2.25 ml Reagent B [concentrated HCl diluted 1:1 with water (pH = 1.00)] was added and the pH tested, using pH paper, to be pH 1.00. Chloroform (3 x 3.0 ml) was used to extract the MA and to transfer it to the test tubes. The samples were vortexed well and the lower phase of each tube was transferred into five vials. The vials were placed on a heat block and the chloroform was evaporated by bubbling with nitrogen (N_2) gas. The samples were then allowed to cool to room temperature. Reagent C [0.15 ml, 2% $KHCO_3$ in methanol-water (1:1)] was added to the dried MA and evaporated at 90^{o}C on the heat block. It was cooled down.

2.4.2.2 Labelling of MA

The 5-BMF (1 mg) was dissolved in 200 µl DMF and added to dry, freshly saponified MA (3 mg). The sample was vortexed well. The 18-crown-6-ether (3.6 mg) was dissolved in 750 µl $CHCl_3$ and added to the sample, which was subsequently vortexed. The sample was heated to 90^{o}C for 60 minutes on a heat block, cooled to room temperature and an additional 1 ml $CHCl_3$ was added. It was further incubated for 60 minutes at room temperature and left overnight.

The sample was washed with 12 x 1.0 ml Reagent E (Reagent [B] mixed 1:1 with methanol). The lower phase was transferred to a dry vial, made up to 2.5 ml with chloroform and aliquoted into vials such that each contained an approximate final weight of 500 µg of MA. Samples were then dried on the heat block at 90^{o}C under a stream of N_2 gas.

2.4.3 Weighing of Sepharose protein A

An empty Eppendorf tube was weighed for each sample to be tested. Sepharose protein A (Seph-prot A) slurry (3 x 20 µl) were added in an Eppendorf tube with a 100 µl micropipette of which the tip was cut to allow for a bigger opening. The Eppendorf tubes were then centrifuged for 3 seconds. Any buffer present in the Eppendorf tubes was removed. The Eppendorf tubes containing Seph-prot A were added to and re-weighed until 0.060 - 0.065 g of Seph-prot A was present. The samples were then stored at 4^{o}C in TRIS buffer (10 mM, 1 ml) till the following morning.

2.4.4 Liposome preparation

The liposomes were prepared a day before an experiment. Cold $CHCl_3$ (1000 µl) was added to 100 mg PC - which was weighed in a clear vial - to make the PC stock solution. To make the cholesterol stock solution 1000 µl cold $CHCl_3$ was added to 100 mg cholesterol. The vials were then capped and vortexed vigorously to ensure that the PC and cholesterol were totally dissolved.

To make PC liposomes, 90 µl of the PC stock solution was transferred to a clean vial. The $CHCl_3$ was evaporated using N_2 gas and 1 ml PBS (pH 7.4) was added. To make cholesterol liposomes, 90 µl of the PC- and 45 µl of the cholesterol-stock solution were transferred to a clean vial. The $CHCl_3$ was evaporated using N_2 gas and 1 ml PBS was added.

To make PC containing 100% fluorescently-labelled MA (100% MA*) liposomes, PC stock solution (238 µl) was added to the MA* vial (660 µg). The $CHCl_3$ was evaporated on the heat block using N_2-gas. Once all the $CHCl_3$ had evaporated PBS (2.64 ml) was added to the vial. To make PC containing 75% fluorescently labelled MA (75% MA*) liposomes, cold $CHCl_3$ (250 µl) was added to an unlabelled MA-containing vial (250 µg). Unlabelled MA (157 µl), after thorough mixing via vortexing, was then transferred to a vial containing 470 µg fluorescently labelled MA. Afterwards, the PC stock solution (225 µl) was added. The $CHCl_3$ was evaporated on the heat block using N_2-gas and PBS (2.5 ml) was added to the vial.

Subsequently, all vials were placed on the heat block at 85°C for 30 minutes. The vials were vortexed for 1 minute and pulse sonified at maximum output 20 and 2% duty cycle for 1 minute.

A two-fold serial dilution of the 100%- and 75%-labelled MA* was done to determine efficiency of labelling. MA* liposomes (200 µl) were placed into 2 wells of a Nunc plate. In subsequent wells, 100 µl PBS was added. The initial 2 wells were mixed well and 100 µl was transferred to the next pair of wells containing 100 µl PBS. It was mixed well and transferred until completion of the dilution series. The fluorescence was then determined at excitation wavelength of 485 nm and emission of 538 nm. The original MA-containing vial was then placed at 4°C overnight. Before an experiment, the vial was vortexed for a further minute.

2.4.5 Sepharose protein A protocol

A summary of the Sepharose protein A protocol adapted from Harlow & Lane (1998) is given in Fig 2.1:

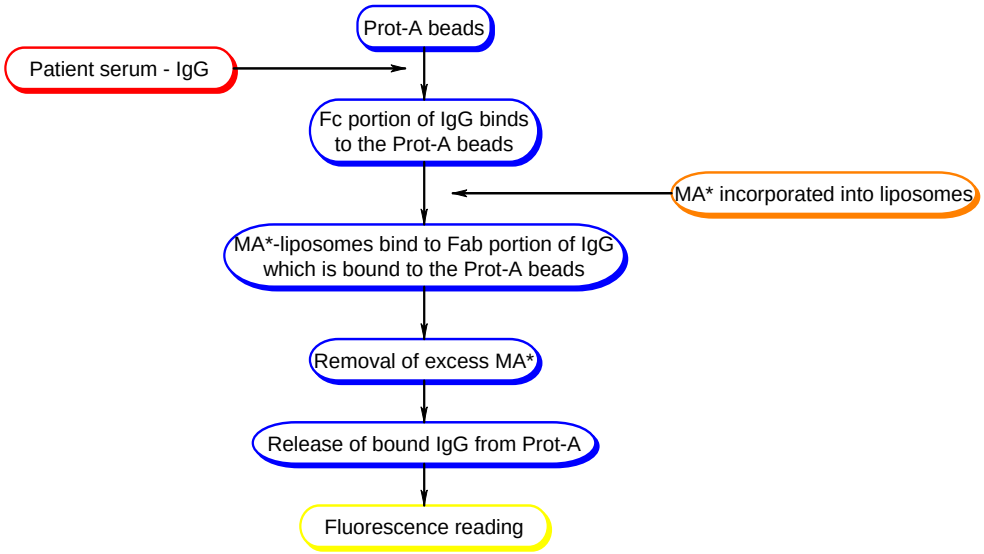


Figure 2.1: An outline of the basic approach used in the Seph-prot A assay.

The patient and healthy control serum samples (50 µl) were first prepared in clean Eppendorf tubes by adding 1 M TRIS (5 µl, pH 8.00). TRIS buffer (100 mM, pH 8.00, 50 µl) as substitute for serum was used as the control. They were then vortexed rapidly

and 50 µl transferred to the Seph-prot A Eppendorf tubes, which already contained 950 µl 10 mM TRIS (pH 8.00).

The samples were incubated for 60 minutes at room temperature with mechanical rotation to allow all of the antibodies (IgG) present in patient serum to bind to Seph-prot A. Subsequently the samples were centrifuged for 7 seconds and the supernatant was removed.

Unbound proteins were removed by washing three times with 1 ml 100 mM TRIS (pH 8.00) and then three times with 10 mM TRIS (pH 8.00). After each addition the samples were vortexed, centrifuged for 7 seconds and the supernatants removed to the waste.

Binding of MA*-liposomes to Seph-prot A-IgG: TRIS buffer (10 mM, pH 8.00, 900 µl) was added to the samples. MA*-liposomes were vortexed for 1 minute before addition to the Seph-A containing tubes. MA*-liposomes (100 µl) were then added to the samples (thus 1:10 liposome dilution). The MA*-liposomes bound to the Fab regions of some of the bound IgG. The samples were rotated-incubated for 60 minutes at room temperature in the dark. They were centrifuged for 7 seconds and 100 µl transferred to the wells of a Nunc^{TM} dark plate. The remainder of the supernatants was then aspirated from the samples.

Any unbound MA* was washed away with 1000 µl 10 mM TRIS (pH 8.00). The samples were vortexed, centrifuged for 7 seconds and 100 µl of the supernatant removed into the wells of a Nunc^{TM} dark plate. The remainder of the supernatants was then aspirated from the samples and resuspended in 1000 µl 10 mM TRIS (pH 8.00). Washing was repeated 12 times, by which time a base line was normally reached when read on the Fluoscan. If the fluorescence reading was still high, the washes were continued.

IgG release: IgG bound to the MA* and Seph-prot A was released by the rotation-incubation of the Seph-A pellet in 500 µl glycine buffer (pH 3.2) for 1 minute at room temperature. Clean Eppendorf tubes were prepared with 65 µl 1 M TRIS (pH 8.00). A volume of 435 µl of centrifuged Seph-A supernatant was removed from the Eppendorf

tubes containing the glycine and transferred into the Eppendorf tubes containing the 1 M TRIS (pH 8.00) to neutralize the acid used in the IgG release step.

The samples were quickly vortexed and 100 µl supernatant was transferred into wells of a Nunc™ dark plate for the determination of fluorescence. This step was repeated once more to determine the efficiency of IgG release. The fluorescence was read at excitation 485 and emission 538 nm.

The supernatants were subsequently combined for the ELISA assay to establish the amounts of IgG captured from the patient samples. Of the pooled IgG released in the supernatants and neutralized in 1 M TRIS, 450 µl was diluted by the addition of 1050 µl 10 mM TRIS. Of this dilution, 200 µl was used in the ELISA in duplicate to create a serial 1:1 dilution in 10 mM TRIS. The sample-IgG coated ELISA plates were incubated overnight at 4°C. The contents of the ELISA plates were flicked out, aspirated and then blocked with Cas-PBS for 1 hour. Goat anti-human IgG (H + L) peroxidase (100 µl) was used as conjugate in a 1:1000 dilution in Cas-PBS and incubated for 30 minutes. OPD and H_2O_2 were dissolved in citrate buffer and added to the ELISA plate after removal of conjugate by 3 wash steps with Cas-PBS. Absorbancies were determined at 450 nm after standing at room temperature for 30 minutes.

2.4.6 Sepharose protein A inhibition protocol

A summary of the Sepharose protein A inhibition protocol is given in Fig 2.2:

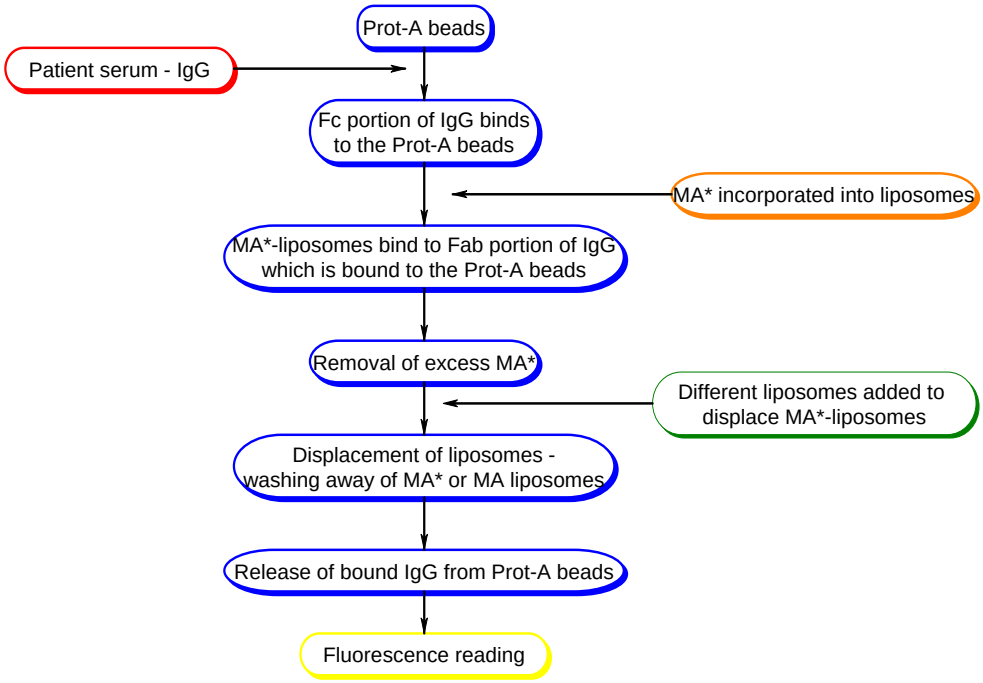


Figure 2.2: An outline of the basic approach used in the Seph-prot A inhibition assay.

The same protocol was followed as in section 2.4.3 for the inhibition experiments till before the release of bound IgG from Prot-A. After removal of excess MA* (100%-labelled MA* was used), different liposomes (i.e. PC-; PC/chol-; PC/MA liposomes) were added (100 ul). The samples were rotated-incubated for 60 minutes at room temperature in the dark. They were centrifuged for 7 seconds and 100 µl supernatant transferred to the wells of a Nunc^{TM} dark plate. The remainder of the supernatants was then aspirated from the samples. Any unbound/displaced liposomes were washed away with 1 ml 10 mM TRIS (pH 8.00) by vortexing, centrifuging for 7 seconds, removing 100 µl supernatant into wells of a Nunc^{TM} dark plate, aspiration and this washing cycle continued until the fluorescence readings stabilized. The same protocol was followed as in section 2.4.3 for ultimate release of IgG and measurement of residual fluorescence.

2.5 RESULTS

To investigate the possibility of using anti-MA Abs in patient sera as surrogate markers for active TB, Seph-prot A was used to immobilize the Abs and fluorescently-labelled MA* was used to determine the specificity of the anti-MA Abs.

2.5.1 Efficiency of mycolic acid labelling

To determine sensitivity of the detection of 5-BMF, a two-fold serial dilution of liposomes containing either 100%- or 75%-labelled MA* was done. The fluorescence was determined at excitation of 485 and emission of 538 nm. A typical dilution graph of liposomes containing either 100%- or 75%-labelled MA* diluted in 1:1 PBS is shown in Fig 2.3.

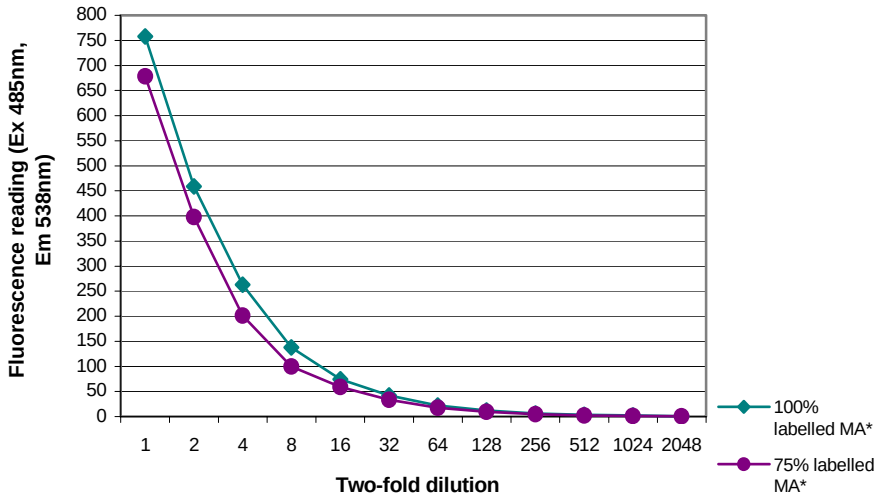


Figure 2.3: Sensitivity of fluorescence detection of 100%- or 75%-labelled MA containing liposomes diluted 1:1 in PBS. MA was labelled with 5-BMF.

