Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
DIAGNOSTICS

Mycobacterium tuberculosis pili (MTP), a putative biomarker for a tuberculosis diagnostic test

Natasha Naidoo, Saiyur Ramsugit, Manormoney Pillay*

Medical Microbiology and Infection Control, School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, South Africa

1. Introduction

There is an urgent need to improve the laboratory turnaround time for the diagnosis of Mycobacteria [1] by the development of a point of care test that is also accessible to resource poor countries [2]. The lack of suitable biomarkers has hindered the development of diagnostic tests that meet all the criteria of a point of care test for the rapid detection of tuberculosis.

Biomarkers, such as epitopes, molecules or genetic materials have been incorporated into assays capable of rapidly and inexpensively identifying active tuberculosis, distinguishing appropriate responses to anti-tuberculous drugs, and those at risk for disease progression [3]. The identification of biomarkers to distinguish among members of the Mycobacterium tuberculosis complex (MTBC) comprising M. tuberculosis, Mycobacterium africanum, Mycobacterium bovis, M. bovis BCG, Mycobacterium microti and Mycobacterium canetti and non-tuberculous Mycobacteria (NTM) has been a challenge. This is due to the high similarity between the genomes of the MTBC members [4]. Examples include the >99.95% homology between M. bovis and M. tuberculosis genomes [5]. There have been conflicting reports on the ability to distinguish MTBC from NTM with the most widely studied potential biomarkers for MTB diagnosis, ESAT-6 and CFP-10 [6–8].

Numerous immunochromatographic tests have been evaluated for use as point of care tests, including few that are based on the MPB64 epitope [9–14], which include the Capilia TB and BIOLINE SD Ag MPT64 tests [12]. The MPB64 antigen is an immunogenic protein found in unheated cultures of M. tuberculosis [9,15–17]. These tests are based on detection from culture with the detection limit of 10.5 cfu/ml [12]. Since the production of this antigen is slow, it is not always detectable in liquid culture compared to solid media [10]. The ICT TB test is based on 5 M. tuberculosis antigens, including Antigen 85 and LAM [18,19]. However, the ability of the ICT test to distinguish between MTBC and NTM has not been evaluated [18,20]. Recently, ESAT-6/CFP-10 has been used in a nanoELIwell assay that integrates on chip culturing of cells, immunoassay and fluorescent imaging [8]. The advantages of this assay are high throughput and rapid culture. However, the...
sensitivity of this test would be reduced since ESAT-6 is secreted by many NTM [6–8]. An immunochromatographic test developed using both ESAT-6/CFP-10 complex showed high specificity and sensitivity of 97% and 97.4% respectively for MTBC organisms [21]. Despite the apparent high specificity and sensitivity compared to sputum smear and tuberculin skin tests, the study showed a greater chance of false negative and false positive results using these epitopes [21].

Adhesins, including pilus, are the first point of contact with the host cell, helping overcome the net repulsive forces on the surfaces of the host and the pathogen [22]. Therefore, pilus represent targets for use in diagnostics, including potential point of care tests. Two types of pili were discovered in M. tuberculosis [23], namely Type IV pili, common to Gram-positive and Gram-negative bacteria [22] and archeal bacteria [24–27] and ‘curli’ pili, common to pathogens such as Escherichia coli and Salmonella typhimurium [28,29]. Type IV pilus possess a hydrophobic amino terminus and a signal peptide [30,31]. They are often observed under the electron microscope as long bundles of filaments, resembling rope-like structures or wicks [30,31]. Curli pili are 2–5 nm wide, coiled and highly aggregative adhesive fibres. These structures are assembled using a nucleation pathway, requiring the major and minor pilin subunits [32].

The curli pili of M. tuberculosis (MTP), encoded by the mtp gene (Rv3312A), has many attributes in common with curli pili of other bacteria [23]. They are highly sticky, aggregative and insoluble fibres that bind laminin [23,33,34] and Congo red dye and contain large numbers of glycine and proline residues. However, there is no similarity between the primary sequence homology of the protein subunits of MTP and curli pili of other bacteria [23].

MTP first identified and characterised by Alteri et al. (2007), are produced during active human infection and facilitate binding to laminin on host cells [23]. Not all Mycobacteria produce pili, although this may be attributed to the response of Mycobacteria to different environmental indicators [30]. With access to complete Mycobacterial genomes, it is now possible to determine the occurrence of the mtp gene in various Mycobacterial species and study the levels of expression of the gene.

In this study, we sought to determine whether the MTP protein is a suitable biomarker for a potential diagnostic test for tuberculosis. Therefore, BLAST analysis on publically available genome databases and sequencing was performed to determine the presence and conservation of the mtp gene in MTBC strains, NTM and other respiratory organisms. In addition, we analysed the protein secondary structure and topology of the pilin subunits of these organisms. We showed that the mtp gene and its encoded protein product, MTP, is unique to MTBC strains with only partial similarity to Mycobacterium marinum.

