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Standardization of natural mycolic acid antigen composition and production for use in biomarker antibody detection to diagnose active tuberculosis

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ABSTRACT

Mycobacterium tuberculosis, the causative agent of tuberculosis, is characterized by the abundance of species specific, antigenic cell wall lipids called mycolic acids. These wax-like molecules all share an identical, amphiphilic mycolic motif, but have different functional groups in a long hydrophobic hydrocarbon mero-chain that divide them into three main classes: alpha-, keto- and methoxy-mycolic acids. Whereas alpha-mycolic acids constitutively maintain an abundance of around 50%, the ratio of methoxy- to keto-mycolic acid types may vary depending on, among other things, the growth stage of *M. tuberculosis*. In human patients, antibodies to mycolic acids have shown potential as diagnostic serum biomarkers for active TB. Variations in mycolic acid composition affect the antigenic properties and can potentially compromise the precision of detection of anti-mycolic acids antibodies in patient sera to natural mixtures. We demonstrate this here with combinations of synthetic mycolic acid antigens, tested against TB patient and control sera. Combinations of methoxy- and α-mycolic acids are more antigenic than combinations of keto- and α-mycolic acids, showing the former to give a more sensitive test for TB biomarker antibodies. Natural mixtures of mycolic acids isolated from mature cultures of *M. tuberculosis* H37Rv give the same sensitivity as that with synthetic methoxy- and α-mycolic acids in combination, in a surface plasmon resonance inhibition biosensor test. To ensure that the antigenic activity of isolates of natural mycolic acids is reproducible, we cultured *M. tuberculosis* H37Rv on Middlebrook 7H10 solid agar plates to stationary growth phase in a standardized, optimal way. The proportions of mycolic acid classes in various batches of the isolates prepared from these cultures were compared to a commercially available natural mycolic acid isolate. LC-MS/MS and NMR data for quantitation of mycolic acids class compositions show that the variation in batches is small, suggesting that the quality of the results for anti-mycolic acid antibody detection in the TB patients should not be affected by different batches of natural mycolic acid antigens if prepared in a standard way.

1. Introduction

Mycolic acids (MA) are high molecular weight (C60-C90) α-alkyl-β-hydroxy fatty acids produced by all mycobacterial species and closely related genera (Minnikin, 1982). MAs are the major components in the heterogeneous mixture of lipids that make up about 40–60% of the mycobacterial cell dry weight (Brennan and Nikaido, 1995). They exist in the cell wall either linked to the arabinogalactan layer by phosphodiester bonds, or bound to trehalose as trehalose monomycolate (TMM) and trehalose dimycolate (TDM) (Barry et al., 1998). All the members of the *Mycobacterium tuberculosis* complex (*Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum* and *Mycobacterium microti*) produce three major classes of MA, alpha-, keto- and methoxy-MA. These three classes are differentiated by the presence of different chemical functional groups on the main hydrophobic chain of MA, called the mero-chain. The most abundant class of MA, alpha-MA, contains two *cis* cyclopropane rings and has no oxygenated functional groups on its mero-chain, compared to keto- and methoxy-MA, which contain respectively oxygenated distal keto and methoxy functional groups. The proximal cyclopropane rings of oxygenated MA can either be *cis* or *trans*. While alpha- and keto-MA are present in various

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mycobacterial species irrespective of growth rate or pathogenic status, methoxy-MA appears to be restricted in distribution, occurring mainly in slow-growing pathogenic species (Barry et al., 1998). Non-tuberculous mycobacterial species that do not contain methoxy-MA but are capable of causing disease in immunocompromised individuals (e.g. HIV-infected) include the slow-growing *Mycobacterium avium* (*M. avium*) and the rapidly-growing *Mycobacterium fortuitum* (*M. fortuitum*). *M. avium* MA comprise alpha- and keto-MA and a so-called wax ester-MA, in which an alpha-methyl ester distal functional group occurs, *M. fortuitum* contains epoxy-MA as the only MA with an oxygenated functional group. During saponification and extraction, the epoxy groups are converted into distal hydroxy-groups (Dobson et al., 1985).

MAs play a role in the maintenance of the structural integrity (Barkan et al., 2009) of mycobacteria and they also have various MA-class specific biological functions (Sambandan et al., 2013; Vander Beken et al., 2011; Dao et al., 2008; Dubnau et al., 2002; Glickman et al., 2000; Yuan et al., 1998). Since the achievement of stereo-controlled chemical synthesis of almost any class of MA, structure-function relationships were determined that demonstrated that not only the type of functional group, but even the stereochemistry of the mero-chain are critical determining factors in the antigenicity of MA as well as their cellular steering functions in innate immunity (reviewed by Verschoor et al., 2012).It has become apparent in recent years that MA is secreted in free-form during the course of mycobacterial infection (Cantrell et al., 2013; Ojha et al., 2010; Ojha et al., 2008), which may explain its immunogenicity by eliciting antibodies. The potential use of MA to detect biomarker antibodies in diagnosis of mycobacterial infections has been explored by several researchers (Chan et al., 2013; Shui et al., 2012; Elhassan et al., 2011; Thanyani et al., 2008; Schleicher et al., 2002; Pan et al., 1999). This derives from their unique properties such as their high abundance in the cell wall, species-specific structure, chemical stability and the ability to induce an antibody response.

Although MA are heavily implicated in TB pathogenesis and would make an ideal diagnostic antigen, a serodiagnostic assay that would detect anti-MA biomarker antibodies has not yet entered the diagnostics market. This is in part due to the limited understanding of the nature of the antigen-antibody interaction that occurs with MA or lipids in general. Understanding the MA-anti-MA antibody interaction is also crucial for standardization of the immunoassay based on MA antigen. Immunoassay standardization is challenging, but important to facilitate clinical interpretation and comparison of results from various studies (Stenman, 2001). Chan et al. (2013) and Beukes et al. (2010) reported that methoxy-MA is the most antigenic class of MA and antibodies raised against MA antigen are primarily specific for methoxy-MA. The use of a single defined antigen such as synthetic methoxy-MA in TB serodiagnostic device is very attractive as it could solve problems associated with standardization of immunoassays. However, Beukes et al. (2010) reported that no single synthetic MA could significantly and reproducibly distinguish the pooled serum of TB positive patients from that of TB negative patients better than the natural mixture of MA. This suggests that the natural mixture of MA may well be the antigen most suitable for use in TB serodiagnostics aimed at detecting MA biomarker antibodies. Nevertheless, the molecular heterogeneous nature of the natural mixture of MA antigen presents a challenge in the standardization of an MA-based immunoassay. The ratio of the three major classes of MA that makes up the natural mixture of MA antigen is known to vary with growth conditions (Watanabe et al., 2001; Yuan et al., 1998; Davidson et al., 1982). Yuan et al. (1998) reported that enhanced production of keto-MA accompanied growth of *M. tuberculosis* in macrophages and that stationary growth phase organisms produce less keto-MA than exponentially growing organisms. Alpha-MA on the other hand maintains itself at around 50% of the total MA under all conditions. The common theme in these studies is that the production of methoxy- and keto-MA is quantitatively variable under different physiologically conditions. However, for the application of natural mixtures of MA in medical diagnosis, a consistent composition is an important requirement, favouring an abundance of methoxy-MA as the most antigenic MA class (Beukes et al., 2010; Chan et al., 2013).