As expected, a higher fluorescence reading was obtained with 100%-labelled MA* than with 75%-labelled MA*, although both are detectable down to sixteen-fold dilution, linearly according to the dilution. All procedures with measurement of fluorescent MA as endpoint therefore had to be contained within the limits of 16-fold dilution maximum of the analyte.

2.5.2 Sepharose protein A immunoglobulin capture assay (SPICA)

The possibility of non-specific hydrophobic association that may occur during an ELISA based assay of Abs binding to hydrophobic Ags such as MA, was sought to be eliminated by the use of Seph-prot A, where patient IgG antibodies were first separated by capturing their Fc portions onto protein A particles in suspension (see 2.4.5). Patients with different TB/HIV status were used (Table 2.2, MATERIALS section). Control and patients' IgG, separately attached to Seph-prot A via their Fc units, were exposed to either 100%- or 75%- labelled MA*. By releasing the Abs and MA* from the Seph-prot A, via a change in pH, one was able to detect how much patient IgG was directed to MA. A high fluorescent reading relative to that of healthy control serum would indicate that a patient is positive for TB or that he/she had been exposed to environmental mycobacteria. In both cases, an immune response would have occurred leading to specific Ab production to MA.

The results in Fig 2.4 show a good variety of signals for different sera, which, if reproducible, would indicate that SPICA is responsive to factors in the sera of patients. The second release of IgG is consistently 77.5% (+/- 4.97) less than the first release, testifying to the acceptable quality of the procedure to release the MA and Abs from the Seph-prot A and the accuracy of measurement of fluorescence.

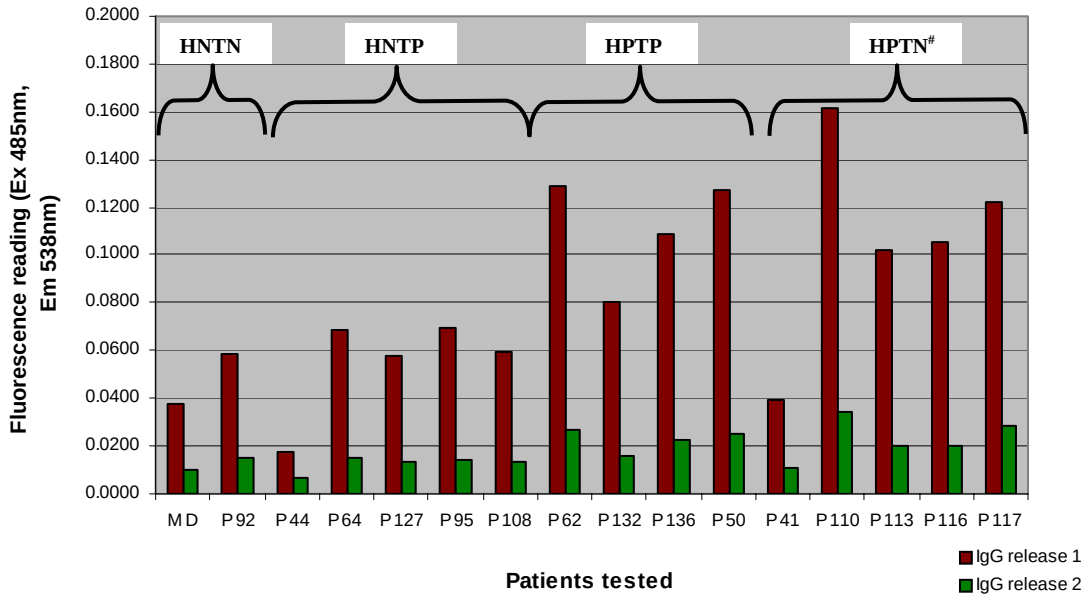


Figure 2.4: Fluorescent readings obtained for the first and second IgG releases from SPICA for the different patients with 100%-labelled MA* as fluorescent Ag. #The TB status of these patients cannot currently be accurately predicted.

The indication of the TB positiveness of the tested patients sera is actually not accurate for the HPTN group, as the sputum culture assay and all other clinical and laboratory assessments for this group of HIV-burdened population have previously been shown to be inaccurate. This group is therefore ignored in the argument that follows: In comparison to the negative (healthy, MD) control, SPICA appears to be 81% accurate (9/11) in predicting active TB, with one false positive (P92) and one false negative (P44). This correlates with the ELISA values determined before (Table 2.2), showing the same false positive and false negative patient serum values. This adds confidence to the ELISA results indicating that they may not be seriously affected by non-specific absorption of Abs to the wells of the ELISA plate.

There was a good correlation between the results obtained using either 75%- or 100%-labelled MA* (Fig 2.5). Would the labelling have caused that the MA lost its antigenicity, the results would have been less predictable. All other experiments were henceforth done with either 75%- or 100%- labelled MA*.

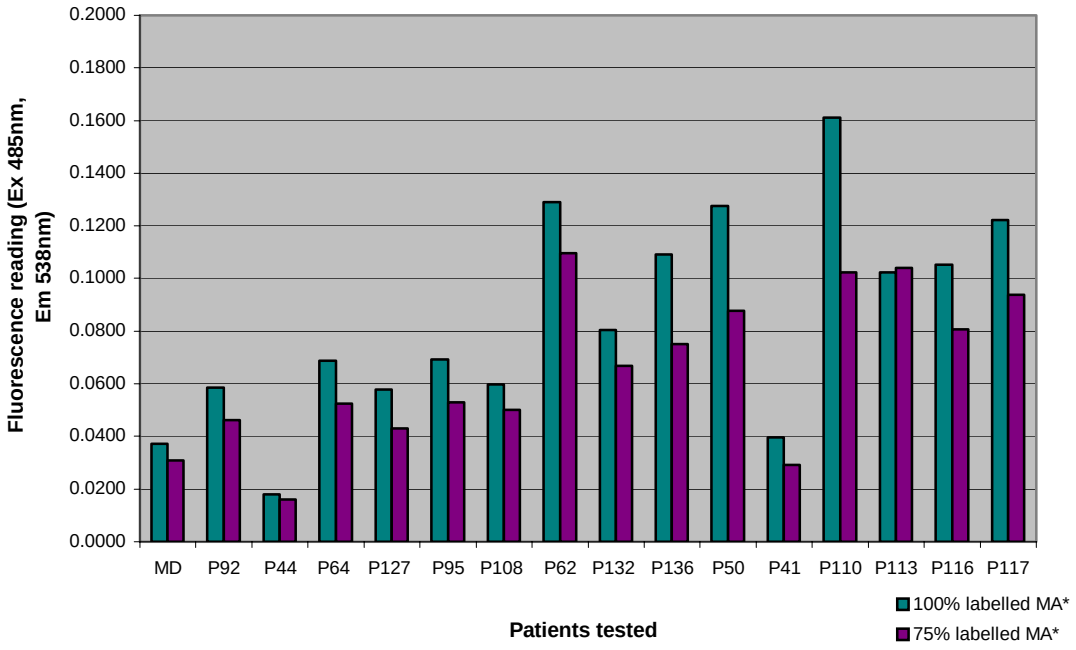


Figure 2.5: A comparison of the fluorescent readings obtained between 100%- and 75%-labelled MA* with the first IgG release from SPICA for the different patients.

2.5.3 Released fluorescence in SPICA indicates Ab specificity for MA

To compare the amounts of immunoglobulin captured by Seph-prot A from various patient and control sera, an ELISA was carried out to quantify the released IgG from SPICA. The released IgG from each patient was pooled and used to coat the wells of an ELISA plate. Anti-IgG peroxidase conjugate was then used as a reporter of the relative amount of sample IgG that bound.

The relative amounts of total IgG captured by Seph-prot A from the patient samples in SPICA were similar irrespective of the specificity of the Abs (Fig 2.6). The varying amounts of fluorescence released from SPICA (Fig 2.4) could therefore not be ascribed to lower IgG capture but to the specificity of the Ab towards the Ag, MA.

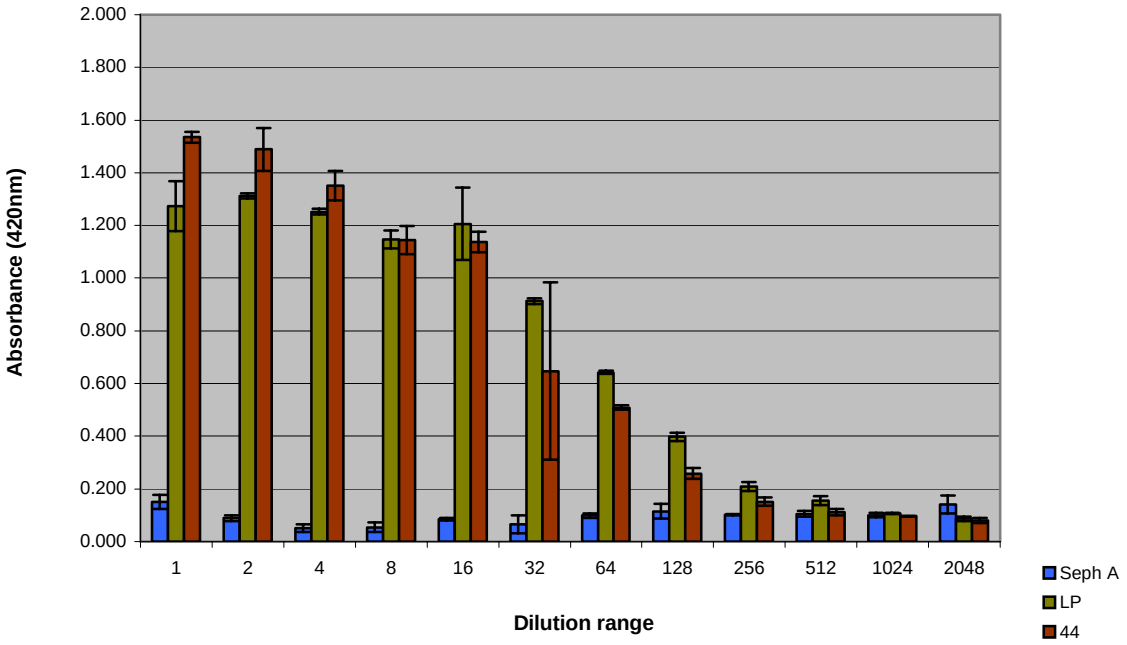


Figure 2.6: Spectrophotometer readings to compare the amounts of immunoglobulin captured by Seph-prot A from various patients and control sera where Seph A = Sepharose protein A and buffer, LP = control serum & P44 = ELISA low positive. An ELISA was done using the released IgG from SPICA. The released IgG from each patient was pooled and used to coat the wells of an ELISA plate. Anti-IgG peroxidase conjugate was then used as a reporter of the relative amount of sample IgG that bound. Error bars indicate standard deviation (n = 3).

In Fig 2.6, Seph-prot A together with buffer was used as a negative control – no Abs were added and therefore an absent or low signal was expected when the ELISA was done. For the serum samples, LP was used as a negative control (see Table 2.2). In a

SPICA experiment using 75%-labelled MA* liposomes, healthy control LP had an anti-MA fluorescent signal of only 0.0285 (Vermaak, 2004) but with the IgG ELISA, it was seen that the total amount of Abs in the sample compared well with that of the patients. P44 (HNTP) had a comparably high anti-IgG ELISA absorbance value but the fluorescent readings were low, which could be attributed to lack of Abs specific towards the MA Ag, as was seen before in the anti-MA ELISA (Table 2.2). Similar results were obtained with other patient sera.

2.5.4 Correlation between SPICA and ELISA

In section 2.5.2 (Fig 2.4), it was observed that qualitatively, SPICA correlated with the anti-MA ELISA values determined before (Table 2.2) showing the same false positive and false negative patient serum values and that SPICA appeared to have an accuracy of 81% in predicting active TB. In order to get a more quantitative/statistical analysis of this correlation, the data obtained, using Seph-prot A and fluorescently labelled MA*, were plotted. The correlation coefficient and the strength of the relationship were determined (see Fig 2.7). For both SPICA and ELISA, 1:20 dilutions of sera were used.

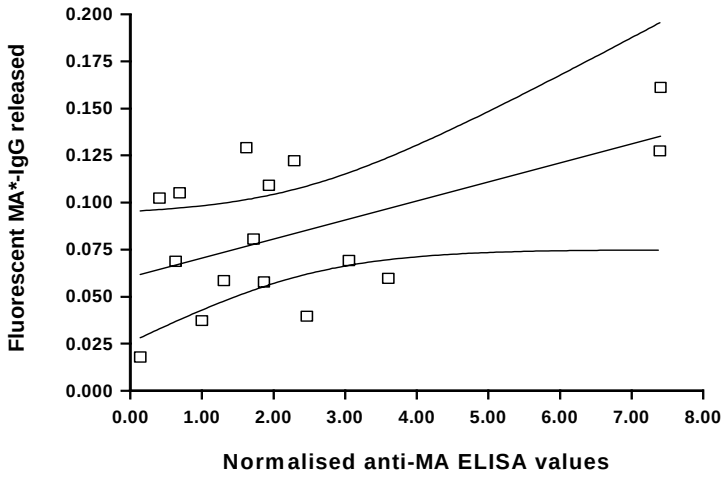


Figure 2.7: Linear regression analysis between ELISA signals on MA coated plates (see Table 2.2; Y Vermaak, 2002) and 100%-labelled MA*-containing liposomes captured on Prot-Seph A immobilized Abs. The sera of 16 human patients were used and consisted of 1 negative control, 1 HNTN, 5 HNTP, 4 HPTP and 5 HPTN. The 95% confidence bands are indicated. The correlation coefficient was determined ($r = 0.556$) and the strength of the relationship ($r^2 = 0.309$).

For the correlation graph (Fig 2.7) all patients tested were plotted and it was observed that SPICA correlates with the ELISA assay with a correlation coefficient of 0.556 and that the strength of the relationship was 0.309 (95% confidence bands are indicated).
It can be concluded that the positive correlation between anti-MA ELISA and SPICA of patient and control sera is weak.

In comparison to the anti-MA ELISA results determined before (Table 2.2), SPICA also registered higher values of anti-MA activity in HIV-positive patients compared to HIV-negative patients, irrespective of whether or not the patients were TB positive (Fig 2.8). In both cases the average readings were about double for HIV-positive patients than with HIV-negative patients, but the results were more consistent with SPICA (lower standard deviation). Using the Student's t-test, a statistical significant difference between the HIV-positive and HIV-negative patients at least 95% confidence level was obtained with SPICA ($P < 0.01$), but not with ELISA.