2. Materials and methods

2.1. Bacterial strains

Mycobacteria and other respiratory organisms used in this study are listed in Table 1. Mycobacterial isolates were cultured for 2–3 weeks at 37 °C, on Middlebrook 7H11 complete agar (BD Difco) containing 10% oleic acid, albumin, dextrose, catalase (OADC) (Becton Dickinson and Company), except for Mycobacterium chelonae that was incubated at 30 °C and Mycobacterium ulcerans at 32 °C. Respiratory organisms were cultured at 37 °C in the presence of 5% CO2 for 48 h on blood agar, with the exceptions of Haemophilus spp. and Legionella pneumophila that were grown on chocolate agar and Buffered Charcoal Yeast Extract (BCYE) agar in moist conditions respectively.

| Table 1 | The MTBC, non-tuberculous Mycobacteria (NTM) and other respiratory organisms selected for sequencing of the mtp gene. |
|---------|------------------------------------------------------------------------------------------|
| Bacterial strain | ATCC number* | n |
| **M. tuberculosis complex strains** | | |
| M. tuberculosis | 91 | |
| F15/LAM4/KZN | 20 | |
| Beijing | 20 | |
| F11 | 10 | |
| F28 | 10 | |
| Unique | 8 | |
| Other clustering strains | 18 | |
| M. africanaum | 1 | |
| M. catenulatum | 1 | |
| M. microti | 1 | |
| M. bovis | 1 | |
| M. bovis BCG | | |
| **Non-tuberculous Mycobacteria** | 34 | |
| M. scrofulaceum | 1 | |
| M. intracellularis | 3 | |
| M. gordonae | 7 | |
| M. fortuitum | 9 | |
| M. avium | 6 | |
| M. chelonae | 4 | |
| M. marinum | 1 | |
| M. abscessus | 23040 | 1 |
| M. smegmatis | 21293 | 1 |
| M. ulcerans | | |
| **Respiratory organisms** | 10 | |
| Gram positive bacteria | | |
| Streptococcus pyogenes | 8668 | 1 |
| Staphylococcus aureus | 25923 | 1 |
| Streptococcus pneumoniae | 49619 | 1 |
| Gram negative bacteria | | |
| Haemophilus influenzae type B | 7901 | 1 |
| Haemophilus parainfluenzae | 33533 | 1 |
| Moraxella catarhalis | | |
| Legionella pneumophila | | |
| Klebsiella pneumoniae | | |
| Pseudomonas aeruginosa | 700623 | 1 |
| Burkholderia cepacia | 27853 | 1 |
| Total | 135 | |

* The organisms that do not have ATCC numbers are clinical isolates.

2.2. Bioinformatics analysis of the mtp gene and corresponding coded amino acid sequence

The complete genome sequences of some Mycobacterial spp. and other respiratory organisms are available on the NCBI website [35] (www.ncbi.nlm.nih.gov), the TB database [36] (www.tbdb.org), the Broad Institute website [37] (www.broad.mit.edu), the GenoList databases [38] (http://genolist.pasteur.fr) and the Sanger Institute website [39] (www.sanger.ac.uk). The degree of homology of the mtp gene and corresponding amino acid sequence to the MTBC was determined by BLAST analysis (blastn and blastp) and multi-sequence alignment (Bioedit). The mtp gene was presumed absent if no hits were obtained for organisms that have the complete genomes present on the database.

2.3. Bioinformatics analysis of proteins

Partial sequence alignment was found in M. marinum, Mycobacterium avium, M. ulcerans, and Mycobacterium abscessus. The function of the hypothetical proteins encoded by these gene sequences is unknown. The amino acid sequences of these partially-aligned sequences were, therefore, analysed for the secondary structure, topology and antigenic epitopes to determine if these proteins resemble pilin protein subunits. This was done using PredictProtein software [40] and the Abie Pro: Peptide Antibody Design version 3.0 program [41], and compared to that of M. tuberculosis.
2.4. Sequencing of the mtp gene in clinical strains of M. tuberculosis, NTM and other respiratory organisms

2.4.1. PCR amplification of the mtp gene

Purified DNA was prepared by the Sodium chloride–Cetyl tri-methylammonium bromide method as previously described [42], with the exception of the M. ulcerans DNA, obtained using the boiling method. The ability of the latter DNA to be amplified was tested using the 16S rRNA housekeeping gene (results not shown). Following optimisation experiments, the DNA concentrations of all MTBC, NTMs and respiratory microbes were quantitated using a Nanodrop 2000 spectrophotometer (Nanodrop Technologies). DNA samples were standardized to 10 ng/µl, that is, the maximum concentration required to give a PCR product if the mtp gene is present in the organism.