Currently the detection of biomarker anti-MA antibodies for TB diagnosis is performed using two methods; a standard indirect enzyme-linked immunosorbent assay (ELISA) and the surface plasmon resonance (SPR) version of the antibody inhibition biosensor test. Schleicher et al. (2002) showed that although ELISA is not accurate enough in detecting anti-MA antibodies in TB positive patient sera, it is at least not affected by co-infection with HIV;(i.e. it was not affected by low CD4 counts of a patient), which is a major challenge with commercially available TB serodiagnostic tests (WHO, 2008). The detection of anti-MA antibodies is achieved more accurately with evanescent field biosensors (Thanyani et al., 2008). The antibody inhibition biosensor test is called MA antibody real-time inhibition (abbreviated MARTI) test and was previously described in detail by Lemmer et al. (2009). Briefly, diluted serum antibodies are allowed to bind to sensor surface immobilized MA antigens. Then, at a higher concentration of serum sample, specific antibody binding to the sensor surface is compared between serum pre-incubated with either MA antigen in liposomes or with just the empty liposomes. A difference in specific antibody binding signal then signifies the presence of specific anti-MA antibodies in the serum, for which a good correlation was found to active TB in the patients (Thanyani et al., 2008). The MARTI-test has not yet undergone a full-scale clinical trial. To prepare for this, the risk of poor reproducibility posed by the heterogeneous nature of the natural mixture of MA antigen was investigated. This study demonstrates the importance of MA antigen composition in the MARTI-test for TB diagnosis using anti-MA antibody detection with stereo-controlled chemically synthetic MAs. It demonstrates on the one hand how standardized natural MA mixture antigens can be reliably produced and prepared to perform optimally in immunoassay, while on the other hand how a chemically synthetic methoxy MA may be applied as a standard antigen preparation to match the accuracy of the immunoassay that can be achieved with extracts of natural MA from *M. tuberculosis*.

2. Materials and methods

2.1. Bacterial strain and preparation of the inoculum

M. tuberculosis laboratory strain H37Rv, ATCC 27294 (Maryland, USA) was used in this study. *M. tuberculosis* cells (subculture 2) cultivated for three weeks were harvested and homogenized in sterile PBS/0.01% Tween 80, pH 7.4. The cell density of the suspension corresponded to a McFarland turbidity standard number 8 (2.4×10^9 cells/mL) to obtain approximately 2.4×10^8 cells/plate as a starting inoculum concentration.

2.2. Growth conditions

M. tuberculosis was cultivated, under four different culture conditions namely: (1) mechanically stirred Middlebrook 7H9 broth medium supplemented with albumin-dextrose-catalase (ADC), (2) stationery Middlebrook 7H9 broth medium supplemented with ADC, (3) Middlebrook 7H10 agar plates supplemented with oleic acid-albumin-dextrose-catalase (OADC) and (4) LJ slants prepared according to the detailed description in the Laboratory Manual of Tuberculosis Methods, Tuberculosis Research Institute of the SA Medical Research Council (Kleeberg et al., 1980). Stirred and stationary Middlebrook 7H9 broth cultures were inoculated with 300 μL of inoculum per 300 mL of medium in conical flasks. The inoculation of both agar plates and LJ slants was performed by spreading 100 μL of the inoculum onto the surface of the agar plates or LJ slants. All the cultures were incubated at 37 °C.

2.3. Harvesting of cultures

The cultures were harvested at seven different time points; days 6, 9, 12, 15, 18, 21 and 24 post inoculations. From three agar plates/LJ slants

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bacterial cells were scraped into a tube containing 0.3 mL saline and vortexed to obtain a homogenous bacterial suspension before they were transferred into Schott bottles and made up to a total volume of 300 mL in saline. Cultures grown as mechanically stirred or stationary cultures in broth were recovered by centrifugation of the cell suspension at 1500 ×*g* for 20 min, using a Heraeus Christ Labofuge. Bacterial cells were resuspended in saline and centrifuged twice to remove any remaining soluble proteins. The cell pellets were resuspended in a small volume of saline and vortexed to form a homogenous suspension, before being transferred into Schott bottles and made up to a total volume of 300 mL with saline. The 300 mL saline cell suspensions were used for the determination of total and viable cell counts and optical density measurements at a wavelength of 486 nm for each cell suspension. Bacteria were inactivated by autoclaving at 121 °C for 60 min.

2.4. Determination of the total number of cells in the harvested cultures

Total cell counts were obtained by counting serial dilutions made in PBS/0.01% Tween 80 using a Neubauer counting chamber and a 400× magnification with a Nikon Labphot 2 light microscope and optical density (OD) measurements at a wavelength of 486 nm using a Novaspec II spectrophotometer. A calibration study was performed first to demonstrate linear range between OD measurements and number of cells using McFarland standard 4 as a reference. McFarland standards were prepared as described by Roberts et al. (1991a, 1991b). To obtain the total number of cells (TNC) the following formula was used:

$$\mathrm{TNC} = \frac{12 \times 10^8 \text{ cells/mL} \times \text{OD reading} \times \text{dilution factor} \times \text{cell suspension volume (mL)}}{\text{OD of the McFarland standard 4}}.$$

Viable cell counts were determined as colony forming units by preparing serial cell dilutions in PBS/0.01% Tween 80. The serial dilutions were inoculated by spreading 100 μL of cells onto the surface of agar plates. Total numbers of bacteria and viable counts were determined for the four different culture conditions investigated.

2.5. Extraction of mycolic acids

Saponification of the bacterial cells was done in autoclavable 2.5 L Schott bottles. To release mycolic acids from mycobacterial cell walls, homogeneous bacterial cell pellets were re-suspended in 800 mL of (25% w/v) potassium hydroxide (KOH) in methanol-water (1:1, v/v). The cell suspensions were saponified in a waterbath at 70 °C, for 16 h. After cooling to room temperature, 600 mL of 32% concentrated HCl diluted in water (1/1, v/v) was added to the cell suspension. The acidified cell suspension was vigorously shaken for 5 min and allowed to cool to room temperature before chloroform was added. Chloroform (800 mL) was added to the cell suspension. The crude cell suspension was then divided into two 2.5 L tightly secured Schott bottles. The suspensions were vigorously shaken and pressure build-up released every 10 s until no further gas was formed. Using a separating funnel the lower phase solution containing crude mycolic acids extract was collected into a suitable glass container. The lower phase solution was concentrated on a Buchi rotary evaporator under vacuum at 85 °C in a pre-weighed round bottom flask. Residual water was removed by adding acetone and completing the drying process. Crude mycolic acids extract thus obtained was stored at 4 °C till further use. The crude mycolic acids extract was purified using the countercurrent distribution method previously described by Goodrum et al. (2001).