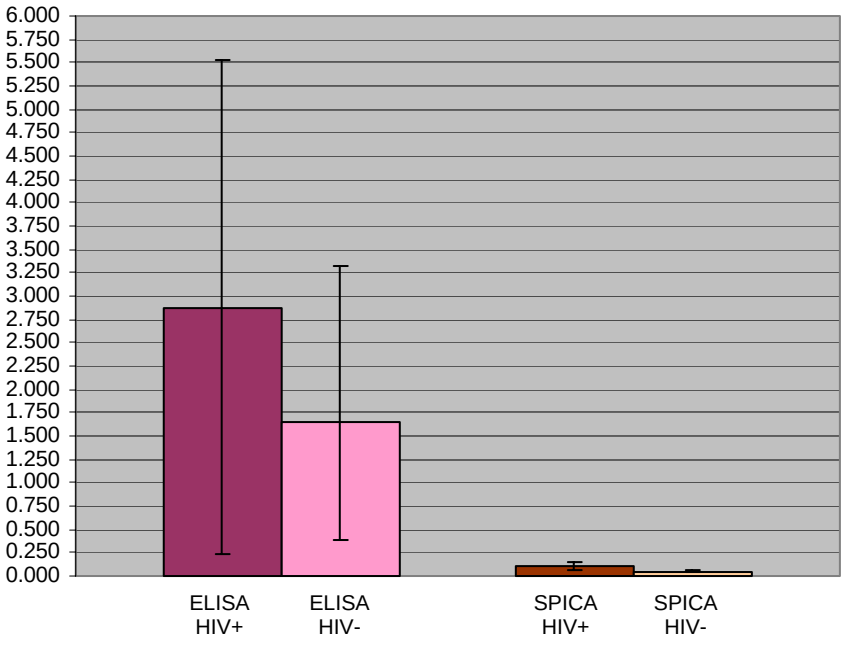


Figure 2.8: Comparison between anti-MA signals of HIV positive (n = 8) and HIV negative patients (n = 7) in ELISA and SPICA.

2.5.4 SPIC inhibition assay

Because quantitatively a poor correlation between anti-MA SPICA and ELISA was found, the SPIC inhibition assay was done to determine whether a better SPICA correlation could be found with clinical TB assessments. To determine this, the IgG attached to Seph-prot A and bound to fluorescently labelled MA* (after thorough washing) was exposed to buffer or different sets of liposomes (containing either PC, PC/chol-; PC/MA) to test the specificity of displacement of the bound MA*-liposomes. If anti-MA Abs bound irreversibly to MA, then the fluorescence signal would be high with IgG release, because no displacement would have taken place. With reversible binding, the fluorescence signal would be low with IgG release because displacement would have taken place.

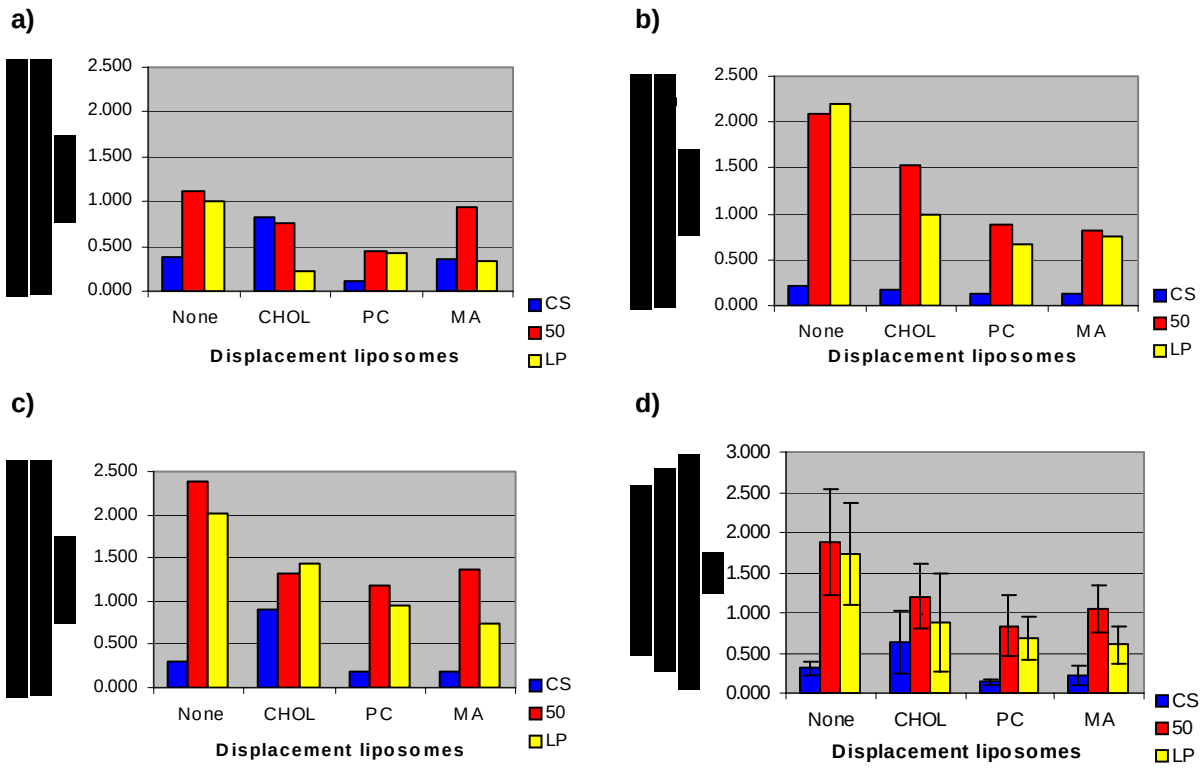


Figure 2.9(a – c): Results obtained with SPIC inhibition assay of 3 independent experiments of released IgG bound to fluorescently labelled MA* in liposomes after displacement with different liposomes. **Figure 2.9(d):** The calculated average and standard deviation of the 3 experiments where CS = buffer, P50 = ELISA high positive & LP = control serum.

When buffer was added instead of liposomes to CS (Seph-prot A together with buffer) no fluorescent signal was expected after IgG release, because no Abs were present and the initial set of liposomes had been washed away (Fig 2.9d blue bar, None). When buffer was added (instead of liposomes) to P50 (HPTP), no displacement was expected

because no liposomes were present to displace the liposomes containing fluorescently labelled MA* already bound to the anti-MA Abs and therefore a high fluorescent reading was expected which was obtained (Fig 2.9d red bar, None). Similarly, buffer added to LP (HNTN), the negative control, gave no displacement as expected (Fig 2.9d yellow bar, None). When PC/chol liposomes were added to CS no displacement was expected because no Abs were present and the initial set of labelled liposomes were washed away. A low fluorescent reading was expected and was obtained (Fig 2.9d, chol). When PC/chol liposomes were added to P50 and LP, lower fluorescent readings were obtained, indicating some displacement that could possibly indicate that a certain percentage of anti-MA Abs are of low affinity and not specific. When PC-containing liposomes were added displacement was found to occur for both P50 and LP, which was not expected. Displacement was expected for P50 when PC/MA-containing liposomes were added, but not for LP because no anti-MA Abs was expected for LP. The extent of the displacement of MA fluorescence was, however, not significantly different between the patient and control sera when either PC, cholesterol or MA were used as displacement agents. Therefore the evidence does not support the hypothesis, and it seems that there is nothing to be gained by attempting competitive displacement of labelled MA from captured IgG from TB patient and healthy control sera, to come to a better correlation between the SPICA and ELISA assays.

2.6 DISCUSSION

In 2002, Schleicher *et al*. investigated the diagnostic potential of an ELISA based on detecting Abs to *Mycobacterium tuberculosis* MA in sera of HIV-seropositive and HIV-seronegative TB patients in a population with a high prevalence of TB. They found that the ELISA had poor sensitivity and specificity (37% and 85% respectively). The reasons for the false positive results obtained could be because 1) there is a high incidence of TB in South Africa with a high exposure level to *Mycobacterium tuberculosis* and 2) some of the TB negative patients had a variety of medical conditions, which could lead to the production of Abs to lipid Ags of generic structure. These Abs would then bind to the lipids, from serum, which may have been captured by the hydrophobic MA-coated ELISA plate and cause an elevation in the absorbance signal.

In order to avoid this hydrophobic surface in a TB serodiagnostic assay, patients' Abs were trapped with Sepharose protein A and the specificity of the Abs was probed with liposomes containing fluorescently labelled MA*. Patients with different TB and HIV status (HNTN, HNTP, HPTN & HPTP) were tested. A TB positive patient was defined as being tested positive by means of best clinical assessment and TB culture assay. Thus free antibodies were supposedly present in serum and able to bind to antigen on the plate. These free antibodies should be able to bind to MA; therefore not associated with MA in circulating immune complexes. Most of the MA present will bind specifically to these antibodies. In contrast, a TB negative patient supposedly has no free Abs present in the serum and therefore no binding to Ag on the plate is expected (Raja *et al*., 1995).

False –positive and –negative results were obtained with SPICA that correlated with those obtained with ELISA. P92 (HNTN) had a higher fluorescence reading than the healthy negative control (MD) and was therefore a false positive result, just as with ELISA (Table 2.2). P44 (HNTP) had a lower fluorescent reading than MD and was therefore a false negative result, as was also registered with ELISA. SPICA appeared to have an accuracy of 81% showing the same false positive and false negative patient serum values. This added confidence to the ELISA results indicating that they may not be that seriously affected by non-specific absorption of Abs to the wells of the ELISA plate. The quantitative correlation between SPICA and ELISA was, however, weak; the

correlation coefficient was 0.556. It remains unsure whether SPICA is actually more accurate than ELISA with the current set of samples analysed.

SPICA, as well as ELISA, registered higher values of anti-MA activity in HIV-positive patients compared to HIV negative patients, irrespective of whether or not the patients were TB positive, but the ELISA results were less consistent (higher standard deviation). Using the Student's t-test, it was determined that there was a much higher statistical significant difference between HIV-positive and HIV-negative patients with SPICA ($P < 0.01$) than with ELISA. HIV infects lymphocytes and macrophages. A wide variety of cytokines derived from these cells appear to alter lipid metabolism (Shor-Posner *et al*., 1993; Grunfeld *et al*., 1989; Dube *et al*., 2003), there is an increase in serum triglycerides levels and a decrease in total cholesterol and high-density lipoprotein cholesterol (HDL) levels. In 2002, Siko proposed a possible mimicry between cholesterol and MA. It was shown, via biosensor experiments, that a MA-coated cuvette surface could attract cholesterol to itself. Could it be that anti-cholesterol Abs (Alving *et al*., 1999; Dijkstra *et al*., 1996) bound fluorescently labelled MA* within the liposomes to result in increases in fluorescent signals? Whereas the ELISA results could hint to the possibility that this might hold true, SPICA with its improved resolution, could statistically support the hypothesis.

In the SPICA inhibition assay, chol, PC and MA containing liposomes all displaced more or less the same amount of fluorescent MA from the antibody-liposome complexes. There was no significant specific displacement of fluorescent mycolic acids from the liposomes captured by the anti-MA antibodies. The possibility exists that lipids exchange freely and non-specifically among IgG captured and free liposomes. Cholesterol in liposomes could exchange with MA containing liposomes, but also stabilize lipid vesicle structure. In the SPICA inhibition assay, lipid exchange among the free and captured liposomes could have occurred that obscured the measurement of displacement of liposomes.

In summary, the SPICA assay showed potential as an effective TB serodiagnostic tool but is not expected to be a dramatic improvement over the much more simple ELISA assay.

In the next chapter, a theoretical model was designed and tested to make use of the MA properties in liposomes in an antibody-independent assay for TB diagnosis.

CHAPTER 3:
MYCOLIC ACIDS AS ANALYTE IN AN ANTIBODY-INDEPENDENT ASSAY IN THE DIAGNOSIS OF TUBERCULOSIS

3.1 INTRODUCTION

Diagnostic assays are normally based either on recognition of the infecting agent or on recognition of the host response to the agent (Daniel, 1990) such as Abs (Lyashchenko *et al*., 1998; Fujita *et al*., 2005), cytokines (Vervenne *et al*., 2004; Lee *et al*., 2006) and T cell responses (Ulrichs *et al*., 1998; Enomoto *et al*., 2005). PCR detects the survival of *Mycobacterium tuberculosis* in Mϕ and is the only approach up to now that gives some promise due to its extreme amplification capacity (> million fold). Another target of detection is lipids, because of their extended lifetime in adipose tissue and lipoproteins in the blood. As a result of the human body living with an abundance of micro organisms in the gut, specificity towards lipids from pathogens is compromised since foreign cell wall lipids land up in the lymph and blood circulation as a continuous, normal activity. MA is a unique pathogen-derived lipid, which is known to act as a fingerprint molecule for the particular mycobacterial species that infects. In addition, it is highly resistant to catabolism due to its large and foreign fatty acid nature. This chapter is dedicated to investigate the possibilities of detecting traces of MA in the serum of patients as an indicator of active TB.

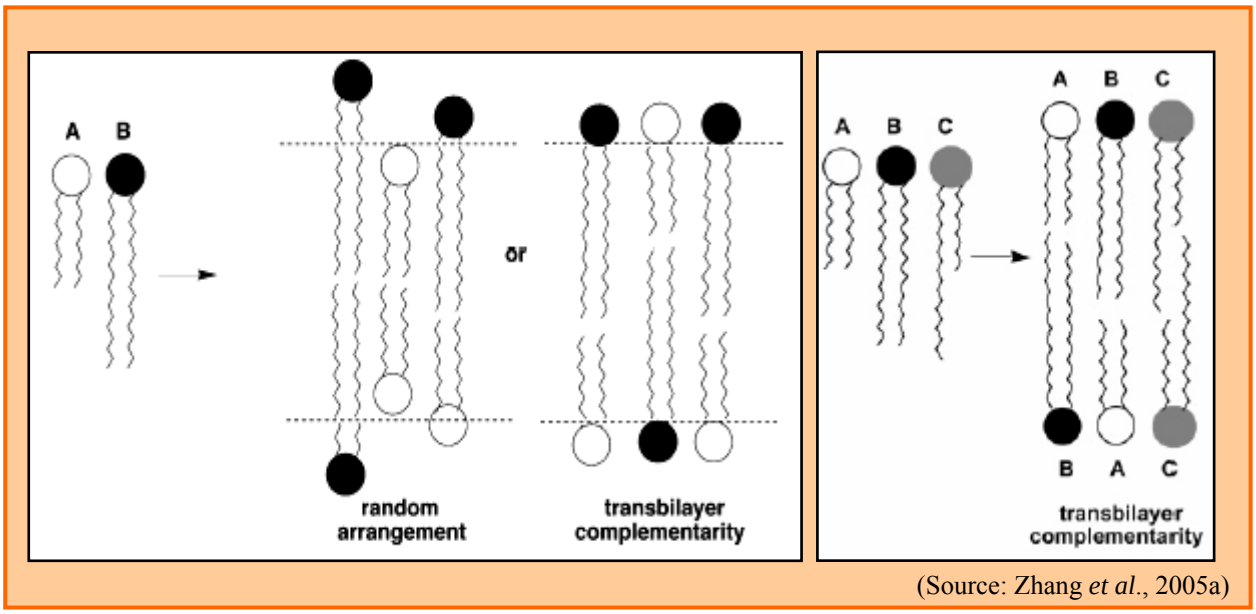
The MA-structure is species specific (Clemens, 1997). It is the most abundant cell wall Ag by far in the mycobacteria and plays a crucial role in determining the fluidity and permeability of the cell walls. MA is hydrophobic due to its long meromycolate chain and attracts cholesterol. Siko (2002) showed that liposomes carrying MA could be immobilised on non-derivatized IAsys (interaction analysis system) biosensor cuvettes. He initially immobilized liposomes containing both MA and cholesterol onto the surface of the hydrophilic cuvette but found that the coated surface was not stable. By first activating the surface of a non-derivatized cuvette with a cationic detergent, cetylpyridiniumchloride (CPC), the hydrophilic surface was made hydrophobic and could be stably coated with MA and cholesterol containing liposomes. He observed that a MA-coated cuvette surface could attract cholesterol from cholesterol-containing

liposomes to itself resulting in a signal of increasing mass accumulation that reached saturation, whilst MA-containing liposomes extracted cholesterol from a cholesterol-coated cuvette surface producing a consistently lower but unstable desorption signal. Siko (2002), Deysel (2007) and Benadie (2007) showed that MA attracts cholesterol with high specificity, which disappears when the carboxylic acid of MA is chemically methylated. They also showed that a structural mimicry between the two molecules (i.e. MA and cholesterol) exists because 1) human TB patient serum anti-MA Abs cross-reacted with cholesterol but not with methylated MA and 2) Amphotericin B, an anti-fungal compound known to bind cholesterol, also bound MA.