The complete mtp gene was amplified using the following primers: forward: 5’-CTC ATG GGT CAC AGC GAG TA-3’, reverse: 5’-ATG ACA GGT TCC CTT CAA GC-3’. This was followed by a denaturation of 1 min at 95 °C, 40 cycles of 20 s at 95 °C, 1 min at 57 °C and 30 s at 72 °C. This was followed by a final extension for 5 min at 72 °C. PCR products were electrophoresed in a 1.5% (w/v) agarose gel stained with ethidium bromide and visualized using the GelDoc system.

2.4.2. Automated DNA sequencing

The PCR products were purified and then sequenced using the primer set described above by Inqaba Biotec, Pretoria, South Africa.

The chromatograms were analysed using Chromas Pro and multiple sequence alignment was performed with Bioedit software.

3. Results

3.1. The mtp gene and corresponding coded amino acid sequence are specific to the MTBC

To determine the value of the mtp gene as a diagnostic marker, it was imperative to determine specificity to M. tuberculosis as compared to other members of the MTBC and NTM as well as other respiratory pathogens and commensals. The mtp gene and corresponding amino acid sequence were aligned against all relevant complete genome sequences available on the publically available databases.

Nucleotide and protein BLAST analysis of the mtp gene and corresponding coded amino acid sequence showed 100% homology in M. tuberculosis C, M. tuberculosis H37Ra, M. tuberculosis Haarlem, M. tuberculosis CDC1551, M. tuberculosis F11, M. bovis, M. bovis BCG, M. canettii and M. microti with that of the laboratory strain H37Rv (Figure 1 and Supplementary Table 1) in contrast to the partial alignment shown by the NTM. The similarity of the mtp gene sequence of the different organisms on the publically available genomic databases was further explored using e-values (Table 2). The lower the e-value, the stronger the homology with that of the mtp gene sequence of the organism. All the MTBC organisms displayed a lower e-value and thus showed 100% homology of the mtp gene. Higher e-values and therefore partial homologies were obtained for the NTM (Table 2).

BLAST analysis of the complete genomes of other respiratory organisms showed that no significant similarity was found for the following bacteria: Streptococcus pyogenes, Haemophilus influenzae, Corynebacterium diphtheriae, Bordetella pertussis, Moraxella bovis BCG Pasteur, M. bovis BCG Tokyo 172, M. bovis ATCC23097, M. africanum CM041182, M. canettii CIPT 140010059, M. microti, M. marinum, M. m. avium 104, M. gilvum Spyr1, M. ulcerans Agy99, M. abscessus, M. smegmatis, M. intracellulare.

Figure 1. A pictorial representation of the BLAST analysis of the mtp gene of MTBC and NTM from the public genome databases. This was modified in the NCBI output diagram to show the organisms adjacent to a colour bar that represents the degree of homology of each organism’s sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The query bar represents the input sequence of 312 base pairs of M. tuberculosis H37Rv that aligned to the complete genomes available on NCBI. The red bar represents the correct alignment of 200 base pairs or more to the query sequence, the pink bar 80–200 base pairs, the green bar 50–80 base pairs, the blue bar 40–50 base pairs and the black bar less than 40 base pairs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
catarrhalis, Streptococcus pneumoniae, Klebsiella pneumoniae, Staphylococcus aureus, Burkholderia cepacia, Chlamydia pneumoniae and Mycoplasma pneumoniae.

The complete genome sequence was not available for L. pneumophila but this isolate was cultured for amplicon sequencing (Table 1).

Viruses and fungi are not likely to possess pilin protein. Nevertheless, the mtp gene sequence was aligned to the complete genome sequences of the following organisms: Rhinovirus, Coronavirus, Enterovirus, Adenovirus, Respiratory Syncytial Virus, parainfluenza virus, Cryptococcus neoformans, Candida albicans, Aspergillus fumigatus and Paracoccidioides brasiliensis. However, no homology was observed.

3.2. Selective amplification of the mtp gene

The mtp gene (Rv3312A) was specifically and consistently amplified as a 582 bp size product from all the MTBC subspecies tested (Figure 2(A)). These included the most prevalent strains in KwaZulu-Natal, a few other cluster strains, some unique strains, M. bovis, M. bovis BCG, M. africanum, M. microti and M. canetti. In contrast, none of the NTM strains showed a positive PCR result (Figure 2(B)), despite extensive optimization (results not shown). The PCR on the NTMs was conducted with a standardised DNA concentration and the negative result was interpreted as the absence of the gene in the organism.

3.3. Sequencing of M. tuberculosis isolates demonstrated a highly conserved gene sequence

No mutations were observed in the mtp gene in 84/86 (97.7%) of M. tuberculosis clinical strains (Figure 3(A)). Two isolates of M. tuberculosis harboured point mutations, a G54C synonymous and a G17T non-synonymous mutation with a C6F amino acid change (Figure 3(B)).