2.6. Determination of the total amount of mycolic acids synthesized at different time points

Roberts et al. (1991a, 1991b) determined the relative number of cells present in the corresponding McFarland standard using a nephelometer. The optical density for each corresponding McFarland standard can be applied to calculate the total number of cells present in bacterial suspension, as long as there is a linear relationship between the bacilli concentration and the OD measurement. In this study, OD measurements were carried out simultaneously with the OD of a single set of McFarland standard 4 (as a reference) prepared as described by Roberts et al. (1991a, 1991b). The average OD value of each standard for the 7 different time points was calculated and a single standard curve was drawn. The standard curve was subjected to linear regression analysis and the corrected curve constructed from the data. The following formula was used to calculate the total number of cells (TNC):

$$\mathrm{TNC} = \frac{\left[12 \times 10^8 \text{ cells/mL} \times \text{OD measurement} \times \text{dilution factor} \times \text{volume of bacterial suspension}\right]}{\text{OD of the McFarland standard 4}}.$$

The bacterial cell pellets were re-suspended in (25% w/v) potassium hydroxide (KOH) dissolved in methanol-water (1:1, v/v) to correspond to a McFarland standard 4. The optical density of each sample suspension as well as a set of McFarland standards were read at a wavelength of 486 nm. Of each cell suspension 1 mL was pipetted into a vial containing internal standard for quantification of the amount of mycolic acids present per sample by HPLC (Goodrum et al., 2001). An additional 1 mL of (25% w/v) potassium hydroxide (KOH) dissolved in methanol-water (1:1, v/v) was added per vial and the derivatisation was carried out.

2.7. Mycolic acid preparation and derivatization

HPLC quantitative analysis of mycolic acids was performed by using 5 μg of an internal standard C-100 *p*-bromophenylacyl ester (Ribi Immuno Research, Inc.). Purified mycolic acids (~0.5 mg) were aliquoted into vials that already contained 5 μg of internal standard. Reagent A (2 mL) was added to the samples, the vials were capped tightly and heated for 10 min at 85 °C. The samples were then cooled to room temperature, acidified by adding 1.5 mL of 6 M HCl and vortex mixed. The sample was extracted three times with 1 mL of CH_2Cl_2 and the CH_2Cl_2 extracts evaporated to dryness under a stream of nitrogen gas. 100 μL of 2% $KHCO_3$ dissolved in methanol-water (1:1) was added to samples and the samples were evaporated to dryness under stream of nitrogen gas. To each vial, 1 mL of CH_2Cl_2 and 100 μL of parabromophenacyl bromide (Pierce Chemical Co., Rockford III) were added successively. The reaction mixtures were tightly closed in vials, vortex mixed for 30 s, heated for 25 min at 85 °C and allowed to cool to room temperatures. The samples were acidified by adding 1 mL of a 12 M HCl-methanol-water mixture (1:2:1) and vortex mixed for 30 s. The phases were allowed to separate completely, derivatised fatty acid were collected and evaporated to dryness under a stream of nitrogen gas. The samples were stored at 4 °C until used.

2.8. HPLC quantification of mycolic acids

The mycolic acid *p*-bromophenacyl esters (5 μL per sample) were applied to 4 μm, C18 *reverse-phase column* (*Novapak*; *3.9 mm by 150 mm*; *60 Å*; *Waters*) *equilibrated in 98%* methanol-2% dichloromethane. After injection of the sample, the solvent gradient was changed linearly to 35% methanol-65% dichloromethane over a period of 8 min, then changed back to 98% methanol-2% dichloromethane over 1.5 min. The separated esters were detected by UV absorption at 260 nm (AL-4500 UV detector). Mycolic acids yield was calculated by using the following formula: Mycolic acids yield (μg) = Peak area of mycolic acids × 5 / peak area of the internal standard (Goodrum et al., 2001).

2.9. Liquid chromatography-tandem mass spectrometry

Four samples from four batches of self-prepared natural MA extracted from *M. tuberculosis* H37Rv strain were analysed together with a

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commercially available MA extract from *M. tuberculosis* (bovine strain, M4537) purchased from Sigma-Aldrich. To obtain four batches of self-prepared natural MA, *M. tuberculosis* H37Rv strain was cultured on Middlebrook 7H10 agar plates, incubated at 37 °C and harvested on day 21. MA extraction and purification was performed as detailed above. The same procedure was repeated four times. For LC-MS analysis, an initial infusion was performed to assess all ionisable compounds present in both the self-prepared natural MA mixture and the commercially available MAs. MA extracts in dichloromethane:methanol (1:1) with 10 mM ammonium acetate were directly infused into the mass spectrometer using negative electro-spray ionisation (ESI) which yielded 19 precursor ions in the 1000–1400 *m/z* range. The optimized parameters of the ion source are shown in the Supplementary data (Table S1). A multiple reaction monitoring (MRM) method was developed which included only the initially observed 19 precursor ions using only the observed Q1 ions (Table S2). Each of these ions were quantified by peak area and classified as alpha-, methoxy- or keto-MA using the *m/z* databases previously reported (Layre et al., 2011; Sartain et al., 2011) and the web based database for lipids at www.lipidmaps.org/tools/ms/Mtb_batch_bulk.html.

Samples were prepared by reconstituting 1 mg of each batch of natural MA extract taken prior to derivatisation or commercial standard as supplied in 3 mL dichloromethane:methanol (1:1) containing 10 mM ammonium acetate followed by brief sonication to ensure complete dissolving. A volume of 200 μL was transferred to vial inserts in wide-neck autosampler vials for injection on the LC-MS/MS. The LC-MS/MS analysis was done on a Shimadzu UHPLC hyphenated to a AB Sciex 3200 QTrap LC-MS/MS. Briefly, 10 μL of each batch of MA extract taken prior to the derivatisation step was injected for analysis onto a C18 Discovery Supelco analytical column (150 mm × 4.6 mm, 3 μm). The mobile phases used were methanol (Solvent A) and dichloromethane:methanol (1:1) (Solvent B) with 10 mM ammonium acetate in both A and B, and a binary gradient elution method implemented. A flow rate of 0.5 mL/min was used with a total run-time of 47 min per injection.

2.10. Nuclear magnetic resonance

NMR spectroscopy was carried out on a Bruker Avance 400 spectrometer, using standard methods published before (Al Dulayymi et al., 2014; Koza et al., 2013).

2.11. Application: biosensor and TB diagnosis methodology

2.11.1. Collection of blood and delivery

Human blood samples were collected from patients not on anti-TB treatment, who were referred for TB testing at the Steve Biko public hospital in Pretoria and from an adult healthy control. Ethics clearance was provided by the University of Pretoria Ethics Committee (159/2011 of 1 September 2011). The study population consisted of 6 tuberculosis-positive (TB^+) patients and 1 tuberculosis-negative (TB^-) healthy control. The TB^+ group comprised of patients diagnosed with smear-positive and/or mycobacterial culture positive pulmonary tuberculosis. The healthy control (JS-09) was from a non-TB exposed, HIV negative, Caucasian student visiting South Africa from the Netherlands. Blood samples were delivered on the same day of collection to the Department Biochemistry, University of Pretoria, for MARTI analysis. All serum samples were collected and stored at −70 °C until use.