In 2000, Gatfield *et al*. reported that cholesterol is essential for the uptake of mycobacteria by macrophages and that it accumulates at the site of mycobacterial entry. Depleting cholesterol from the plasma membrane specifically inhibited mycobacterial uptake. Cholesterol also mediated the phagosomal association of TACO (tryptophan aspartate-containing coat protein) preventing degradation of mycobacteria in lysosomes. Av-Gay *et al*. (2000) found that pathogenic mycobacteria are able to take up, modify and accumulate cholesterol from liquid growth media and form a zone of clearance around a colony when plated on solid media containing cholesterol. Siko (2002) proposed that MA in the mycobacterial cell wall may be responsible for the cholesterol attraction.

Driver (BSc Hons report, 2006) observed that when two sets of liposomes; one containing fluorescently labelled MA, the other cholesterol or unlabelled MA; are mixed together (at 37^{o}C) and fluorescence activated cell sorting (FACS) is performed on the liposomes within five minutes after mixing the sets, the fluorescence of the resulting liposomes was reduced by half. The size of the original cholesterol containing liposomes was bigger in comparison to the size of the original MA-containing liposomes. After mixing of cholesterol-containing and MA-containing liposomes at 37^{o}C, the resultant liposomes obtained after five minutes were intermediate in size to that of the two parental liposome populations, suggesting a rapid and complete re-distribution of cholesterol and MA among all liposomes. The results of Driver imply that MA can exchange quickly between liposomes at body temperature and that the specific interactions between MA and cholesterol, and/or by MA and MA among different liposomes may rule this dynamic exchange.

Zhang *et al*. (2005a) examined whether communication exists between the inner and outer leaflets of fluid bilayers by doing a series of nearest-neighbour recognition (NNR) experiments. They found that phospholipids have the ability to select complementary phospholipids from adjoining monolayers as nearest neighbours. NNR involves the chemical equilibration and analysis of phospholipid homodimers [molecules composed of two identical phospholipid units (monomers) that are covalently bonded through their head groups] and heterodimers (composed of two different covalently bonded phospholipids). The thermodynamic preference for a phospholipid to become a nearest neighbour of another in the bilayer state was then assessed. Zhang *et al*. (2005b) investigated lipid-lipid interactions across cholesterol-rich phospholipid bilayers by measuring nearest-neighbour preferences of exchangeable phospholipids.



(Source: Zhang *et al*., 2005a)

Figure 3.1: Liposomes composed of "long" and "short" phospholipids favor a transbilayer arrangement in which the short phospholipid lies directly across from the long one (Source: Zhang *et al*., 2005a).

Liposomes composed of 'long' and 'short' homodimeric and heterodimeric phospholipids favoured an arrangement where the short phospholipid homodimer lies directly across from the long one forming a smooth surface that maximizes hydrophobic interactions. Similarly, heterodimeric phospholipids tended to arrange themselves. In the absence of cholesterol, transbilayer complementarity (a preference for the pairing of a long phospholipid with a short phospholipid across the bilayer) was observed but to a lesser degree. Transbilayer complementarity (Fig 3.1) of phospholipids may play a vital role in stabilizing biological membranes. Sugahara *et al*. (2001) observed that sterol-

phospholipid affinity increases as the length of the phospholipid increases; therefore cholesterol favours high-melting lipids as nearest neighbours.

Liu *et al*. (1996) showed that MA play a crucial role in determining the fluidity and permeability of the mycobacterial cell walls. The melting temperatures for the cell walls of *Mycobacterium tuberculosis* and *Mycobacterium avium* are higher than that of other mycobacterial species because their mycolates are longer and because of the presence of a *cis*-cyclopropane in the proximal position of the meromycolate. This may enhance the attraction of cholesterol to *Mycobacterium tuberculosis* and *Mycobacterium avium*.

As a result of the above observations, the cholesterol-MA and the MA-MA interactions were investigated which lead to the possibility of detecting traces of MA in the serum of patients as an indicator of active TB.

The following assumptions were made that led to the subsequent experimental design:

1) MA circulates the TB patient body in lipoproteins, and are not immediately cleared from the blood like protein antigens (Simonney *et al*., 1997; Raja *et al*., 1995; Samuel *et al*., 1984).
2) Due to the stability of MA and its foreignness, they are not metabolised like normal fatty acids but remain as antigens as a reporter of active TB, i.e. the stage where *Mycobacterium tuberculosis* replicates in the body (Xu *et al*., 1994).
3) Liposomes containing fluorescently labelled MA in a test vial, will rapidly exchange its labelled MA with lipoproteins from TB patients but not with healthy or non-TB patient serum lipoproteins.

In this chapter, it was determined whether labelled MA from immobilized liposomes will exchange label with MA-containing liposomes, but not with non-MA containing liposomes. Liposomes containing fluorescently labelled MA*, cholesterol and PC (MA*/chol/PC) were adsorbed to a glass surface of a glass-bottomed Nunc^{TM} dark plate as coat liposomes. They were subsequently contacted with suspensions of liposomes (test liposomes) that contained either nothing (PC); cholesterol (PC/chol); or non-fluorescently labelled MA and cholesterol (MA/chol/PC). As a result of lipid-lipid interactions, fluorescence was expected to be released/extracted and the residual fluorescence remaining in the coat was determined using a fluorimeter.

In this model, the PC/chol test liposomes represented the lipoproteins from healthy people and the MA/chol/PC test liposomes those of humans infected with *Mycobacterium tuberculosis*. The residual fluorescence in the coat liposomes after contact with the test liposomes was expected to correlate inversely with the amount of MA in the test liposomes, which in turn will supposedly correlate to the degree of infection of the host with actively proliferating *Mycobacterium tuberculosis*, if the test liposome preparation is substituted with human TB patient serum.

3.2 AIMS

It is to be determined here whether the:

1) Exchange of MA between coat- and test- liposomes is specific, i.e. whether labelled MA from immobilized liposomes will exchange label with MA-containing liposomes, but not with non-MA containing liposomes.
2) If specific, then whether such a model would also hold when an immobilized MA-containing liposome surface is contacted with sera from TB and non-TB patients to enable diagnosis of the former.

3.3 MATERIALS

3.3.1 Mycolic acids

Non-fluorescently labelled MA [*Mycobacterium tuberculosis* H37Rv, ATCC 27294; isolated from human lung tissue and purchased from the American Type Culture Collection (ATCC), Maryland, USA] was obtained from S van Wyngaardt (Department of Biochemistry, University of Pretoria). The TB culture was maintained at the National Tuberculosis Institute of the MRC of South Africa, Pretoria. The MA was then purified from the mycobacterial cell wall as described by Goodrum *et al.* (2001). Already isolated aliquoted MA was then used for fluorescence labelling with 5-BMF.

3.3.2 Apparatus used

The Model B-30 Branson sonifier (Sonifier Power Company, USA) was used to sonify the liposomes. The Fluoscan Ascent Fluorimeter FL Type 374 (Thermo Labsystems, Oy) was used to read the fluorescence in glass-bottomed Nunc^{TM} dark plates (obtained from Nulge Nunc International, Denmark). The ELISA plate shaker (Titertek, Flow Laboratories) was used during the incubation periods.

3.3.3 Reagents

Ultra-pure double distilled, de-ionised water (dddH_2O) was used throughout for the preparations of aqueous solutions. The following reagents, all of analytical grade, were obtained from Merck (NT laboratories, SA): sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium hydrogen phosphate (Na_2HPO_4) and potassium hydroxide (KOH). Chemically pure chloroform ($CHCl_3$) and ethylene diamine tetra-acetic acid (EDTA) were purchased from Saarchem (SA). Cholesterol (5-cholesten-3β-ol) (chol), L-α-phosphatidylcholine (L-α-Lecithin) (PC), sodium azide (NaN_3) and cetylpyridinium chloride (CPC) were obtained from Sigma (St Louis, MO – USA). 5-bromofluorescein (5-BMF) was purchased from Molecular Probes (Leiden, Netherlands).

3.3.4 Buffers

Phosphate buffered saline-azide EDTA buffer (PBS/AE): 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 1.05 g $NaHPO_4$ per liter dddH_2O with 1 mM EDTA and 0.02% (m/v) NaN_3, adjusted to pH 7.4.

3.3.5 Human sera

Serum samples were collected for another study by Schleicher *et al*. (2002). These patients were admitted to the general medical wards of the Helen Joseph Hospital, Johannesburg (South Africa) for various conditions including active pulmonary TB. The sera were collected from the hospital between August and December 2000. Informed consent was obtained from all patients before enrolment. The Institutional Ethics Committee approved the study (University of Witwatersrand).

The TB-positive (TP) group consisted of patients with recently diagnosed smear-positive pulmonary TB of which some were HIV-seropositive. The TB-negative (TN) group used for control had medical conditions other than TB and was recruited from the general medical wards. They had no clinical, radiological or microbiological evidence of active infection with *Mycobacterium tuberculosis*. The negative control serum denoted NC was obtained from healthy students in the Biochemistry Department and pooled.

Vermaak (2002) did an ELISA where MA were coated to the wells of an ELISA plate. Patient sera dilutions were subsequently added and the activity of Abs towards MA was detected using an antihuman IgG peroxidase reporter Ab, enzyme substrate that converts to a chromogenic product and a spectrophotometer. NC (negative control) had a low ELISA signal against MA indicating the absence or low activity of anti-MA Abs. P132 was HIV positive TB positive (HPTP) and P129 HIV negative TB positive (HNTP) and they had ELISA signals of 1.720 and 1.592 respectively, which is a ratio in respect of the negative control used. These patients (P132 & P129) have little/low amounts of anti-MA Abs present in their sera that may be important to prevent interference with the assay and MA that are still present in the sera in the lipoproteins.

3.4 METHODS

3.4.1 Preparations of solutions

CPC (0.02 mg/ml) was prepared in PBS/AE and saline (0.9% sodium chloride) was prepared in dddH_2O.

3.4.2 Liposomes preparation

Cholesterol containing liposomes were prepared by combining 45 µl cholesterol and 90 µl PC stock solutions. MA (100% labelled or no label) containing liposomes were prepared by adding 90 µl PC stock to 1 mg dried MA. Empty liposomes, those containing no cholesterol or MA, were prepared by taking 90 µl PC of the stock solution. The vials were quickly vortexed and placed on a heat block at 85^{o}C where the liposome ingredients were dried using N_2 gas. Liposome formation was induced by the addition of 2 ml saline (0.9% NaCl) and placing in a heat block for 20 minutes at 85^{o}C with a quick vortex after minutes. The liposomes were then vortexed for 1 minute and subsequently pulse sonified for 1 minute at 20% duty cycle at an output of 2%. The liposomes were then aliquoted into 10 vials (200 µl/vial) and kept at –20^{o}C overnight before freeze-drying. The liposomes were subsequently stored at –70^{o}C until needed.

On the day of the experiment, 2 ml PBS/AE was added to the different liposome sets and then placed in the heat block for 30 minutes at 85^{o}C. Subsequently, the liposomes were vortexed for 1 minute and pulse sonified for 1 minute at 30% duty cycle at an output of 3%.

3.4.3 Regeneration protocols

The regeneration protocol involved the usage of either 1 M KOH or 0.1 M HCl. In the wells of a glass-bottomed Nunc^{TM} dark plate, 300 µl KOH (1 M) or 300 µl HCl (0.1 M) was added and left to incubate for 5 minutes. The wells were then rinsed 3 times using 300 µl PBS/AE. EtOH (300 µl, 96%) was then added in each well and left to incubate for 15 minutes. The plate was rinsed thrice with 300 µl PBS/AE. The remaining buffer was flicked out and the plate was allowed to dry overnight.

3.4.4 Test liposomes dilutions

For the test liposome dilutions, 200 µl of test liposomes (PC, PC/chol, MA/chol/PC) were placed into 3 wells of an ELISA plate each. In subsequent wells, 100 µl PBS was added. The initial 3 wells were mixed well and 100 µl was transferred to the next set of wells containing 100 µl PBS. It was mixed well and transferred until completion of the dilution series. All the dilutions were then transferred from the ELISA plate to the glass-bottomed Nunc™ dark plate which was coated with MA*/chol/PC liposomes. The test liposomes were incubated for 20 minutes with shaking. The wells were then washed five times using 100 µl PBS/AE and the remaining fluorescence was read using the Fluoscan Ascent Fluorimeter.

3.4.5 Protocol

A summary of the MA liposome adsorption and desorption (MALADE) assay is given in Fig 3.2:

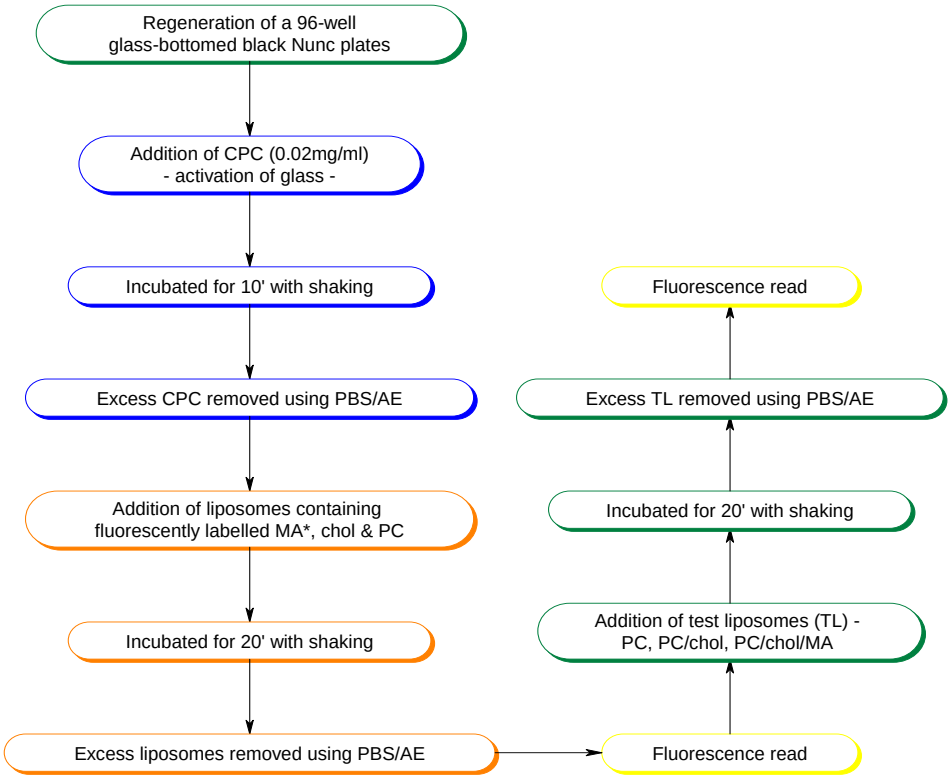


Figure 3.2: An outline of the basic approach used in the MALADE assay.