3.4. Pili protein structure and topology

Partial sequence alignment was found in M. marinum, M. avium, M. ulcerans and M. abscessus. The function of the hypothetical proteins encoded by these gene sequences is unknown. The amino acid sequences of these partial sequences were therefore analysed for secondary structure and topology using the PredictProtein software. The predicted protein structure of the encoded gene sequences of M. avium, M. ulcerans and M. abscessus differed from that of the M. tuberculosis, which was 67% similar to M. marinum (Figure 4).

3.4.1. Bioinformatics analysis of amino acid sequences of the MTP and NTMs

MTP protein coded for by the mtp gene contained a transmembrane region, one antigenic epitope, and comprised 19.42% α-helix, with 80.58% random coil. The amino acid sequence that had the highest sequence similarity (67%) and pairwise sequence identity (55%) to the MTP amino acid sequence was that of M. marinum. The protein of M. marinum contained a transmembrane region, but possessed 2 epitopes and a different secondary structure (27.66% α-helix and 72.34% random coil). M. abscessus amino acid sequence had a 44% pairwise sequence identity and 59% sequence similarity to the MTP amino acid sequence. Whilst the secondary structure was similar, the protein contained no transmembrane region. The M. avium amino acid sequence had a 43% pairwise sequence identity and 57% sequence similarity to the MTP amino acid sequence. However, the protein lacked a transmembrane region and contained 4 epitopes. The secondary structure comprised 63.4% strand structure and no α-helix. M. ulcerans amino acid sequence had the

| Mycobacterial species | e-values |
|-----------------------|---------|
| M. tuberculosis CDC1551 | e^{-176} |
| M. tuberculosis F11 | e^{-176} |
| M. tuberculosis H37Ra | e^{-176} |
| M. tuberculosis KZN 1435 | e^{-176} |
| M. bovis AF2122/97 | e^{-176} |
| M. bovis BCG str. Tokyo 172 | e^{-176} |
| M. bovis BCG str. Pasteur | e^{-176} |
| M. africanum GM041182 | e^{-176} |
| M. canetti | e^{-176} |
| M. microti | e^{-176} |
| M. marinum | 6e^{-15} |
| M. paratuberculosis paratuberculosis K-10 | 0.087 |
| Mycobacterium sp. MCS | 0.087 |
| Mycobacterium sp. JLS | 0.087 |
| Mycobacterium sp. KMS | 0.087 |
| M. avium 104 | 0.087 |
| M. avium subsp. paratuberculosis K-10 | 0.087 |
| M. smegmatis str. MC2 155 | 0.34 |
| M. vanbaalenii | 0.34 |
| M. ulcerans Agg99 | 0.34 |
| M. leprae Br4923 | 1.4 |
| M. gilvum | 1.4 |
| M. gilvum PYR-GCK | 1.4 |
| M. abscessus ATCC 19977 | 1.4 |
| M. vaccae | No hits |
| M. lflandii | No hits |
| M. celatum | No hits |

* e-values represent the number of hits one can “expect” to see by chance when searching a database. The lower the e-value, or the closer it is to “0”, the higher is the “significance” of the match.
lowest similarity to the MTP amino acid sequence with 41% pairwise sequence identity and 53% sequence similarity. The protein contained no transmembrane region, 4 epitopes, and a highly looped secondary structure (Figure 4). It is therefore apparent that the proteins of *M. abscessus*, *M. avium* and *M. ulcerans* do not resemble the pilin subunit protein structure. The protein of *M. marinum* however, does resemble the pilin subunit protein of *M. tuberculosis*.

![Figure 3](image)

**Figure 3.** (A) Multiple sequence alignment of the *mtp* gene sequences of representative isolates in KwaZulu-Natal (KZN). The KZN, Beijing, F28, F11 and clusters 5–8 strains represent strain families dominant in KZN. The unique strain was isolated from a single patient in KZN. The alignment is shown as a dot plot, each dot representing the same nucleotide as the reference *mtp* gene sequence of H37Rv. A change in nucleotide would result in the nucleotide standing out from the rest of the dot plot. Thus, it is apparent that no mutations were found in the strains represented in the figure. (B) Point mutations in the *mtp* gene occurred within the first 60 bp. The mutants were sequenced three times for confirmation. The point mutations stand out from the dot plot. In mutant 1, at position 17, the nucleotide change of guanine to thymine brings about change in amino acid from cysteine to phenylalanine. In mutant 2, at position 54, the nucleotide change of guanine to cytosine brings about no amino acid change.