2.11.2. Procedure for serum separation

Blood samples were received in sterile tubes, with prior patient consent as per the ethics clearance provided. Samples were first processed to serum by allowing it to stand for 4 h. Serum was then aspirated into clean 1.5 mL Eppendorf tubes and centifuged at 4 °C to remove any contaminating red blood cells. Serum (35 μL) was aliquoted into each of thirty 600 μL Eppendorf tubes. The remaining serum was aliquoted in 500 μL volumes into 1.8 mL cryo tubes (NUNC™ Brand products, Nunc international, Denmark) and stored at −70 °C until used.

2.11.3. MA antigens

Natural mycolic acid mixture was isolated from mycobacterial culture extracts as described above, while single synthetic mycolic acids of the alpha- (Al-Dulayymi et al., 2005), methoxy- (Al-Dulayymi et al., 2007) and keto- (Koza et al., 2013) classes were synthetized at School of Chemistry, University of Bangor, UK. The synthetic antigens were used as combinations of alpha- + methoxy-MA and alpha- + keto-MA. The structures of each are provided in Fig. 1.

2.11.4. Preparation of liposome using sterol modified lipids

To determine the effect of MA class composition in both the immobilized antigen and the inhibitory antigen carrying liposome, three MA compositions were prepared, consisting of 1 mg MA natural mixture, or 1 mg of a 1:1 mixture of α-MA **1** and keto-MA **3**, or 1 mg of a 1:1 mixture of α-MA **1** and methoxy-MA **2** in 180 μL of chloroform. A stock solution of phosphatidylcholine (PC) (66 mg/mL) and sterol modified lipid (SML, 33 mg/mL of 1-palmitoyl-2-cholesteryl carbonoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids, USA) was prepared by dissolving and combining the weighed amounts in chloroform. Equal volumes of MA solution and SML-PC solutions were mixed together and evaporated by heating and nitrogen displacement for 5–10 min using a Reacti-Vap® (Thermo Scientific, Newington, USA). The formation of liposomes was then induced by adding saline (2 mL, 0.9% NaCl) to the dried liposome ingredients followed by heating on a heat block at 85 °C for 20 min with vortex mixing after every 5 min. The liposomes were sonicated for 5 min at 40% duty cycle with an output control of 2 using a UD-201 ultrasonic disruptor (Tomy Kogyo Co. Ltd., Tokyo, Japan). The sonicator tip was washed with chloroform, acetone and distilled water before and after use. Liposomes were then distributed into 200 μL aliquots and kept at −70 °C for 30 min, then placed in the freezedrier (Virtis, SP Industries, Gardiner, NY, USA). After freezedrying, the liposomes were reconstituted by re-suspending in PBS/AE buffer (2 mL), then placed on a heat block for 20 min at 85 °C and sonicated as before. The liposome solution was then used for analysis on the biosensor.

2.11.5. Detection of anti-mycolic acid antibody with ESPRIT SPR biosensor (MARTI test)

The biosensor analysis applied was according to the standard procedure used in the MARTI biosensor test as described by Lemmer et al.

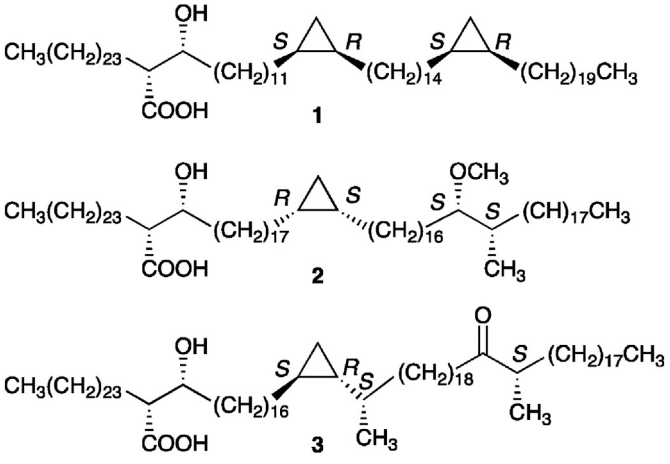

Fig. 1. Exact stereochemical structures of the three synthetic mycolic acid (MA) classes that were investigated for antigenicity against TB patient sera, α-MA **1**, methoxy-MA **2** and keto-MA **3**. For keto-MA, the keto-α-methyl group is shown in the *S* configuration, whereas the racemic *S*,*R*-mixture was used in the experiment. The saponification procedure used during sample preparation and extraction for isolation of the natural MAs would also have caused epimerisation at this position of the natural keto-MA.

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(2009). The ESPRIT biosensor (Autolab, Utrecht, Netherlands) functions under a pre-automated programme sequence that controls the addition of all the samples and liquids into the cuvette. Integrity of the sensor surface coat was monitored by determining the SPR resonance after each step. The samples were aspirated from a 384 multi-well plate (Bibby Sterilin Ltd., Stone, UK) to the cuvette surface. First the baseline of the ESPRIT biosensor was set with 10 μL wash buffer, followed by addition of 50 μL MA liposomes on the octadecylthiol coated gold disc for 20 min. The immobilized liposomes were then washed five times with 100 μL wash buffer and a baseline reading obtained. A twin cuvette's contents were then substituted with 50 μL of saponin (0.125 mg/mL) and incubated for 8 min. The cells were then washed five times with 100 μL wash buffer, before the content of each cell was substituted with 50 μL of wash buffer and left to achieve a stable baseline. After obtaining a stable baseline, a 1/500 dilution of serum sample (10 μL) in wash buffer was added in each cell to calibrate the response signals of the two cells over 10 min. A pre-incubation of 1/250 dilutions of serum with solutions of liposomes containing mycolic acids (SML-MAPC) or empty liposomes (SML-PC alone) were allowed for 20 min at room temperature. These were then added (10 μL) for binding inhibition determination between the two cells. The percentage inhibition signal was calculated from the initial slope differences of the inhibited and non-inhibited sensor readings.

2.12. Statistical analysis

Data were analysed using Student's *t*-test. *P* values < 0.05 were considered significant.

3. Results and discussion

3.1. Comparison of growth of M. tuberculosis H37Rv under four different culture conditions

To assess the growth of mycobacteria in different growth conditions of (1) mechanically stirred liquid culture, (2) stationary liquid culture or (3) Middlebrook 7H10 agar solid culture and (4) LJ slant solid media, the numbers of bacterial cells in harvested cell suspensions were quantified. Three methods were compared namely; total bacterial counts using a counting chamber and optical density measurements using a spectrophotometer and viable bacterial counts using the standard plate count. Total bacterial counts using the counting chamber were hindered by the tendency towards formation of mycobacterial clumps, which made it difficult to obtain accurate counts. Viable cells gave more accurate results, but since MAs are mycobacterial cell wall products, they are present in both viable and dead cells. Quantifying the total number of bacteria using optical density measurements was therefore applied.