The wells of a glass-bottomed Nunc™ dark plate were activated using 100 µl/well 0.02 mg/ml CPC. The plate was then incubated for 10 minutes with shaking on the ELISA

shaker and subsequently washed thrice, using 100 µl PBS/AE. The last wash volume was substituted with 100 µl PBS/AE to read the background fluorescence in the wells of the glass-bottomed Nunc™ dark plate. Liposomes containing fluorescently labelled MA*, chol and PC were added into the wells (100 µl/well) and incubated for 20 minutes with shaking. The non-immobilized liposomes were removed and the wells washed with 100 µl PBS/AE until the baseline fluorescence reading were stable. Test liposomes (90 µl; PC, PC/chol & MA/chol/PC liposomes) were loaded into each well and incubated for 20 minutes with shaking. The wells were then washed five times using 100 µl PBS/AE and the remaining fluorescence was read using the Fluoscan Ascent Fluorimeter.

3.4.6 Field testing of MALADE

The same protocol was followed as in section 3.4.4 except substituting test liposomes with TB patient sera in the experimental tube. The patient sera were prepared as follows: the sera were thawed at 37°C for 5 minutes. The sera were then diluted 1:500, 1:1000 and 1:2000 using PBS/AE. The serum dilutions were loaded into each respective well. The test liposomes (100 µl; PC, PC/chol & MA/chol/PC liposomes) were added separately into their respective wells and incubated for 20 minutes with shaking. The wells were then washed five times using 100 µl PBS/AE and the remaining fluorescence was read using the Fluoscan Ascent Fluorimeter.

3.5 RESULTS

3.5.1 Optimization of the test liposome dilution

To determine at which dilution the test liposomes were most stable, undiluted 100% labelled MA*/chol/PC liposomes were used for coating the bottom of a glass-bottomed Nunc^{TM} dark plate and a dilution range of test liposomes (PC, PC/chol, MA/chol/PC) were titrated.

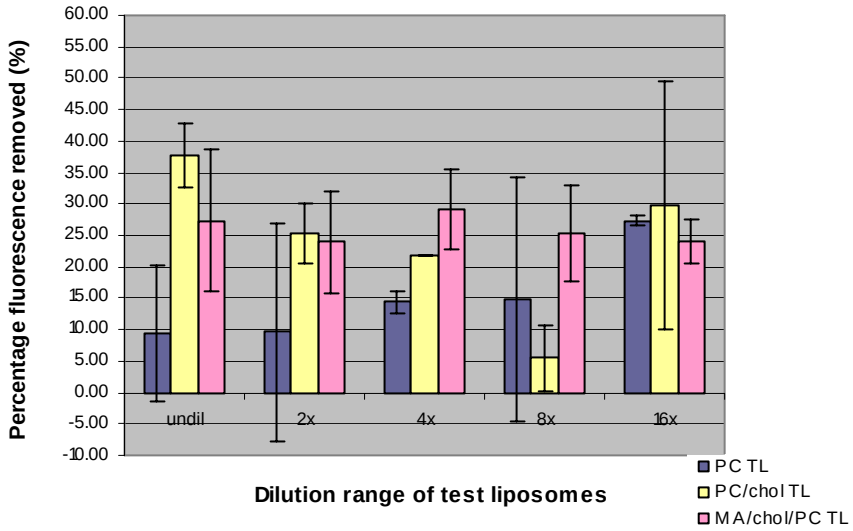


Figure 3.3: Percentage of fluorescence removed (%) from coated MA*/chol/PC liposomes with a dilution range of test liposomes (TL) containing PC or PC/chol or MA/chol/PC in the MALADE test.

With the undiluted and 2x diluted test liposomes, the empty PC test liposomes hardly removed any fluorescent MA-label from the coat liposomes on the surface of the glass wells, whilst both the cholesterol only and the cholesterol and MA-containing test liposomes affected the MA*/chol/PC coat surface significantly (Fig 3.3). With higher dilutions (i.e. 8x and 16x), the pattern observed suggested possible decreased liposome stability and perishing of the coat liposomes, as fluorescence was lost irrespective of the type of test liposome that it was contacted with. PC liposomes showed the instability of the coat liposomes as dilution increased while PC/chol demonstrated that when extreme concentrations (either low or high) are used the specificity of the assay is compromised due to the presence of cholesterol. This model for detecting MA in test samples only holds in a critical window of dilution. Anything higher or lower than this critical window is out of range for resolution between cholesterol and MA. With higher dilutions the c. m. c. is passed and therefore decreased liposome stability occurs. The 4x dilution complied best with the expected result, based on the assumption that MA would

better exchange with MA* in liposomes than cholesterol and also that at this dilution the c. m. c. of liposomes is met. It also gave the best reproducibility. The model was therefore tested to work using 4x diluted test liposomes.

3.5.2 Comparison of regeneration protocol

In order to make this a feasible diagnostic test, it would have to be cheaper than current tests and one way would be to regenerate the glass surface of the plates so that they can be used more than once. A protocol would have to be established where one would be certain that the plates could be reused without the fear of possible contamination from previous tests and usage. The regeneration protocol developed and tested was based on that used previously with biosensor experiments (Siko, 2002). The wells of a glass-bottomed Nunc^{TM} dark plate were regenerated with either 1 M KOH or 0.1 M HCl, washed with PBS/AE, rinsed with EtOH and washed again with PBS/AE. The plate was allowed to dry overnight. A new plate that was not pre-treated with a regeneration protocol was designated NR, no regeneration.

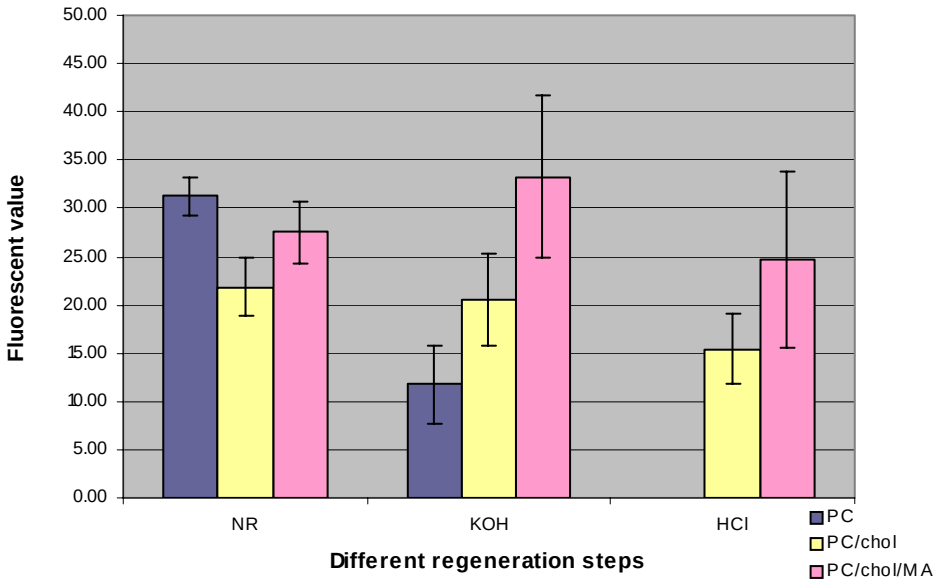


Figure 3.4: Comparison of different regeneration protocols in the MALADE test where NR = no regeneration of glass-bottomed Nunc^{TM} dark plate; KOH = plate regenerated with KOH and HCl = plate regenerated with HCl. Undiluted MA*/chol/PC liposomes were used for coating the wells of the plate and the 4x diluted TL (PC, PC/chol, MA/chol/PC) were used to test the coated surface. Error bars indicate standard deviation (n = 3).

The results are shown in Fig 3.4. With no regeneration (NR), the empty PC test liposomes extracted the most fluorescence from the coat liposomes compared to the

PC/chol- and the MA/chol/PC-containing test liposomes. Regenerating the plate using KOH resulted in the empty test liposomes (PC) removing the least fluorescence and the MA/chol/PC test liposomes removing the most. With the HCl regeneration protocol, the PC test liposomes removed no fluorescence from the coat liposomes and the MA/chol/PC test liposomes extracted the most fluorescence. The plates would therefore be regenerated using either KOH or HCl.

3.5.3 Validation of MALADE

To determine the reproducibility whereby data can be obtained with the MALADE test model, the experimental procedure was optimized and repeated.

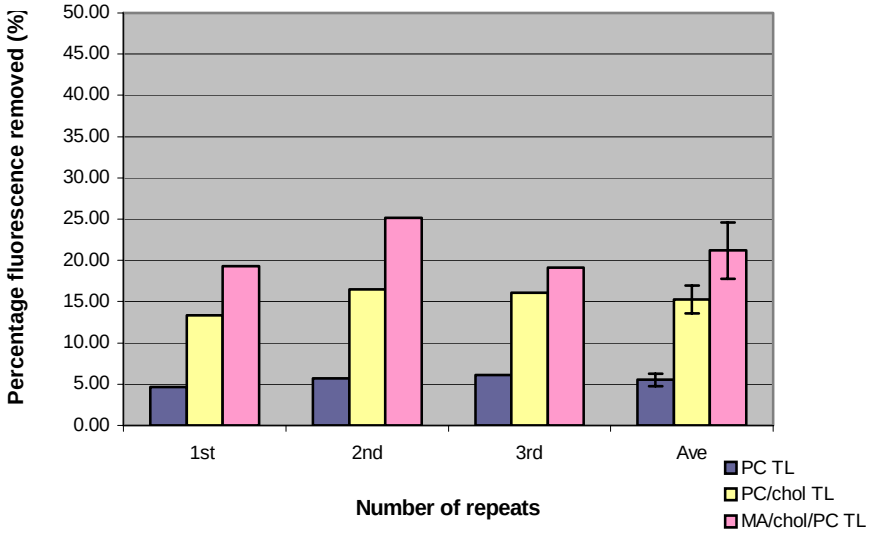


Figure 3.5: Results obtained in the MALADE test with a 0.1M HCl regenerated glass-bottomed Nunc™ dark plate. The wells were coated with undiluted MA*/chol/PC liposomes, washed with PBS/AE and the 4x diluted test liposomes (PC, PC/chol, MA/chol/PC) were then added, washed with PBS/AE and the remaining fluorescence read. The calculated average and standard deviation of the 3 repeats is shown in the last column – error bars indicate standard deviation.

In Fig 3.5, the test liposomes containing MA, chol and PC removed the most fluorescence from a liposome coat containing MA*, chol and PC whilst the cholesterol and PC containing liposomes removed less. The PC-containing test liposomes removed the least fluorescence. The above results (Fig 3.5) suggest that the theoretical model works. However, the assay appears to be quite fragile and restricted to narrow concentration limits. Using the Student's t-test, it was determined that there was a

statistical significant difference of MA/chol/PC test liposomes removing more fluorescence than the PC/chol test liposomes at a 95% confidence level.

3.5.5 Applying MALADE to patient sera

To get a cursory indication whether this model could be used as a possible diagnostic test for TB, patient sera and 4x diluted test liposomes were used. Undiluted MA*/chol/PC coat liposomes were used for coating the bottom of a glass-bottomed Nunc^{TM} dark plate that had been regenerated using the 0.1 M HCl protocol and activated using 0.02 mg/ml CPC. The coat liposomes were incubated and then washed using PBS/AE. The test liposomes were subsequently added as well as the patient sera, which were diluted 1:500, 1:1000 and 1:2000. Sera from healthy individuals in the Biochemistry Department, denoted NC, were pooled and used as the negative control. P129 (HNTP) and P132 (HPTP) were tested. The test liposomes and sera were incubated and subsequently washed with PBS/AE and the fluorescence read (Fig 3.6).

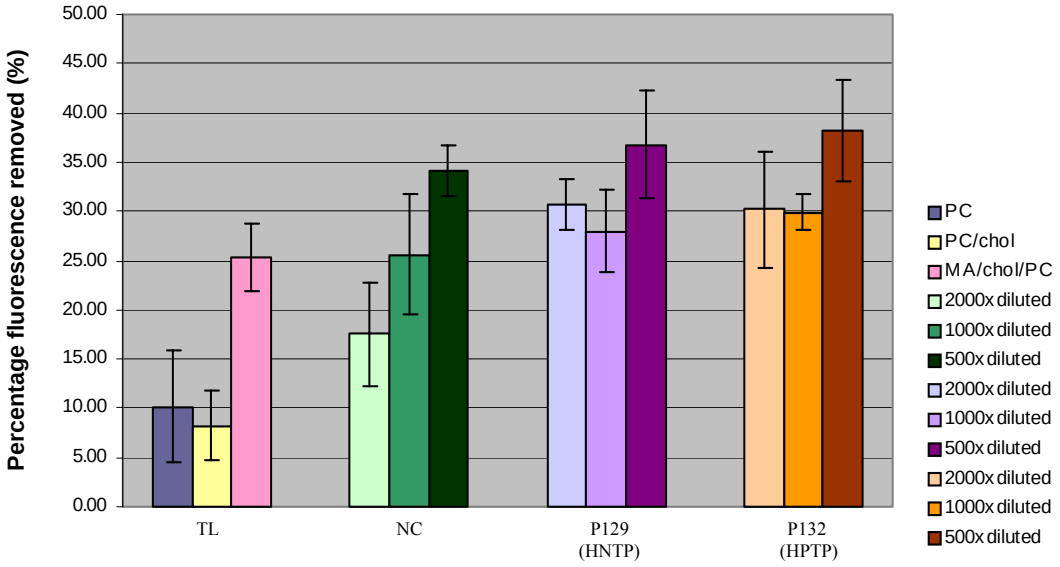


Figure 3.6: Testing the MALADE assay with human patient serum samples. A 0.1M HCl regenerated glass-bottomed Nunc^{TM} dark plate was used. The wells were coated with undiluted MA*/chol/PC liposomes, and incubated with either 4x diluted test liposomes (PC, PC/chol, MA/chol/PC), or patient sera (TB negative NC, and TB positive P129, P132) diluted either 1:500 or 1:1000 and 1:2000 and washed with PBS/AE. The remaining fluorescence was subsequently read.