![Figure 4](image)

**Figure 4.** Bioinformatics analysis using Protein Predict and Abie Pro software of the *M. tuberculosis* pili (MTP) protein and the proteins of the NTMs that showed a partial homology upon BLAST analysis. (A) *M. tuberculosis* pilin protein has a transmembrane region (t), contains one antigenic epitope (a) from amino acids 7–80, and comprises 19.42% α-helix (h) and 80.58% random coil (l). NTMs: (B) *M. marinum* protein has a transmembrane region (t), contains 2 antigenic epitopes (a) from amino acids 54–89 and 82–91, 27.66% α-helix (h) and 72.34% random coil (l). (C) *M. abscessus* protein does not contain a transmembrane region (t) and contains a large antigenic epitope at the N-terminal. (D) *M. avium* protein does not contain a transmembrane region (t), contains 4 smaller antigenic epitopes (a) and has 6.30% strand (s) structure, with no α-helix (h). (E) *M. ulcerans* protein contains no transmembrane region (t), 4 antigenic epitopes (a), and a highly looped (l) secondary structure.
4. Discussion

The role of MTP in adhesion of *M. tuberculosis* to the host was first reported in 2007 [23]. MTP were proposed to be the first point of contact with the host and unique to pathogenic Mycobacteria. MTP were recently viewed by Atomic Force Microscopy in XDR-TB and TDR-TB strains [43]. It has been conclusively proven that the *mtp* gene is required for pilus formation in *M. tuberculosis* [23,44]. In this study, BLAST analysis of the complete genome sequences of various Mycobacteria and other respiratory pathogens and commensals [35–37,39], as well as amiplocus sequencing has proven conclusively that the *mtp* gene is unique to members of the MTBC. The very low frequency of mutations in the *mtp* gene indicated that it is highly conserved among the clinical strains of *M. tuberculosis*. Furthermore, the partial homology detected in NTN has not been linked to proteins similar to those encoding pilin subunits, with the exception of *M. marinum*. This is the first study providing evidence that the *mtp* gene is highly conserved, suggestive of the potential use of its encoded product, the MTP protein, as a biomarker for MTBC pathogens.

Further support for this is provided by the bioinformatics analysis, suggesting that the partial protein homologies in other NTMs occurred by chance, with high e-values and very low sequence similarity. The predicted protein structure encoded by the gene sequences of *M. avium*, *M. ulcersans*, and *M. abscessus* differed from that of *M. tuberculosis*. The low pairwise sequence identity and the fact that these are not transmembrane proteins and differ in secondary structure decreases the probability that these proteins code for pilin subunits or are involved in pilin assembly. Also, the probability of cross-reactivity with MTP in an antigen based diagnostic assay for MTBC is unlikely.

In contrast to these NTMs, the transmembrane protein of *M. marinum* has greater sequence identity and secondary structural similarity to *M. tuberculosis*. Whilst this does not necessarily dictate a similar function, pilin proteins from groups of Gram-positive organisms have been shown to be clearly related. Ancillary protein1 (AP1), encoded by pilus islands 1 and 2 of group B Streptococci share 42% sequence identity with each other and 50% identity with AP1 of *S. pneumoniae* [45]. It is likely that this partially-aligned protein of *M. marinum* may be a pilus-like protein, similar to that of MTP but the presence of these cell-surface structures in this organism still needs to be confirmed experimentally. However, the secondary structural similarity of *M. marinum* is unlikely to pose an obstacle to MTBC diagnostics as this organism does not cause NTM lung disease. *M. marinum* has been isolated from subcutaneous lesions on the skin of humans and can only grow at very low temperatures [46,47].

MTP are similar to curli pili produced by *E. coli* and *Salmonella enterica* in their morphology as well as their ability to bind to Congo Red and laminin [23]. However, they differ in their secondary structure, in that the MTP amino acid sequence consists of an α-helix, whereas, that of *E. coli* pilin is a β-barrel structure [22]. Curli pili in general are non-branching, β-sheet rich fibres that are resistant to protease digestion and 1% SDS [22]. In *E. coli*, curli pili are assembled via the nucleation precipitation pathway [32]. Little is known about the structure and assembly of MTP [23]. The *mtp* gene does not belong to an operon like the pilin biogenesis genes of bacteria such as *E. coli*, *Salmonella* spp. and Gram-positive bacteria [48–51]. The *mtp* gene is located in between genes involved in intermediary metabolism (*add, deoA* and *cdd*) as well as *Rv3312c*, a gene of unknown function [23,38,44].