Using the optical density measurement method to assess the growth of bacteria, mechanically stirred Middlebrook 7H9 proved to be the best growth medium for cultivation of *M. tuberculosis* with the optimum harvesting time between days 9 and 24 (Fig. 2). The increased bacterial growth rate that is seen with stirred liquid cultures can be attributed to aeration that is provided by mechanically stirred media (Wayne, 1976; Bowles and Segal, 1965; Lyon et al., 1961).) In contrast, stationary Middlebrook 7H9 cultures showed decreased bacterial growth rate compared to stirred cultures (Fig. 2). This was expected and is due to bacterial cells settling at the bottom of the flask resulting in oxygen deprivation, which ultimately causes slow replication rates (Wayne, 1976). Middlebrook 7H10 agar based medium showed the best growth of the non-stirred cultures between days 15 and 24 (Fig. 2).

3.2. Determination of the total amount of mycolic acids synthesized at different time points

The next step was to determine the total amount of MA that can be recovered from *M. tuberculosis* H37Rv cultivated in different media.

Comparison of MA volumetric yields obtained by HPLC from the same volumes of inoculum indicate that the best MA yield was obtained from mechanically stirred Middlebrook 7H9 cultures with a maximum yield of ± 50 mg. This was followed by cultures grown in Middlebrook 7H10 agar plates with a maximum yield of ± 11 mg. LJ-slants had the least MA yield with a maximum of ± 5 mg (Fig. 3).

Although the best MA yield was obtained from bacteria harvested from mechanically stirred Middlebrook 7H9 cultures, a decision to use Middlebrook 7H-10 agar based medium for the production and standardized preparation of MA antigen was made. This was motivated by the fact that it is safer and more affordable to use Middlebrook 7H-10 agar based medium and that potential microbial contamination can be easily detected and controlled. Fig. 3 shows that the optimum MA yield in bacteria harvested from Middlebrook 7H-10 agar based medium is obtained on day 18 but MA were extracted from bacteria harvested on day 21, since methoxy-MA is reported to be predominantly produced during the later growth of *M. tuberculosis* (Davidson et al., 1982).

3.3. Comparison of ratios of MA classes in self-prepared and commercial MA

From the four batches of *M. tuberculosis* H37Rv grown on Middlebrook 7H-10 agar, the MA was isolated using a standardized method (Goodrum et al., 2001).The ratios of alpha-, keto- and methoxy-MA in the total mycolate extracts for each batch were calculated from LC-MS/MS analysis using only the area of the most dominant

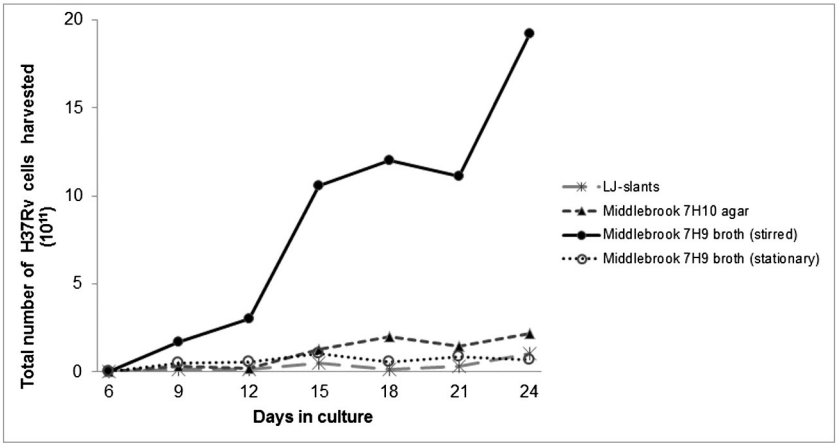

Fig. 2. Quantification of the total number of *M. tuberculosis* H37Rv cells harvested from 300 mL saline suspension.

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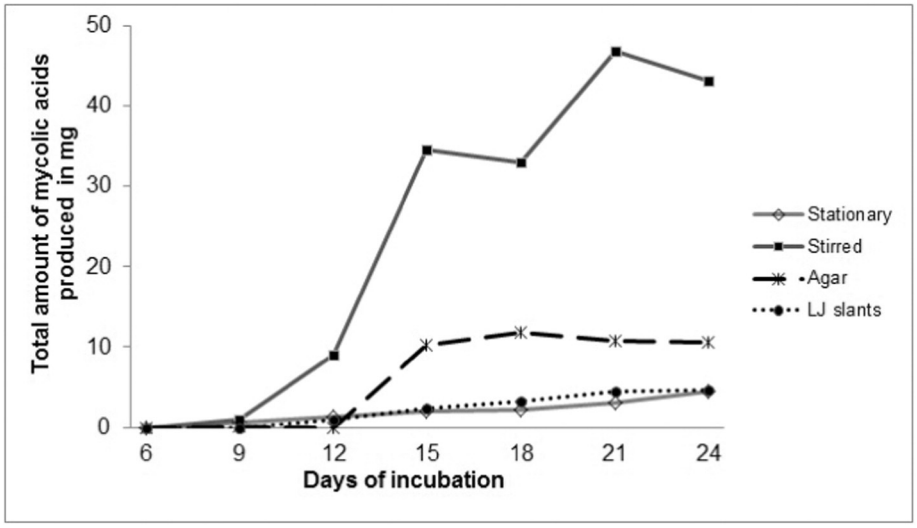

Fig. 3. HPLC quantification of the total amount of MA produced by *Mtb* H37Rv strain harvested at different time points from Middlebrook 7H9 broth stationary cultures, Middlebrook 7H9 broth stirred cultures, Middlebrook 7H10 agar plates and LJ Slants (ODs measured using McFarland standard 4 as a reference).

peak from each MA class in the chromatographically separated MA total ion chromatogram. These peaks were selected from the extracted ion chromatograms using both retention time and precursor mass to assign the identity of the selected MA (Fig. 4). The most abundant molecular ions (*m/z*) observed for each class of MA were used as representative molecular ions for each MA class. Determining the ratio of MA classes assumed that each MA ion is ionized similarly and that there is little or no ion suppression. It should be noted that there is co-elution of some MA peaks from different MA subclasses that cannot be well differentiated by low resolution mass spectrometers in the chromatogram (Fig. 4). This LC-MS/MS method is limited to providing an estimate of MA class ratios and not accurate quantification of each independent MA. Certified reference material (CRM) or preferably deuterated standards for each MA subclass would be required for mass spectrometric platforms to accurately quantify all MAs. Nonetheless, MS-based methods have been used by several researchers to quantify lipids (Sartain et al., 2011; Sandra et al., 2010; Shui et al., 2007; Koivusalo et al., 2001). The principal outcome was to determine the ratio of alpha-MA versus methoxy-MA and keto-MA.