The PC and PC/chol test liposomes removed no fluorescence while MA/chol/PC test liposomes removed the most. All the sera tested removed fluorescence, including NC. MALADE was applied to three different concentrations of human serum samples –

healthy and patient sera samples. Fluorescence was removed by the different concentrations of the NC and both patients. The concentrated serum samples (500x diluted) removed more fluorescence and no significant difference was observed between the healthy control and patient sera. However, a difference was observed between the healthy control and patients' sera at a 2000x dilution. At this dilution, significantly more labelled MA was removed from the MA* coat by the TB positive patient sera compared to the pooled negative serum control ($P < 0.1$). Higher dilutions may either provide better data, or lead to the destabilization of the coat liposomes due to decreased c. m. c.

3.6 DISCUSSION

In this chapter, the experimental approach taken was an attempt to create a TB-serodiagnostic assay that aimed at the direct detection of compounds of *M. tuberculosis* in the circulation as an indication of active TB. The unique structure of MA was thought to provide the required specificity. Sensitivity was achieved by using fluorescently labelled MA. MA was not detected directly, e.g. as with HPLC, but indirectly through its presumed physical properties of interaction with other MA.

The experimental design was first tested on a model. This model was composed of 1) coat liposomes that contained fluorescently labelled MA, cholesterol and PC and 2) test liposomes that either contained PC only, PC and cholesterol or PC, non-labelled MA and cholesterol. It was observed that test liposomes containing non-labelled MA, PC and cholesterol extracted or removed the most fluorescence whilst the test liposomes containing PC or PC and cholesterol removed less fluorescence. It was also observed that the model assay is quite fragile and is restricted to a narrow concentration window. Once the model was proven to work, the experimental design was tested on two human TB patient serum samples in order to detect possible traces of MA as an indicator of active TB.

With the 500x- and 1000x- diluted sera samples, no differences were observed for the different types of patients; i. o. w. the different concentrations (500x- and 1000x-dilutions) of sera removed fluorescence and was not dependent on whether the patients were healthy (NC), HIV negative TB positive (P129) or HIV positive TB positive (P132). However when a lower concentration of serum (2000x dilution) was used, a difference was observed between the healthy control and the patient sera. Patient sera removed more fluorescently labelled MA* from the coat liposomes than the healthy control.

In flow cytometric analysis, Driver (BSc Hons report, 2006) showed that the absorption of cholesterol into MA-containing liposomes increased their size up to 40%. She also showed that cholesterol's concentration in excess of 40% could bring liposomes to exceed their maximum size, leading to fission. This may explain why cholesterol-containing test liposomes and serum also removed labelled MA from the coated

liposomes. The improved resolution that was obtained between control and TB patient sera at very high serum dilutions supported this assumption.

Even though TB patient and control sera could be resolved at high dilution using the MALADE test, this was merely an exploratory investigation and more work still needs to be done: The c. m. c. needs to be taken into consideration – any concentration extreme, too concentrated or too dilute, can destabilize the coat liposome and affect the assay. The c. m. c. decreases with decreasing temperature and the micellar size increases (Haberland & Reynolds, 1973) – temperature will have to be taken into consideration too. It appears to be important to maintain c. m. c. by doing extreme dilutions of sera or test liposomes with dilution buffer containing PC. The necessity for extreme dilutions of sera appears to be necessary to lower the concentration of cholesterol in the sera or test liposomes. Although each patient's cholesterol levels are different, extreme dilution may thwart the interference of cholesterol in the assay. The stage of TB infection (Fujita *et al*. 2005) may be important to know whether the MA antigen is still present in the system for the detection as a marker of disease. This will need to be determined statistically with an optimized assay. Hypercholesterolemia and HIV could also have an effect on the outcome of the MALADE assay (Shor-Posner *et al*., 1993; Grunfeld *et al*., 1989; Dube *et al*., 2003) but the prospects appear good that the MALADE assay can be optimized to accommodate all these variables.

In the MALADE assay, TB patient and control sera could be resolved at high dilution. If subsequent studies confirm these findings, then this concept may be converted into a simple, rapid and affordable TB diagnostic test that may allow the determination of the presence of MA in the patients' systems as a marker of disease and therefore the stage of TB infection.

CHAPTER 4: CONCLUDING DISCUSSION

As a result of the development of drug-resistant strains of *Mycobacterium tuberculosis* and the breakdown of the immune system of its host by HIV, TB, is no longer a 'controlled' disease and has become a major health problem in both developed and developing countries (Houghton *et al*., 2002). Lapses in public health programmes and the fact that diagnosis of TB is not 100% reliable contribute to the increase of TB infection. Therefore, early, affordable and unsophisticated diagnosis of TB and its timely and proper treatment have become of highest priority to public health (Chan *et al*., 2000).

A great deal of research has gone into the improvement of current TB diagnostic tests. Different techniques have been utilized and a variety of antigens have been employed. In this study, the development of a totally different approach to TB diagnosis was attempted using either anti-mycolic acids antibodies as surrogate marker of active TB, or exploiting the particular properties of the mycolic acids to detect traces of the antigen in the serum of patients as an indicator of active TB.

MA are α-alkyl-β-hydroxy fatty acids of exceptional length and complexity that are unique to mycobacteria and closely aligned genera (Yuan *et al*., 1998). Although the basic α-alkyl-β-hydroxy structure is highly conserved, there is considerable variation in the functional groups that interrupt the long mero branch of these acids at two positions (Minnikin *et al*., 1984; Barry *et al*., 1998; Watanabe *et al*., 2001). These functional groups will affect the packing of the long hydrocarbon mycolate chains thereby influencing the intrinsic physiological functions of the cell envelope and ultimately the packing when placed in liposomes. Villeneuve *et al*. (2005) analyzed the temperature effect on the Langmuir monolayer packing of all three subclasses of *Mycobacterium tuberculosis* and showed that MA of different chemical structures form Langmuir monolayers having distinctive physicochemical features and each MA exhibits multiple phase transitions depending on the temperature and surface pressure. Dubnau *et al*. (2000) observed that permeation rate is lowered when a cell wall-linked mycolate consists solely of α-MA. A recombinant mycobacterial strain showed poor growth in

macrophages and a decreased rate of permeation for hydrophilic substances when the keto-MA was completely replaced by methoxy-MA (Yuan *et al*., 1998). It is now thought that different mycolates have different effects on the cell function and permeability. Mycolic acids have been shown to be unique antigens for TB diagnosis (Fujiwara *et al*., 1999), but warrant the investigation of new technologies of detection in order to break through the current glut of inadequacy of TB serodiagnosis in general (Bloom *et al*., 1992; Kaufmann, 2004). In particular, methods of MA immobilization could be improved as well as the possible exploitation of the unique physical properties of MA.

In chapter 2, the problem of the hydrophobic nature of MA for immobilization was avoided by first trapping patient antibodies with protein-A. The anti-MA antibodies were then quantified by probing with liposomes containing fluorescently labelled MA*. This assay was called the Sepharose protein A immunoglobulin capture assay (SPICA). Although it generally worked well, false –positive and –negative results were obtained with SPICA. In the exploratory experiment using 11 TB-positive and –negative sera, SPICA appeared to have an accuracy of 81%, which correlated with the ELISA values determined before by Y. Vermaak (2002), showing the same number and identity of false positive and false negative patient serum values. The quantitative correlation between SPICA and ELISA was, however, only 0.556. It did not fall in the scope of this study to determine which of SPICA or ELISA provided the best quantitative data to indicate the levels of anti-MA antibodies in patients. As it stands now, both SPICA and ELISA give the same qualitative result in distinguishing between TB-positive and TB-negative cases.

SPICA, as well as ELISA, registered higher values of anti-MA activity in HIV-positive patients compared to HIV-negative patients irrespective of whether or not the patients were TB positive but the ELISA results had higher standard deviations. Using the Student's t-test, it was determined that there was a much higher statistical significant difference of the results between HIV-positive and HIV-negative patients with SPICA ($P < 0.01$) than with ELISA. This may suggest that the SPICA test is more accurate than ELISA. SPICA is, however, more labour intensive and not even remotely as amenable to large-scale screening and automation as ELISA. In addition, the higher signals generated with sera from HIV-positive patients were thought to be due to lipid

disturbances caused by HIV and/or anti-retroviral treatment that could give rise to MA-cross-reactive antibodies, e.g. anti-cholesterol antibodies. For this reason, the further validation of SPICA was not undertaken.

In order to achieve useful levels of sensitivity and specificity it has been suggested by Kulshrestha *et al*. (2005) to include several antigens in a diagnostic assay since there is great heterogeneity in humoral response in TB patients. Immunoassays represent attractive approaches for the detection of any infection (Lyashchenko *et al*., 1998; Gennaro, 2000). Immunoassays can be developed in simple and robust formats and can be easily implemented under the conditions commonly encountered in developing countries (Kulshrestha *et al*., 2005). However, for these assays to be successful in TB, immunodominant mycobacterial antigen(s) need to be identified and produced in large, highly pure quantities, essential for the development of cost-effective test kits. Unfortunately, expression of mycobacterial proteins in heterologous hosts has proven to be difficult and most mycobacterial proteins are insoluble when over expressed and purified. The choice of antigens for TB serological tests is also complicated due to the heterogeneity in response of different individuals to the same antigen. Moreover, the heterogeneous response to the same antigen in different populations can further add to the difficulty to formulate a robust universal diagnostic test for different clinical settings.

Another important point that needs to be taken into consideration is that the sensitivity of serologic tests for TB is dependent on the origin of the sample and the clinical spectrum of the disease groups prevalent in the area where the patient lives (McConkey *et al*., 2002). Therefore each new serodiagnostic test should be validated with cases and control specimens from the countries or regions in which it will be used.

In chapter 3, a novel technique was assessed in an attempt to create a TB-serodiagnostic assay that aimed at the direct detection of the lipid antigen, MA, of *Mycobacterium tuberculosis* in the circulation as an indication of active TB. MA was not detected directly, e.g. as with HPLC, but indirectly through its perceived unique physical properties of interaction with other MA using fluorescently labelled MA. The assay was called the mycolic acids liposome adsorption and desorption (MALADE) assay. The principle is based on the release of fluorescent MA from immobilized liposomes on

glass by means of the specific attraction that MA in test liposomes or TB patient serum was perceived to have on the immobilized MA. Contact between the test liposomes or TB patient serum and the immobilized fluorescent MA was then to leach the latter from the surface into the test liposomes or serum to be washed away, while non-TB serum was supposed to be unable to extract any MA from the surface by lack of any traces of mycolic acid in the serum of healthy individuals. The end-point measured was the remaining fluorescent MA on the surface.

The MALADE assay was first tested on a model and it was observed that test liposomes containing non-labelled MA, PC and cholesterol extracted or removed the most fluorescence whilst the test liposomes containing PC or PC and cholesterol removed less fluorescence. This model/assay is quite fragile and is restricted to a narrow window of MA concentration in the test liposomes of the model. To get a cursory indication whether this model could be used as a possible diagnostic test for TB, two patient sera were tested. Only with a very high (2000X) dilution could differences be observed between the control and patients' sera. Patient sera removed more fluorescently labelled MA* from the coat liposomes than the healthy control. No differences were observed between the HIV negative TB positive and HIV positive TB positive patients.

Even though TB patient and control sera could be resolved at high dilution using the MALADE test, this was merely an exploratory investigation and more work still needs to be done before the test is ready for validation with large numbers of serum samples. Specificity and sensitivity are important parameters in developing and marketing serodiagnostic assays. These two parameters need to be taken into consideration and investigated for the MALADE test.

Specificity of the MALADE assay will be determined by using the different MA subclasses of *Mycobacterium tuberculosis* as well as MA from other *Mycobacterium* species and working at a constant temperature where the liposomes are most stable.

Laser light sources in conjunction with fluorescence detection have been utilized to achieve detection limits at low femtomolar and attomolar ranges for compounds of pharmaceutical and biomedical interest. Mukherjee *et al*. (1995) evaluated 5-BMF for determining the detection limit of palmitic acid and achieved a detection limit of 7.56 x

10^{-10} M of palmitic acid at a signal to noise ratio of 3, which corresponds to 38 femtomoles of palmitic acid on-column. Such sensitivity is expected with the MALADE assay that can be further amplified by the ability of MA in patient sera to rapidly increase its ability to extract fluorescent MA from the immobilised layer by positive co-operativity as the accrued MA in the serum may enhance further extraction of MA up to equilibrium. Should such a mechanism apply to the MALADE test, then the sensitivity of the assay may become comparable to that of PCR that allows in vitro amplification of target DNA to a detectable level and which is also being used for the detection of *Mycobacterium tuberculosis* in patient samples, but in a much more elaborate and expensive process.

Two vastly different techniques were investigated in this study – one making use of antibodies and antigen, the other, just antigen and its unique physical properties of interaction with other MA. Although the latter appeared to be more successful in this study, anti-MA antibody based serodiagnosis may still hold great potential for the future. Thanyani (2003) demonstrated that the IAsys affinity biosensor could be a valuable alternative method for the detection of anti-MA antibodies in patient serum, even among patients co-infected with HIV and other diseases. The biosensor assay bases its success on the measurement of antigen-antibody interaction in real-time, thereby gaining the ability to detect antibodies of even low affinity. This is not possible with ELISA and appears to be of key importance to gain sensitivity of detection to acceptable levels.

In the MALADE assay, TB patient and control sera could be resolved at high dilution. If subsequent studies confirm these findings, then this concept may be converted into a simple, rapid and affordable TB diagnostic test or be used in combination with the IAsys affinity biosensor to provide a more thorough diagnosis.

SUMMARY

Tuberculosis has become one of the world's most devastating diseases, with more than two million deaths and eight million new cases occurring annually due to the development of drug-resistant strains of *Mycobacterium tuberculosis*, the breakdown of the immune system of its host by HIV, lapses in public health programmes and the fact that diagnosis of TB is not 100% reliable. Early, affordable, unsophisticated and accurate diagnosis of TB to facilitate timely and proper treatment has become of highest priority to public health.

Mycolic acid (MA) is the major lipid cell wall component of *Mycobacterium tuberculosis* and is unique to mycobacteria and closely aligned genera. Mycolic acids have been shown to be unique antigens for TB diagnosis and have been utilized in standard serodiagnostic techniques, but sensitivity and specificity was found to be unsatisfactory.

Two vastly different techniques were investigated in this study – one making use of antibodies and MA, the other, just MA and its unique physical properties of interaction with other MA using fluorescently labelled MA.

In the first approach, Sepharose protein-A was employed to trap patient IgG antibodies. The anti-MA antibodies were then quantified by probing with liposomes containing fluorescently labelled MA. Although it generally worked well, a few false –positive and –negative results were obtained. This assay appeared to be more accurate than the standard ELISA immunoassay but it is more labour intensive and not even remotely as amenable to large-scale screening and automation as ELISA.

The second approach is based on the release of fluorescent MA from immobilized liposomes on glass by means of the specific attraction that MA in test liposomes or TB patient serum was perceived to have on the immobilized MA. The end-point measured was the remaining fluorescent MA on the surface. Differences were observed between the control and patients' sera at a very high dilution but not between the HIV negative,

TB positive and HIV positive, TB positive patients. This was merely an exploratory investigation and more work still needs to be done before the test is ready for validation with large numbers of serum samples. If subsequent studies confirm these findings, then this concept may be converted into a simple, rapid and affordable TB diagnostic test or be used in combination with the IAsys affinity biosensor to provide a more thorough diagnosis.