Novel TB biomarkers are urgently needed as targets for the development of rapid point of care diagnostics as well as therapeutic interventions and vaccines. Previous studies identified an arsenal of novel *M. tuberculosis* antigens including 38-kDa antigen, 19-kDa lipoprotein, 16-kDa antigen, MTB81, ESAT-6, antigen 85B, MPT51, MPT32, 14-kDa antigen, A60, HBHA, PE and PPE antigens and the glycolipid antigens for use in serodiagnostic assays as reviewed by Abebe et al., 2007 [52]. The low and variable sensitivity of these assays based on the inclusion of a single biomarker led to the proposal of various combinations of these immunodominant antigens for the detection of different stages of TB infection or disease [53]. However, serodiagnostic tests aimed at measuring antibody levels in biological samples have proved unsuccessful [54–58]. Commercial point of care diagnostic tests that measure the presence of *M. tuberculosis* antigens such as MPT64 [9–13], ESAT-6/CFP-10 [7,8,21,59,60] and Antigen 85 [61] are limited as they are based on culture. The MPT64 antibodies showed a limited sensitivity of 64.4% when tested on sputum specimens in a sandwich Enzyme Linked Immunosorbent Assay (ELISA) for the diagnosis of pulmonary tuberculosis [62]. LAM antigens, either alone or with other epitopes have shown limited accuracy on clinical specimens [18,20,63]. Other epitopes that have been evaluated for diagnostic purposes include the IP-10 [64], B cell antigens [65], malate synthase and MPT51 [66–68].

A limitation of most biomarker studies is the lack of extensive evaluation of an epitope prior to screening of biological samples. In addition to providing evidence of the conserved nature and specificity of the *mtp* gene to the MTBC in this study, we have detected the *mtp* gene transcript in broth and agar grown clinical MTBC strains in preliminary experiments using RT-PCR. Further studies are in progress to verify these results and to detect the presence of MTP in these strains. Furthermore, gene knockout and complementation studies have proved the essentiality of the *mtp* gene for MTP production [23,44]. We therefore propose MTP as a relevant diagnostic marker for the MTBC. Studies are underway to screen for anti-pili antibodies in patients with various stages of tuberculosis and to generate monoclonal antibodies for the detection of MTP antigen in clinical specimens of patients with tuberculosis.

Acknowledgements

We are grateful to Miss Nafissah Bibi Chotun and Miss Nonhlanhla Mhlongo for their contribution to the amplification of the *mtp* gene for sequencing of some of the clinical strains as part of their honours research projects. We are also grateful to the National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital for the donation of isolates of respiratory pathogens. We are thankful to Dr Deepak Almeida of the KwaZulu-Natal Research Institute for Tuberculosis and HIV-co-infection (K-RITH) for the *M. ulcersans* strain. We appreciate the donation of the DNA of *M. africanum*, *M. microti* and *M. canettii* from Dr. Philip Supply of the Institut Pasteur de Lille, France.

Ethical approval: The study was approved by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (BE 245-11).

Funding: N. Naidoo gratefully acknowledges scholarships received from the National Research Foundation (NRF); College of Health Sciences, UKZN and K-RITH, Howard Hughes Medical Research Institute. We thank the Medical Research Council, NRF (grant no. 77286), K-RITH and the College of Health Sciences, UKZN for project running costs.

Competing interests: None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2014.03.004.
References

[1] Parsons LM, Somoskovi A, Gutierrez C, Lee E, Paramasivam CN, Abinumi A, Spencer SE, Rosciglione G, Nkeganhaj S. Laboratory diagnosis of tuberculosis in resource-poor countries: challenges and opportunities. Clin Microbiol Rev 2011;24:314–50.

[2] World Health Organization. TB diagnostics in the spotlight. http://www.who.int/news-room/2010/tb-diagnostics/en/index.html [accessed 13.06.13].

[3] Nyendik MR, Lewinsohn DA, Lewinsohn DM. New diagnostic methods for tuberculosis.Curr Opin Infect Dis 2009;22:174–82.

[4] Brosch R, Pym AS, Gordon SV, Cole ST. The evolution of mycobacterial pathogenicity: clues from comparative genomics. Trends Microbiol 2001;9:452–45.

[5] Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C, Monsime P, Simon S, Harris B, Atkin R, Daggett J, Mayes R, Keating L, Wheeler PR, Parkhill J, Barrett BG, Cole ST, Gordon SV, Hewson RC. The complete genome sequence of Mycobacterium bovis. Proc Natl Acad Sci U S A 2003;100:7877–82.

[6] van Puitens NGC, Warren RM, van Helden PD. ESAT-6 and CFP-10: what is the diagnosis? Infect Immun 2002;70:6509–11.

[7] Arend SM, van Meijgaarden KE, de Boer K, de Palou FC, van Sooeling D, Ottenhoff TH, van Dissel JT. Tuberculin skin testing and in vitro T cell responses to ESAT-6 and culture filtrate protein 10 after infection with Mycobacterium tuberculosis or M. kansasi. J Infect Dis 2002;186:1757–807.

[8] Nguyen YH, Ma X, Qin L. Rapid identification of Mycobacterium tuberculosis by a novel susceptibility screening of ESAT-6 secreting Mycobacteria by a NanoELWell assay. Sci Rep 2012;2:635.

[9] Abe C, Hirano K, Tomiyama T. Simple and rapid identification of the Mycobacterium tuberculosis complex by immunochromatographic assay using anti-MBP64 monoclonal antibodies. J Clin Microbiol 1995;37:3689–93.