Natural MA extract batches yielded similar patterns of MA class ratios to a commercially available MA purchased from Sigma-Aldrich (Table 1). Data from the analysis of the mass spectra of the various mycolates from Mtb H37Rv were in accordance with published MA molecular masses (Sartain et al., 2011; Layre et al., 2011). The major peaks corresponding to alpha-MA were at *m/z* 1108, 1136 and 1164. Peaks at *m/z* 1252, 1280, 1308 and 1336 corresponded to methoxy-MA and

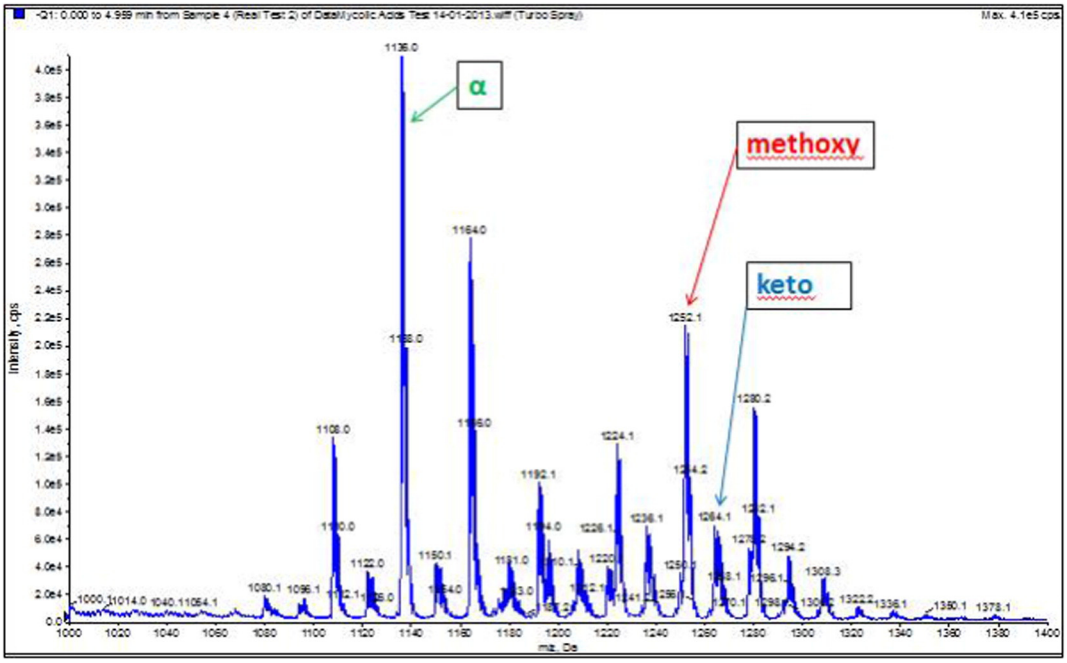

Fig. 4. Continuous infusion-based negative ionisation mode mass spectrum of the mass range 1000–1400 *m/z* showing the nineteen MA ions typically detected in the natural MA extracts on an entry level triple quadrupole mass spectrometer. The three specific α-, methoxy - and keto - MA subclass ions (1136, 1252 and 1264 *m/z* respectively) selected to determine the relative class ratio are also indicated on the mass spectrum.

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Table 1
Estimation of MA compositions of self-prepared and commercially MA (Sigma).

m/z used	MA class	Batch 1 MA ratio	Batch 2 MA ratio	Batch 3 MA ratio	Batch 4 MA ratio	Batch mean	Batch SEM	Sigma-MA ratio
1136	Alpha-MA	53	54	51	53	52.75	0.63	53
1264	Keto-MA	8	10	9	7	8.50	0.65	15
1252	Methoxy-MA	39	36	40	39	38.50	0.87	32

m/z spectra of most abundant MA peak of each MA class.

peaks at *m/z* 1208, 1236 and 1264 were assigned to keto-MA based on published data (Layre et al., 2011; Sartain et al., 2011; Shui et al., 2007). The most dominant MA representing each MA class were *m/z* 1136, 1264 and 1252 corresponding to alpha-, keto- and methoxy-MA respectively. These results agree with results of Shui et al. (2013), who reported the same major MA type for MA extracted from bacterial cultures.

The results obtained from the mass spectrum (Fig. 4 and Table 1) were correlated with that obtained from NMR data. Both proton and carbon NMR spectra provide fingerprints of the composition of the natural MA extracts and provide a means of determining the batch to batch variation, highlighting any significant changes in the ratios of the MA classes or the presence of other components such as alkene mycolates. The 400 MHz proton NMR spectrum of 1 mg of Batch 1 MA extract isolated above is shown in Fig. 5. Expansions of the various parts of the carbon NMR spectrum are presented in the Electronic Supplementary information.

The ratio of different MA classes can be calculated from a number of key signals in the proton NMR spectrum. These include signals between 5.1–5.4 ppm which show no signal, indicating the absence of alkene mycolate. If there were alkene mycolates, the signal's shape would provide the cis/trans ratio and the integral would determine the overall alkene content. The total MA content is given from the signal at 3.7 ppm for the single proton adjacent to the secondary alcohol. It is important to note, however, that this signal will move (normally downfield) if the alcohol is protected; an example would be when this group is protected as an acetate. The total *cis*-cyclopropane content can be determined from the signal at −0.4 ppm (one hydrogen, but there are two such cyclopropanes in an alpha-MA). The *trans*-cyclopropane can be determined from the three hydrogen multiplet at 0.05–0.2 ppm. The total methoxy-MA content can be determined from the methyl singlet (3H) at 3.3 ppm. The total keto-content can be determined from the doublet for the adjacent methyl group appearing at ca. 1 ppm.

In the case of this sample, the total relative integral for the MA —CH(OH)— at 3.7 ppm is 1.0 (see Supplementary data). Given that each MA in *M. tuberculosis* includes a proximal and a distal substituent the total integral for one proton in each of these positions should therefore be 2.0. The *cis*-cyclopropane integral for one proton at −0.4 ppm is