REFERENCES

Al-Hajjaj, M. S., Gad-El-Rab, M. O., Al-Orainey, I. O. and Al-Kassimi, F. A. (1999). "Improved sensitivity for detection of tuberculosis cases by a modified Anda-TB ELISA test." *Tuber. Lung Dis.* **79**: 181-185.

Alving, C. R. and Wassef, N. M. (1999). "Naturally occurring antibodies to cholesterol: a new theory of LDL cholesterol metabolism." *Immunol. Today* **20**: 362-366.

Amicosante, M., Houde, M., Guaraldi, G. and Saltini, C. (1999). "Sensitivity and specificity of a multi-antigen ELISA test for the serological diagnosis of tuberculosis." *Int. J. Tuberc. Lung Dis.* **3**: 736-740.

Amicosante, M., Richeldi, L., Monno, L., Cuboni, A., Tartoni, P. L., Angarano, G., Orefici, G. and Saltini, C. (1997). "Serological markers predicting tuberculosis in human immunodeficiency virus-infected patients." *Int. J. Tuberc. Lung Dis.* **1**: 435-440.

Andersen, A. B. and Hansen, E. B. (1989). "Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*." *Infect. Immun.* **57**: 2481-2488.

Arias-Bouda, L. M., Kuijper, S., van Deutekom, H., van Gijlswijk, R., Pekel, I., Jansen, H. M. and Kolk, A. H. J. (2003). "Enzyme-linked immunosorbent assays using immune complexes for the diagnosis of tuberculosis." *J. Immunol. Meth.* **283**: 115-124.

Ashtekar, M. D. and Samuel, A. M. (1985). "Circulating immune complexes in pulmonary tuberculosis." *J. Clin. Lab. Immunol.* **18**: 91-95.

Attallah, A. M., Osman, S., Saad, A., Omran, M., Ismail, H., Ibrahim, G. and Abo-Naglla, A. (2005). "Application of a circulating antigen detection immunoassay for laboratory diagnosis of extra-pulmonary and pulmonary tuberculosis." *Clin. Chim. Acta* **356**: 58-66.

Av-Gay, Y. and Sobouti, R. (2000). "Cholesterol is accumulated by mycobacteria but its degradation is limited to non-pathogenic fast-growing mycobacteria." *Can. J. Microbiol.* **46**: 826-831.

Barnes, P. F., Bloch, A. B., Davidson, P. T. and Snider Jr., D. E. (1991). "Tuberculosis in patients with human immunodeficiency virus infection." *N. Engl. J. Med.* **324**: 1644-1650.

Barry III, C. E., Lee, R. E., Mdluli, K., Sampson, A. E. and Schroeder, B. G. (1998). "Mycolic acids: Structure, biosynthesis and physiological functions." *Prog. Lipid Res.* **37**: 143-179.

Beckman, E. M., Porcelli, S. A., Morita, C. T., Behar, S. M. and Furlong, S. (1994). "Recognition of a lipid antigen by CD1-restricted αβ+ T cells." *Nature* **372**: 691-694.

Benadie, Y. (2007). Amphotericin B as a mycolic acid specific targeting agent in tuberculosis. MSc. Dissertation, Department of Biochemistry, Faculty of Biological and Agricultural Sciences, University of Pretoria.

Benítez, J., Palenzuela, D., Rivero, J. and Gavilondo, J. V. (1998). "A recombinant protein based immunoassay for the combined detection of antibodies to HIV-1, HIV-2 and HTLV-I." *J. Virol. Meth.* **70**: 85-91.

Bloch, H. (1950). "Studies on the virulence of tubercle bacilli: Isolation and biological properties of a constituent of virulent organisms." *J. Exp. Med.* **91**: 197-218.

Bloom, B. R. and Murray, C. J. L. (1992). "Tuberculosis: Commentary on a re-emergent killer." *Science* **257**: 1055-1064.

Boelaert, M., Rijal, S., Regmi, S., Singh, R., Karki, B., Jacquet, D., Chappuis, F., Campino, L., Desjeux, P., Le Ray, D., Koirala, S. and van der Stuyft, P. (2004). "A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis." *Am. J. Trop. Med. Hyg.* **70**: 72-77.

Brennan, P. J. (1995). "The envelope of mycobacteria." *Annu. Rev. Biochem.* **64**: 29-63.

Brennan, P. J. (2003). "Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*." *Tuberculosis* **83**: 91-97.

Chan, E. D., Heifets, L. and Iseman, M. D. (2000). "Immunologic diagnosis of tuberculosis: A review." *Tuber. Lung Dis.* **80**: 131-140.

Chang, Z., Choudhary, A., Lathigra, R. and Quiocho, F. A. (1994). "The immunodominant 38-kDa lipoprotein antigen of *Mycobacterium tuberculosis* is a phosphate-binding protein." *J. Biol. Chem.* **269**: 1956-1958.

Chiang, I. -H., Suo, J., Bai, K. -J., Lin, T. -P., Luh, K. -T., Yu, C. -J. and Yang, P. -C. (1997). "Serodiagnosis of tuberculosis: A study comparing three specific mycobacterial antigens." *Am. J. Respir. Crit. Care Med.* **156**: 906-911.

Cimitan, S., Lindgren, M. T., Bertucci, C. and Danielson, U. H. (2005). "Early absorption and distribution analysis of anti-tumour and anti-AIDS drugs: Lipid membrane and plasma protein interactions." *J. Med. Chem.* **48**: 3536-3546.

Clemens, D. L. (1997). "*Mycobacterium tuberculosis*: bringing down the wall." *Trends Microbiol.* **5**: 383-385.

Clemens, D. L., Lee, B. -Y. and Horwitz, M. A. (2002). "The *Mycobacterium tuberculosis* phagosome in human macrophages is isolated from the host cell cytoplasm." *Infect. Immun.* **70**: 5800-5807.

Cocito, C. and Van Linden, F. (1988). "Subcellular localization and sedimentation behaviour of antigen 60 from *Mycobacterium bovis* BCG." *Med. Microbiol. Immunol.* **177**: 15-25.

Cole, R. V., Lazarus, A. W. and Hedrick, H. G. (1972). "Development and evaluation of a simple latex agglutination test for diagnosis of tuberculosis." *Appl. Microbiol.* **24**: 525-534.

Daniel, T. M. (1989). "Rapid diagnosis of tuberculosis: Laboratory techniques available in developing countries." *Rev. Infect. Dis.* **11**: S471-S478.

Daniel, T. M. (1990). "The rapid diagnosis of tuberculosis: A selective review." *J. Lab. Clin. Med.* **116**: 277-282.

Daniel, T. M., Sippola, A. A., Okwera, A., Kabengera, S. and Hatanga, E. (1994). "Reduced sensitivity of tuberculosis serodiagnosis in patients with AIDS in Uganda." *Tuber. Lung Dis.* **75**: 33-37.

De Charnace, G. and Delacourt, C. (2001). "Diagnostic techniques in paediatric tuberculosis." *Pediatr. Resp. Rev.* **2**: 120-125.

Deysel, M. (2007). Structure-function relationship of mycolic acid in tuberculosis. PhD thesis, Department of Biochemistry, Faculty of Biological and Agricultural Sciences, University of Pretoria.

Dijkstra, J., Swartz Jr., G. M., Raney, J. J., Aniagolu, J. and Toro, L. (1996). "Interaction of anti-cholesterol antibodies with human lipoproteins." *J. Immunol.* **157**: 2006-2013.

Djavad, N., Bas, S., Shi, X., Schwager, J., Jeannet, M., Vischer, T. and Roosnek, E. (1996). "Comparison of rheumatoid factors of rheumatoid arthritis patients, of individuals with mycobacterial infections and of normal controls: evidence for maturation in the absence of an autoimmune response." *Eur. J. Immunol.* **26**: 2480-2486.

Driver, C. H. S. (2006). Towards TB serodiagnosis by Flow Cytometric Analysis of mycolic acid exchange among liposomes. Hons. Report, Department of Biochemistry, Faculty of Biological and Agricultural Sciences, University of Pretoria.

Dube, M. and Fenton, M. (2003). "Lipid abnormalities." *Clin. Infect. Dis.* **36**: S79-83.

Dubnau, E., Chan, J., Raynoud, C., Mohan, V. P., Lanèelle, M. -A., Yu, K., Quemard, A., Smith, I. and Daffé, M. (2000). "Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice." *Mol. Microbiol*. **36**: 630-637.

Enomoto, Y., Sugita, M., Matsunaga, I., Naka, T., Sato, A., Kawashima, T., Shimizu, K., Takahashi, H., Norose, Y. and Yano, I. (2005). "Temperature-dependent biosynthesis of glucose monomycolate and its recognition by CD1-restricted T cells." *Biochem. Biophys. Res. Comm.* **337**: 452-456.

Espitia, C. and Mancilla, R. (1989). "Identification, isolation and partial characterization of *Mycobacterium tuberculosis* glycoprotein antigens." *Clin. Exp. Immunol.* **77**: 378-383.

Fätkenheuer, G., Taelman, H., Lepage, P., Schwenk, A. and Wenzel, R. (1999). "The return of tuberculosis." *Diagn. Microbiol. Infect. Dis.* **34**: 139-146.

Fifis, T., Costopoulos, C., Radford, A. J., Bacic, A. and Wood, P. R. (1991). "Purification and characterization of major antigens from a *Mycobacterium bovis* culture filtrate." *Infect. Immun.* **59**: 800-807.

FitzGerald, J. M., Grzybowski, S. and Allen, E. A. (1991). "The impact of human immunodeficiency virus infection on tuberculosis and its control." *Chest* **100**: 191-200.

Fujita, Y., Doi, T., Sato, K. and Yano, I. (2005). "Diverse humoral immune responses and changes in IgG antibody levels against mycobacterial lipid antigens in active tuberculosis." *Microbiology* **151**: 2065-2074.

Fujiwara, N., Pan, J., Enomoto, K., Terano, Y., Honda, T. and Yano, I. (1999). "Production and partial characterization of anti-cord factor (trehalose-6,6P-dimycolate) IgG antibody in rabbits recognizing mycolic acid subclasses of *Mycobacterium tuberculosis* or *Mycobacterium avium*" *FEMS Immunol. Med. Microbiol.* **24**: 141-149.

Ganju, L., Batra, H. V., Talwar, G. P. and Mukherjee, R. (1991). "A rapid latex agglutination test for detection of antibodies in tuberculosis and Hansen's disease." *J. Immunoassay* **12**: 579-595.

Garza-Gonzalez, E., Guerrero-Olazaran, M., Tijerina-Menchaca, R. and Viader-Salvado, J. M. (1998). "Identification of mycobacteria by mycolic acid pattern." *Arch. Med. Res.* **29**: 303-306.

Gatfield, J. and Pieters, J. (2000). "Essential role for cholesterol in entry of mycobacteria into macrophages." *Science* **288**: 1647-1650.

Geluk, A., van Meijgaarden, K. E., Franken, K. L. M. C., Wieles, B., Arend, S. M., Faber, W. R., Naafs, B. and Ottenhoff, T. H. M. (2004). "Immunological cross-reactivity of the *Mycobacterium leprae* CFP-10 with its homologue in *Mycobacterium tuberculosis*." *Scand. J. Immunol.* **59**: 66-70.

Gennaro, M. L. (2000). "Immunologic diagnosis of tuberculosis." *Clin. Infect. Dis.* **30**: S243-S246.

Ghetie, V., Nilsson, K. and Sjöquist, J. (1974). "Detection and quantitation of IgG on the surface of human lymphoid cells by rosette formation with protein A-coated sheep red blood cells." *Eur. J. Immunol.* **4**: 500-505.

Goding, J. W. (1978). "Use of staphylococcal protein A as an immunological reagent." *J. Immunol. Meth.* **20**: 241-253.

Goodrum, M. A., Siko D.G.R, Niehues T., Eichelbauer D. and Verschoor J.A. (2001). "Mycolic acids from *Mycobacterium tuberculosis*: purification by countercurrent distribution and T-cell stimulation." *Microbios.* **106**: 55-67.

Goren, M. B. (1972). "Mycobacterial lipids: Selected topics." *Bacteriol. Rev.* **36**: 33-64.

Gray, J. W. (2004). "Childhood tuberculosis and its early diagnosis." *Clin. Biochem.* **37**: 450-455.

Grunfeld, C., Kotler, D. P., Hamadeh, R., Tierney, A., Wang, J. and Pierson, R. N. (1989). "Hypertriglyceridemia in the acquired immunodeficiency syndrome." *Am. J. Med.* **86**: 27-31.

Haberland, M. E. and Reynolds, J. A. (1973). "Self-association of cholesterol in aqueous solution." *Proc. Natl. Acad. Sci. USA* **70**: 2313-2316.

Hamasur, B., Bruchfeld, J., Haile, M., Pawlowski, A., Bjorvatn, B., Källenius, G. and Svenson, S. B. (2001). "Rapid diagnosis of tuberculosis by detection of lipoarabinomannan in urine." *J. Microbiol. Meth.* **45**: 41-52.

Harlow, E. and Lane, D. (1998). Using antibodies: A laboratory manual: Portable protocol no. 1. Cold Spring Harbour Laboratory Press, NY.

Helbert, M., Robinson, D., Buchanan, D., Hellyer, T. and McCarthy, M. (1990). "Mycobacterial infection in patients infected with the human immunodeficiency virus." *Thorax* **45**: 45-48.

Houghton, R. L., Lodes, M. J., Dillon, D. C., Reynolds, L. D., Day, C. H., McNeill, P.D., Hendrickson, R. C., Skeiky, Y. A. W., Sampaio, D. P., Badaro, R., Lyashchenko, K. P. and Reed, S. G. (2002). "Use of multi-epitope polyproteins in serodiagnosis of active tuberculosis." *Clin. Diagn. Lab. Immunol.* **9**: 883-891.

Hunter, S. W., Gaylord, H. and Brennan, P. J. (1986). "Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli." *J. Biol. Chem.* **261**: 12345-12351.

Jelinek, T., Wastlhuber, J., Pröll, S., Schattenkirchner, M. and Löscher, T. (2000). "Influence of Rheumatoid Factor on the specificity of a rapid immunochromatographic test for diagnosing Dengue infection." *Eur. J. Clin. Microbiol. Infect. Dis.* **19**: 555-556.

Julián, E., Matas, L., Ausina, V. and Luquin, M. (1997). "Detection of lipoarabinomannan antibodies in patients with newly acquired tuberculosis and patients with relapse tuberculosis." *J. Clin. Microbiol.* **35**: 2663-2664.

Julián, E., Matas, L., Pérez, A., Alcaide, J., Lanèelle, M. -A. and Luquin, M. (2002). "Serodiagnosis of tuberculosis: Comparison of immunoglobulin A (IgA) response to sulfolipid I with IgG and IgM responses to 2,3-diacyltrehalose, 2,3,6-triacyltrehalose and cord factor antigens." *J. Clin. Microbiol.* **40**: 3782-3788.