[10] Marzouk M, Kahla IH, Hanchani N, Ferjani A, Salwa WM, Chezal S, Bouakdja J. Validation of an immunochromatographic assay for rapid identification of Mycobacterium tuberculosis complex in clinical isolates. Diagn Microbiol Infect Dis 2011;69:396–9.

[11] Ngamert K, Sinthuwattanawibool C, McCarthy KD, Sohn H, Starks A, Kanjanamongkolpis P, Anek-vorangop R, Tanasaneypan T, Monkongdee P, Diem L, Varma JK. Diagnostic performance and costs of Capilia TB for Mycobacterium tuberculosis complex identification from broth-based culture in Bangkok, Thailand. Trop Med Int Health 2009:14:748–53.

[12] Park MY, Kim YJ, Hwang SH, Kim HH, Lee YE, Jeong SH, Chang CL. Evaluation of an immunochromatographic assay kit for rapid identification of Mycobacterium tuberculosis complex in clinical isolates. J Clin Microbiol 2009;47:481–4.

[13] Tothi AH, Rasolofo V, Andrianarisoa SH, Ranjalalhy GM, Ramarokoto H. Validation of an immunochromatographic assay kit for the identification of Mycobacterium tuberculosis complex. Mem Inst Oswaldo Cruz 2011;106:777–80.

[14] Vadwai V, Sadani N, Bhas T, Chavan A, Balan K, Naik A, Kambli P, Dhane V, Toihir AH, Rasolofo V, Andrianarisoa SH, Ranjalahy GM, Ramarokoto H. Identification of two protein-binding and functional regions of curli, a surface organelle and virulence determinant of Escherichia coli. J Biol Chem 2002;277:13568–72.

[15] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.

[16] Reddy TB, Riley R, Wymore F, Montgomery P, DeCaprio D, Engels R, Celisches M, Hubble J, Den J, H, Koehrens M, Larson L, Mao M, Nitzberg M, Sisk D, Stolte C, Weinig R, Magowan J, Zacharias JD, Burkhard P, Aebi U, Müller SA. Schoolnik GK. TB database: an integrated platform for tuberculosis research. Nucleic Acids Res 2009;37(Database issue):D499–508.

[17] Kocic AS, Djesna AL, Jurek J, Verba RG, GC, Meyerson MS, Patte, S. PathSeq: software to identify or discover microbes by deep sequencing of human tissue. Nat Biotechnol 2011;29:393–6.

[18] Lew JM, Kaapoopoulou A, Jones LM, Cole ST. Tuberculosis — 10 years after. (Edinb) 2011;191:1–7.

[19] Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B. Artemis: sequence visualization and annotation. Bioinformatics 2000;16:444–5.

[20] Bret J, Yachdav G, Liu S. The PredictProtein server. Nucleic Acids Res 2004;32(Web server issue):W321–6.

[21] Chang Bioscience. Abi Pro 3D: Peptide antigen design. http://www.changbioscience.com/abi_pro.htm [accessed 15.06.12].

[22] Vais P, Kleinling D, de la Huerga SL, van Embden JD. DNA fingerprinting of Mycobacterium tuberculosis. Methods Enzymol 1994;235:196–205.

[23] Velayati AA, Farnia P, Masjedi MR. Pili in totally drug resistant Mycobacterium tuberculosis (TDR-TB). Int J Mycobact 2012;1:57–8.

[24] Ramsugit S, Guma S, Pillay B, Jain P, Larsen MH, Danaviah S, Pillay M. Pili contribute to biofilm formation in vitro in Mycobacterium tuberculosis. Antonie Van Leeuwenhoek 2013;104:725–35.

[25] Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. Nat Rev Microbiol 2006;4:509–19.

[26] Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, Johnson PDR, Abdellah Z, Arrowsmith C, Chillingworth T, Churcher C, Clarke K, Cronin A, Davis P, Goodhead I, Holroyd N, Jagels K, Lord A, Moule S, Mangall K, Norbeck H, Qual M, Edelin) 2013;93:239–9.

[27] Small PL, Brosch R, Naraharakhan L, Fischbach MA, Parkhill J, Cole ST. Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. Genome Res 2008;18:729–41.

[28] Clark HP, Shepard C, Dschirla C, Burkard P, Hamborg B. Environmental infections on infection with Mycobacterium marinum (balone) of mice and a number of poikilothermic species. J Bacteriol 1963;86:1057–69.

[29] Barnhart MM, Chapman MR. Curli biogenesis and function. Annu Rev Microbiol 2006;60:131–47.

[30] Collinson SK, Parker JM, Hodges RS, Kay WW. Structural predictions of AgfA, an immunochromatographic assay and smear morphology. J Clin Microbiol 2007;45:3101–6.

[31] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.