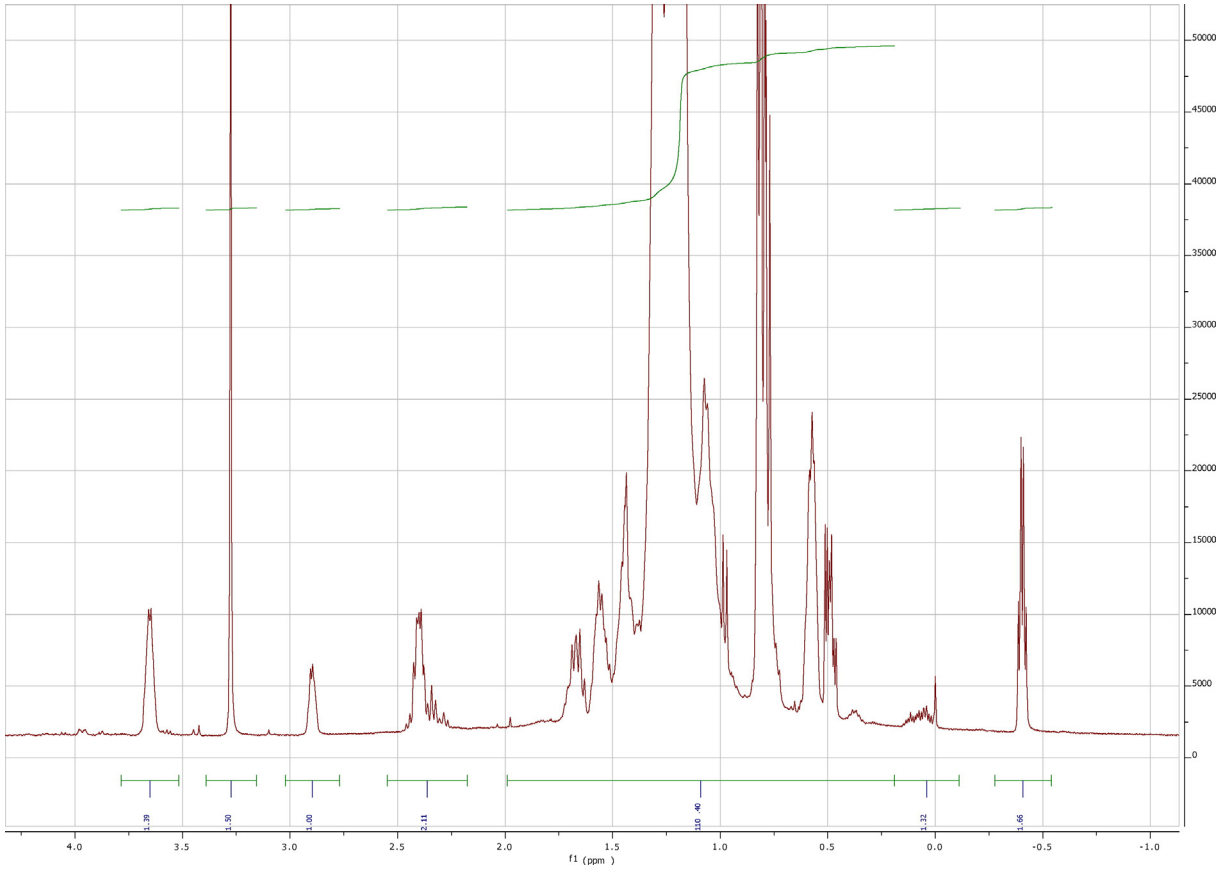

Fig. 5. An expanded view of a proton NMR spectrum (400 MHz) of Batch 1 of the natural MA extract. Further expansions are provided in the Supplementary data.

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1.28, the *trans*-cyclopropane is 0.27 per hydrogen, one proton of the methoxy-MA at 3.3 ppm integrates to 0.39 and the three protons of the keto-methyl (difficult to quantify exactly at 400 MHz, because the keto-methyl group lies on the edge of a broad multiplet which is present in the spectrum of the alpha-MAs.) is ca. 0.28 (0.09 per hydrogen in keto MA). The proportions can therefore be calculated as: *cis*-cyclopropane 1.28, *trans*-cyclopropane 0.27, methoxy-MA 0.39, keto-MA 0.09. The total relative integral for these (1.28 + 0.27 + 0.39 + 0.9) adds up to 2.03. This matches the total MA signal above well, the largest error probably arising from the keto-MA figures. Since all the *trans*-cyclopropane will be in the proximal position, the *cis*/*trans*-ratio at that position can be calculated as 0.73/0.27, leaving a distal *cis*-cyclopropane signal area of 0.55 (1.28–0.73), i.e. around 55% dicyclopropane α-MA. Assuming that only the three classes of MA reported to be present are indeed present, the MA class ratio is therefore ca. methoxy 39 (0.39/1.0): keto 9 (0.09/1.0): alpha 55. The integrals are subject to an error of around ±2% and assume no interference from impurities. They do match reasonably well those determined by MS (Table 1). The overall *cis*/*trans* cyclopropane ratio is 4:1, though from this single spectrum of mixture it is not possible to determine whether this is the same in all three classes.

In all batches of self-prepared natural MA and commercial MA, alpha-MA was the major component, while methoxy-MA was the most dominant oxygenated MA (Table 1). Watanabe et al. (2001) reported a high proportion of methoxy-MA in MA extracted from extended cultures of *Mycobacterium bovis* BCG Tokyo. Previous reports suggest that methoxy-MA is the most antigenic while keto- and alpha-MA have been shown to be weak antigens (Beukes et al., 2010). Our data show that the ratio of methoxy:keto is 4:1 on average, consistent from batch to batch, taking into account the approximately 2% error of detection. The 4:1 ratio of methoxy:keto-MA is in agreement with reported late growth stage ratios where other authors have shown that late growth stage cultures predominantly produce higher ratios of methoxy-MA compared to keto-MA (Yuan et al., 1998).

3.4. The effect of MA class composition on the antigenicity of MA

To determine the effect of MA class composition in both the immobilized antigen and the inhibitory antigen carrying liposome suspension on the accuracy of the SPR-based MARTI test, stereo-controlled, chemically synthetic MA combinations were used as antigens and their performance compared to natural isolated MA mixtures, typically composed of approximately equal amounts of α-MA and oxygenated (combined methoxy- and keto-) MA. Accordingly, the combinations were made up of synthetic MA in equimolar amounts of α-MA with either keto-MA or methoxy-MA, thus representing the extremes of the keto- or methoxy-MA composition possibilities. A total of 6 serum samples from confirmed TB positive patients were selected to test the antigenicity of synthetic mycolic acid combinations in comparison to the isolated natural mycolic acid mixture. As a negative control, a non-TB exposed, HIV negative human serum (JS-09) sample was obtained from a Caucasian student visiting South Africa from the Netherlands. The cohort characteristics for the TB positive patients and healthy control are summarized in Table 2. The results for the TB sputum smears were graded according to a technical guide document of International Union Against Tuberculosis and Lung Disease (IUATLD) (Akhtar et al., 2000).

All TB patients were confirmed TB positive by either sputum smear and/or mycobacterial culture. TB sputum smear negative represents positive mycobacterial culture from sputum, but with no AFB counts in at least 100 fields, TB positive represents sputum with 1 to 9 AFB counts in 100 fields, positive 1+ represents 10 to 99 AFB counts in 100 fields and positive 3+ represents greater than 10 AFB counts per field in at least 20 fields (Akhtar et al., 2000).

Fig. 6A & B shows the antigenicity of the three MA combinations against the TB patient sera in the SPR based MARTI assay. First, the functional operation of the MARTI assay was confirmed for all TB positive patient sera and the TB negative serum on the assay when isolated natural MA mixture (NatMA, Fig. 6A & B) was used as immobilized antigen and liposomal antigen inhibitor. A corresponding similar outcome is demonstrated when the synthetic α- and methoxy-MA combination was used as antigen, with the synthetic antigen either providing the same (MB-07, JC-08, AZ-11), stronger (BD-04), or weaker (VM-10, BM-12) signals than were obtained with the natural MA mixture as antigen. False negative results were only observed when the synthetic α-/keto-MA combination was used as antigen (Fig. 6B). In cases where synthetic α- and keto-MA did confirm the positive outcome, the MARTI signal was either similar (BD-04, MB-07, JC-08) or weaker (VM-10) than when natural MA mixture was used as antigen.

Within this limited sample group it seems that the natural mixtures of MA give approximately equivalent results to a mixture of the synthetic α- and methoxy-MA antigens in the MARTI-test for detecting anti-MA antibodies in patient serum. The results also show that high keto-MA ratios such as the one studied here should not be used, as this may lead to false negative results.