Kaplan, A. V. and Quimby, F. W. (1983). "A radiolabelled staphylococcal protein A assay for detection of anti-erythrocyte IgG in warm agglutinin autoimmune haemolytic anemia of dogs and man." *Vet. Immunol. Immunopath.* **4**: 307-317.

Karakousis, P. C., Bishai, W. R. and Dorman, S. E. (2004). "*Mycobacterium tuberculosis* cell envelope lipids and the host immune response." *Cell. Microbiol.* **6**: 105-116.

Kato, M. (1972). "Antibody formation to trehalose-6, 6'-dimycolate (cord factor) of *Mycobacterium tuberculosis*." *Infect. Immun.* **5**: 203-212.

Kaufmann, S. H. E. (2004). "New issues in tuberculosis." *Ann. Rheum. Dis.* **63**: ii50-ii56.

Khomenko, A. G., Bayensky, A. V., Chernousova, L. N., Kulikovskaya, N. V., Demianenko, N. V. and Litvinov, V. I. (1996). "Serodiagnosis of tuberculosis: Detection of mycobacterial antibodies and antigens." *Tuber. Lung Dis.* **77**: 510-515.

Klewitz, T., Gessler, F., Beer, H., Pflanz, K. and Scheper, T. (2005). "Immunochromatographic assay for determination of botulinum neurotoxin type D." *Sensors and Actuators* 113: 1-8.

Kulshrestha, A., Gupta, A., Verma, N., Sharma, S. K., Tyagi, A. K. and Choudhary, V. K. (2005). "Expression and purification of recombinant antigens of *Mycobacterium tuberculosis* for application in serodiagnosis." *Prot. Exp. Purifi.* **44**: 75-85.

Lara, M., Servín-González, L., Singh, M., Moreno, C. and Cohen, I. (2004). "Expression, secretion and glycosylation of the 45- and 47-kDa glycoprotein of *Mycobacterium tuberculosis* in *Streptomyces lividans*." *Appl. Environ. Microbiol.* **70**: 679-685.

Laval, F., Lanèelle, M. -A., Deon, C., Monsaraat, B. and Daffé, M. (2001). "Accurate molecular mass determination by MALDI-TOF mass spectrometry." *Anal. Chem.* **73**: 4537-4544.

Lee, J. -S., Son, J. W., Jung, S. -B., Kwon, Y. -M., Yang, C. -S., Oh, J. -H., Song, C. -H., Kim, H. -J., Park, J. -K., Paik, T. -H. and Jo, E. -K. (2006). "*Ex vivo* responses for interferon-gamma and pro-inflammatory cytokine secretion to low-molecular-weight antigen MTB12 of *Mycobacterium tuberculosis* during human tuberculosis." *Scand. J. Immunol.* **64**: 145-154.

Lee, R. E., Brennan, P. J. and Besra, G. S. (1996). "*Mycobacterium tuberculosis* cell envelope." *Curr. Topics Microbiol. Immunol.* **215**: 1-27.

Liu, J., Barry III, C. E., Besra, G. S. and Nikaido, H. (1996). "Mycolic acid structure determines the fluidity of the mycobacterial cell wall." *J. Biol. Chem.* **271**: 29545-29551.

Lyashchenko, K., Colangeli, R., Houde, M., Al Jahdali, H., Menzies, D. and Gennaro, M. L. (1998). "Heterogeneous antibody responses in tuberculosis." *Infect. Immun.* **66**: 3936-3940.

McConkey, S. J., Youssef, F. G., Azem, E., Frenck, R. W. and Weil, G. J. (2002). "Evaluation of a rapid-format antibody test and the tuberculin skin test for diagnosis of tuberculosis in two contrasting endemic settings." *Int. J. Tuberc. Lung Dis.* **6**: 246-252.

McGill, A., Marsh, R., Craft, A. W. and Toms, G. L. (2005). "Analysis of the binding of monoclonal and polyclonal antibodies to the glycoproteins of antigenic variants of human respiratory syncytial virus by surface plasmon resonance." *J. Immunol. Meth.* **297**: 143-152.

Minnikin, D. E., Minnikin, S. M., Parlett, J. H., Goodfellow, M. and Magnusson, M. (1984). " Mycolic acid patterns of some species of *Mycobacterium*." *Arch. Microbiol.* **139**: 225-231.

Mukherjee, P. S., DeSilva, K. H. and Karnes, H. T. (1995). "5-bromomethyl fluorescein (5-BMF) for derivatization of carboxyl containing analytes for use with laser-induced fluorescence detection." *Pharma. Res.* **12**: 930-936.

Murray, J. F. (1990). "Cursed duet: HIV infection and tuberculosis." *Respiration* **57**: 210-220.

Noordhoek, G. T., Kolk, A. H. J., Bjune, G., Catty, D., Dale, J. W., Fine, P. E. M., Godfrey-Faussett, P., Cho, S. -N., Shinnick, T., Svenson, S. B., Wilson, S. and van Embden, J. D. A. (1994). "Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: A blind comparison study among seven laboratories." *J. Clin. Microbiol.* **32**: 277-284.

Otsyula, M. G., Yee, J. L., Suleman, M. A., Marz, P. A. and Jennings, M. B. (1996). "Immunoassay for detection of antibodies to simian immunodeficiency virus and human immunodeficiency virus in serum." *Lab. Animal Sci.* **46**: 198-201.

Pan, J., Fujiwara, N., Oka, S., Maekura, R., Ogura, T. and Yano, I. (1999). "Anti-cord factor (trehalose 6,6'-dimycolate) IgG antibody in tuberculosis patients recognizes mycolic acid subclasses." *Microbiol. Immunol.* **43**: 863-869.

Pape, J. W., Jean, S. S., Ho, J. L., Hafner, A. and Johnson Jr., W. D. (1993). "Effect of isoniazid prophylaxis on incidence of active tuberculosis and progression of HIV infection." *The Lancet* **342**: 268-272.

Pereira, J. M., Madureira, A. J., Vieira, A. and Ramos, I. (2005). "Abdominal tuberculosis: Imaging features." *Eur. J. Radiol.* **55**: 1-8.

Pickering, L. K. (2000). Red Book: Report of the Committee on Infectious Diseases 25th Edition. Elk Grove Village, IL.

Piehler, J. (2005). "New methodologies for measuring protein interactions *in vivo* and *in vitro*." *Curr. Opin. Struct. Biol.* **15**: 4-14.

Raja, A., Narayanan, P. R., Mathew, R. and Prabhakar, R. (1995). "Characterization of mycobacterial antigens and antibodies in circulating immune complexes from pulmonary tuberculosis." *J. Lab. Clin. Med.* **125**: 581-587.

Rapoport, B. L., Morrison, R. C. A., Sher, R. and Dos Santos, L. (1990). "A study of auto-antibodies in chronic mycobacterial infections." *Int. J. Lep.* **58**: 518-525.

Rawlinson, W. D. and Basten, A. (1989). "Cross-reactivity of *Mycobacterium leprae* and *M. tuberculosis* and the implications for assessment of *in vitro* T cell function in leprosy patients." *Trans. Royal Soc. Trop. Med. Hyg.* **83**: 557-559.

Rodrigo, G., Källenius, G., Hoffmann, E. and Svenson, S. B. (1992). "Diagnosis of mycobacterial infections by PCR and restriction enzyme digestion." *Lett. Appl. Microbiol.* **15**: 41-44.

Sada, E., Aguilar, D., Torres, M., Herrera, T. (1992). "Detection of lipoarabinomannan as a diagnostic test for tuberculosis." *J. Clin. Microbiol*. **30**: 2415-2418.

Sada, E., Brennan, P. J., Herrera, T., Torres, M. (1990). "Evaluation of lipoarabinomannan for the serological diagnosis of tuberculosis." *J. Clin. Microbiol.* **28**: 2587-2590.

Samuel, A. M., Ashtekar, M. D. and Ganatra, R. D. (1984). "Significance of circulating immune complexes in pulmonary tuberculosis." *Clin. Exp. Immunol.* **58**: 317-324.

Schleicher, G. K., Feldman, C., Vermaak, Y. and Verschoor, J. A. (2002). "Prevalence of anti-mycolic acid antibodies in patients with pulmonary tuberculosis co-infected with HIV." *Clin. Chem. Lab. Med.* **40**: 882-887.

Shafer, R. W. and Edlin, B. R. (1996). "Tuberculosis in patients infected with human immunodeficiency virus: perspective on the past decade." *Clin. Infect. Dis.* **22**: 683-704.

Shor-Posner, G., Basit, A., Lu, Y., Cabrejos, C., Chang, J., Fletcher, M., Mantero-Atienza, E. and Baum, M. K. (1993). "Hypocholesterolemia is associated with immune dysfunction in early human immunodeficiency virus-1 infection." *Am. J. Med.* **94**: 515-519.

Siko D.G.R. (2002). Mycobacterial mycolic acids as immunoregulatory lipid antigens in the resistance to tuberculosis. Ph.D. Thesis, Department of Biochemistry, Faculty of Biological and Agricultural Sciences, University of Pretoria.

Simonney, N., Molina, J. M., Molimard, M., Oksenhendler, E. and Lagrange, P. H. (1997). "Circulating immune complexes in human tuberculosis sera: demonstration of specific antibodies against *Mycobacterium tuberculosis* glycolipid (DAT, PGLTb1, LOS) antigens in isolated circulating immune complexes." *Eur. J. Clin. Invest.* **27**: 128-134.

Sjöquist, J., Movitz, J. and Johansson, I. -B. (1972). "Localization of protein A in the bacteria." *Eur. J. Biochem.* **30**: 190-194.

Sugahara, M., Uragami, M., Yan, X. and Regen, S. L. (2001). "The structural role of cholesterol in biological membranes." *J. Am. Chem. Soc.* **123**: 7939-7940.

Tasaka, H., Shigeto, E., Matsuo, K., Yamaguchi, R., Haga, S., Yamazaki, A., Yamazaki, T., Nagai, S. and Nakamura, R. M. (1995). "Secretion of MPB64 antigen by a recombinant clone of *Mycobacterium smegmatis*: characterization and application for the diagnosis of tuberculosis." *Scand. J. Immunol.* **42**: 487-492.

Tessema, T. A., Hamasur B., Bjune, G., Svenson, S. and Bjorvatn, B. (2001). "Diagnostic evaluation of urinary lipoarabinomannan at an Ethiopian tuberculosis centre." *Scand. J. Infect. Dis.* **33**: 279-284.

Tessema, T. A., Bjune, G., Assefa, G., Svenson, S., Hamasur, B. and Bjorvatn, B. (2002b). "Clinical and radiological features in relation to urinary excretion of lipoarabinomannan in Ethiopian tuberculosis patients." *Scand. J. Infect. Dis.* **34**: 167-171.

Tessema, T. A., Bjune, G., Hamasur, B., Svenson, S., Syre, H. and Bjorvatn, B. (2002a). "Circulating antibodies to lipoarabinomannan in relation to sputum microscopy, clinical features and urinary anti-lipoarabinomannan detection in pulmonary tuberculosis." *Scand. J. Infect. Dis.* **34**: 97-103.

Thanyani T.S. (2003). A novel application of affinity biosensor technology to detect antibodies to mycolic acid in tuberculosis patients. MSc. Dissertation, Department of Biochemistry, Faculty of Biological and Agricultural Sciences, University of Pretoria.

Tiwari, R. P., Tiwari, D., Garg, S. K., Chandra, R. and Bisen, P. S. (2005). "Glycolipids of *Mycobacterium tuberculosis* strain H37Rv are potential serological markers for diagnosis of active tuberculosis." *Clin. Diagn. Lab. Immunol.* **12**: 465-473.

Tsao, K. -C., Chen, G. -W., Huang, C. -G., Huang, Y. -L., Lin, J. -Y., Keng, M. -C., Sun, C. -F. and Shih, S. -R. (2005). "False positive antibody results against human T-cell lymphotropic virus in patients with severe acute respiratory syndrome." *J. Med. Virol.* **77**: 1-6.

Ulrichs, T., Munk, M. E., Mollenkopf, H., Behr-Perst, S., Colangeli, R., Gennaro, M. L. and Kaufmann, S. H. E. (1998). "Differential T cell responses to *Mycobacterium tuberculosis* ESAT6 in tuberculosis patients and healthy donors." *Eur. J. Immunol.* **28**: 3949-3958.

Van Regenmortel, M. H., Altschuh, D., Chatellier, J. Christensen, L., Rauffer-Bruyere, N., Richalet-Secordel, P., Witz, J. and Zeder-Lutz, G. (1999). "Measurement of antigen-antibody interactions with biosensors." *J. Mol. Recognit.* **11**: 163-167.

Vermaak, Y. (2002). Properties of anti-mycolic acid antibodies in human tuberculosis patients. MSc. Dissertation, Department of Biochemistry. Faculty of Biological and Agricultural Sciences, University of Pretoria.

Vervenne, R. A. W., Jones, S. L., van Soolingen, D., van der Laan, T., Andersen, P., Heidt, P. J., Thomas, A. W. and Langermans, J. A. M. (2004). "TB diagnosis in non-human primates: comparison of two interferon-γ assays and the skin test for identification of *Mycobacterium tuberculosis* infection." *Vet. Immunol. Immunopath.* **100**: 61-71.

Villeneuve, M., Kawai, M., Kanashima, H., Watanabe, M. and Minnikin, D. E. (2005). "Temperature dependence of the Langmuir monolayer packing of mycolic acids from *Mycobacterium tuberculosis*." *Biochim. Biophys. Acta* **1715**: 71-80.

Wang, S., Zhang, C. and Zhang, Y. (2005). "Development of a flow-through enzyme-linked immunosorbent assay and a dipstick assay for the rapid detection of the insecticide carbaryl." *Analyt. Chim. Acta* **535**: 219-225.

Watanabe, M., Aoyagi, Y., Ridell, M. and Minnikin, D. E. (2001). "Separation and characterization of individual mycolic acids in representative mycobacteria." *Microbiology* **147**: 1825-1837.

Watterson, S. A. and Drobniewski, F. A. (2000). "Modern laboratory diagnosis of mycobacterial infections." *J. Clin. Pathol.* **53**: 727-732.

Wilson, K. and Walker, J. (1994). Practical Biochemistry 4th Edition: Principles and techniques. Cambridge University Press, Great Britain.

Xu, S., Cooper A, Sturgill-Koszycki, S., van Heyningen, T., Chatterjee, D., Orme, I., Allen, P., Russell, D. G. (1994). "Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages." *J. Immunol.* **153**: 2568-2578.

Yuan, Y., Zhu, Y. Q., Crane, D. D. and Barry III, C. E. Y. (1998). "The effect of oxygenated mycolic acid composition on cell wall function and macrophage growth in *Mycobacterium tuberculosis*." *Mol. Microbiol.* **29**: 1449-1458.

Zhang, J., Jing, B. and Regen, S. L.B. (2005a). "Transbilayer complementarity of phospholipids: Proof of principle." *Langmuir* **21**: 8983-8986.

Zhang, J., Jing, B., Tokutake, N. and Regen, S. L.B. (2005b). "Transbilayer complementarity of phospholipids in cholesterol-rich membranes." *Biochemistry* **44**: 3598-3603.