[32] Weiser NJ, van Embden JD. DNA fingerprinting of Mycobacterium tuberculosis. Methods Enzymol 1994;235:196–205.

[33] Velayati AA, Farnia P, Masjedi MR. Pili in totally drug resistant Mycobacterium tuberculosis (TDR-TB). Int J Mycobact 2012;1:57–8.

[34] Ramsugit S, Guma S, Pillay B, Jain P, Larsen MH, Danaviah S, Pillay M. Pili contribute to biofilm formation in vitro in Mycobacterium tuberculosis. Antonie Van Leeuwenhoek 2013;104:725–35.

[35] Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. Nat Rev Microbiol 2006;4:509–19.
Steingart KR, Henry M, Laal S, Hopewell PC, Ramsay A, Menzies D, Cunningham J, Weldingh K, Pai M. Commercial serological antibody detection tests for the diagnosis of pulmonary tuberculosis: a systematic review. PLoS Med 2007;4:202.

Steingart KR, Dendukuri N, Henry M, Schiller I, Nahid P, Hopewell PC, Ramsay A, Pai M, Laal S. Performance of purified antigens for serodiagnosis of pulmonary tuberculosis: a meta-analysis. Clin Vaccine Immunol 2009;16:216–76.

Zhang L, Wang Q, Wang W, Liu Y, Wang J, Yue J, Xu Y, Xu W, Cui Z, Zhang X, Wang H. Identification of putative biomarkers for the serodiagnosis of drug-resistant Mycobacterium tuberculosis. Proteome Sci 2012;10:12.

Arend SM, Geluk A, van Meijgaarden KE, van Dissel JT, Theisen M, Andersen P, Ottenhoff THM. Antigenic equivalence of human T-cell responses to Mycobacterium tuberculosis-specific RD1-encoded protein antigens ESAT-6 and culture filtrate protein 10 and to mixtures of synthetic peptides. Infect Immun 2000;68:3314–21.

Waters WR, Nonnecke BJ, Palmer MV, Robbe-Austermann S, Bannantine JP, Stabel JR, Whipple DL, Payer JB, Estes DM, Pitzeer JE, Minion FC. Use of recombinant ESAT-6: CFP-10 fusion protein for differentiation of infections of cattle by Mycobacterium bovis and by M. avium subspp. avium and M. avium subspp. paratuberculosis. Clin Diag Lab Immunol 2004;11:729–35.

Kashyap RS, Shekhawat SD, Nayak AR, Purohit HJ, Taori GM, Daginawala HF. Diagnosis of tuberculosis infection based on synthetic peptides from Mycobacterium tuberculosis antigen 85 complex. Clin Neurol Neurosurg 2013;115:678–83.

Zhu C, Liu J, Ling Y, Yang H, Liu Z, Zheng R, Qin L, Hu Z. Evaluation of the clinical value of ELISA based on MPT64 antibody aptamer for serological diagnosis of pulmonary tuberculosis. BMC Infect Dis 2012;12:96.

Muletwa R, Boehme C, Dimairo M, Bandason T, Munyati SS, Mangwanyana D, Mungofa S, Butterworth AE, Mason PR, Corbett EL. Diagnostic accuracy of commercial urinary lipoarabinomannan detection in African tuberculosis suspects and patients. Int J Tuberc Lung Dis 2009;13:1253–9.

Goletti D, Raja A, Kabeer BSA, Rodrigues C, Sodha A, Carrara S, Vernet G, Longuet C, Ippolito G, Thangaraj S, Leportier M, Girardi E, Lagrange PH. Is IP-10 an accurate marker for detecting M. tuberculosis-specific response in HIV-infected persons? PLoS One 2010;5:12577.

Sartain MJ, Slayden RA, Singh KK, Laal S, Belisle JT. Disease state differentiation and identification of tuberculosis biomarkers via native antigen array profiling. Mol Cell Proteomics 2006;5:2102–13.

Achkar JM, Dong Y, Holzman RS, Belisle J, Kourbeti IS, Sherpa T, Condos R, Rom WN, Laal S. Mycobacterium tuberculosis malate synthase- and MPT51-based serodiagnostic assay as an adjunct to rapid identification of pulmonary tuberculosis. Clin Vaccine Immunol 2006;13:1291–3.

Achkar JM, Jenny-Avital E, Yu X, Burger S, Leibert E, Bilder PW, Almo SC, Casadevall A, Laal S. Antibodies against immunodominant antigens of Mycobacterium tuberculosis in subjects with suspected tuberculosis in the United States compared by HIV status. Clin Vaccine Immunol 2010;17:384–92.

Wanchu A, Dong Y, Sethi S, Myneedu VP, Nadas A, Liu Z, Belisle J, Laal S. Biomarkers for clinical and incipient tuberculosis: performance in a TB-endemic country. PLoS One 2008;3:2071.