When using the self-prepared MA for comparing its antigenicity to that of combinations of synthetic alpha- and oxygenated keto- or methoxy MAs in SPR biosensor analysis, the MA antigen, whether in its natural mixture state or in its synthetic combination of α- and methoxy MA, showed antibody responses that could distinguish equally well between six TB positive patients (by sputum smear and/or culture) and a healthy individual using the MARTI-biosensor test. The MA combination of the synthetic α- and methoxy-MA tested was more antigenic than the combination of α- and the *trans*-cyclopropane keto-MA, which did not correctly identify two TB+ samples.

Table 2
Tuberculosis (TB) and control serum samples source and characteristics.

Sample identity	TB sputum smear outcome	HIV infection status	Age	Gender	Race
TB positive					
BD-04	Positive	Negative	59	Male	Black
MB-07	Positive	Negative	54	Male	Black
JC-08	Negative	Negative	85	Male	White
VM-10	Positive 1+	Positive	35	Male	Black
AZ-11	Negative	Negative	52	Male	White
BM-12	Positive 3+	Negative	19	Male	Black
TB negative					
JS-09	Negative	Negative	24	Female	White

4. Conclusion

The commercialisation of diagnostic devices based on detection of anti-MA antibodies as a biomarker of active disease caused by diverse mycobacteria is imminent. The need for particular MA class antigens appropriate to detect infection with opportunistic mycobacterial pathogens such as *M. avium* may require the use of synthetic MAs. For instance, *M. avium* MA does not contain the most antigenic methoxy-MA which is present in *M. tuberculosis*. Instead it has a meromycolic wax ester group which cannot be isolated intact from the bacilli by saponification and extraction. If the *M. avium* wax ester turns out to be the most antigenic MA, then the specific patient biomarker antibodies will have to be detected using chemically synthetic MA. For diagnosis of active TB, we conclude that natural mycolic acids extracted from *M. tuberculosis* can be prepared as a reproducible mixture for use as target antigen for detection of anti-MA antibodies in active TB patients. Although the sensitivity of detection of anti-MA antibodies of patient sera is affected by the MA antigen composition with respect to the MA class ratio,

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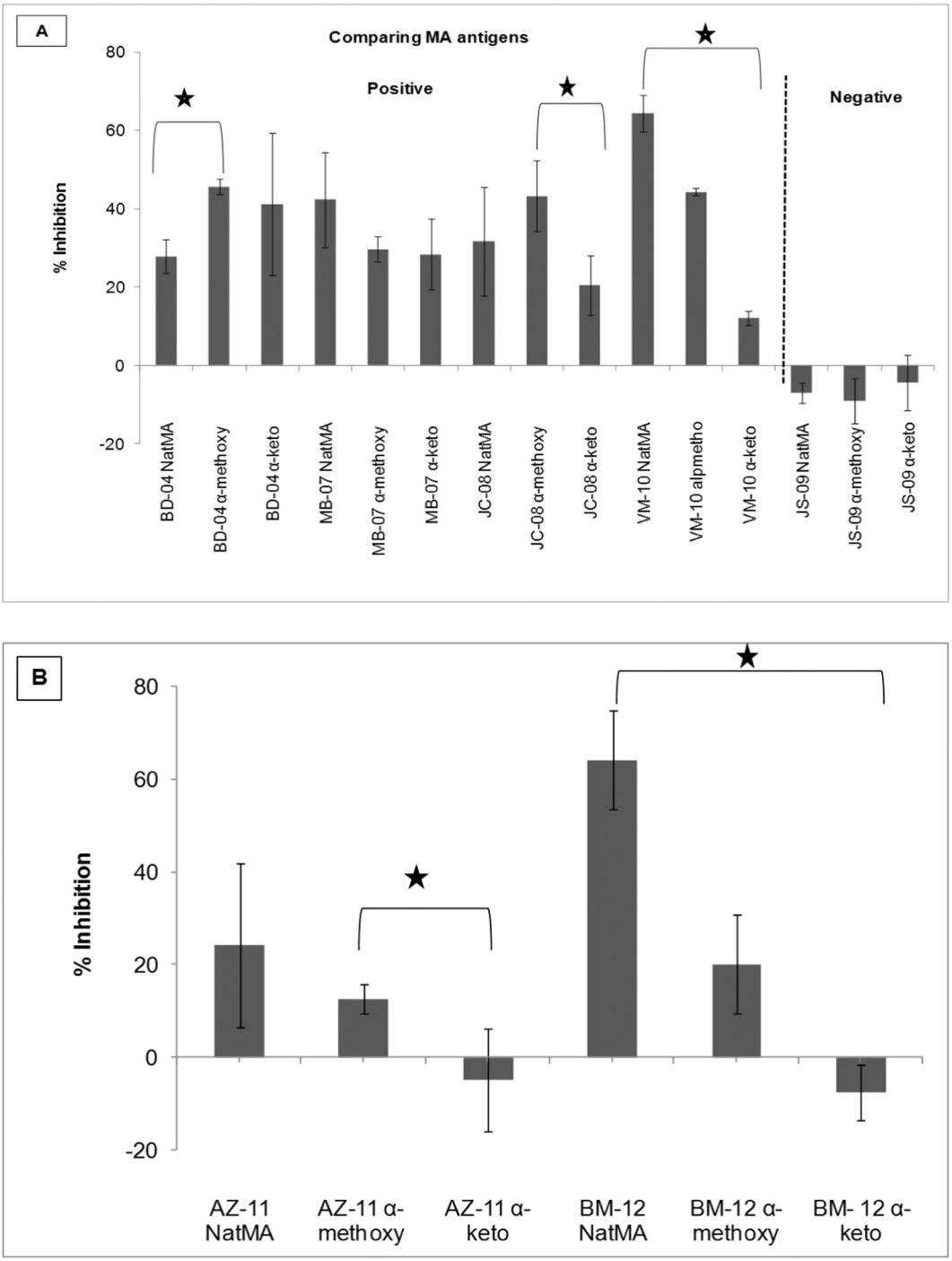

Fig. 6. SPR-MARTI analysis of TB patient and control human sera on combinations of synthetic MA compared to the natural isolated MA mixture. A: Set with no false negative results. B: Set with false negative results. False negative results were only observed using synthetic α-/keto-MA. Synthetic α-/methoxy MA gave SPR-MARTI signals that were equal to (MB-07, JC-08, AZ-11), higher (BD-04) or lower (VM-10, BM-12) than those obtained with natural isolated MA mixture. Synthetic α-/keto-MA consistently gave the same (BD-04, MB-07, JC-08, AZ-11) or lower (VM-10, BM-12) signals compared to the natural isolated MA mix. (*) indicates statistical difference at $P < 0.05$ for averages obtained with three runs in a two tailed Student's *t* test.

the natural mixture of MA isolated from late stage mycobacterial growth shows little batch to batch variation. It contains an adequate ratio of the MA classes to ensure that TB positive and TB negative patients can be distinguished at least as well as can be achieved with the combination of the synthetic MAs. The MA ratios in the natural extracted mixtures tested in this work are typical of the late stationary growth phase of the *M. tuberculosis* pathogen. The absence of alkenes noted in the natural mixture of MAs implies that it should not be prone to peroxidation upon extended storage or exposure to air.

Conflict of interest

Authors have no conflict of interest.

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