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DIAGNOSTICS

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

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SUMMARY

Novel biomarkers are urgently needed for point of care TB diagnostics. In this study, we investigated the potential of the pilin subunit protein encoded by the *mtp* gene as a diagnostic biomarker. BLAST analysis of the *mtp* gene on published genome databases, and amplicon sequencing were performed in *Mycobacterium tuberculosis* Complex (MTBC) strains and other organisms. The protein secondary structure of the amino acid sequences of non-tuberculous Mycobacteria that partially aligned with the *mtp* sequence was analysed with PredictProtein software. The *mtp* gene and corresponding amino acid sequence of MTBC were 100% homologous with H37Rv, in contrast to the partial alignment of the non-tuberculous Mycobacteria. The *mtp* gene was present in all 91 clinical isolates of MTBC. Except for 2 strains with point mutations, the sequence was 100% conserved among the clinical strains. The *mtp* gene could not be amplified in all non-tuberculous Mycobacteria and respiratory organisms. The predicted MTP protein structure of *Mycobacterium avium*, *Mycobacterium ulcerans* and *Mycobacterium abscessus* differed significantly from that of the *M. tuberculosis*, which was similar to *Mycobacterium marinum*. The absence of the *mtp* gene in non-tuberculous Mycobacteria and other respiratory bacteria suggests that its encoded product, the pilin subunit protein of *M. tuberculosis* may be a suitable marker for a point of care TB test.

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 canettii* 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

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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 pili, 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, pili 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 pili 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% CO_2 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**		91
F15/LAM4/KZN		20
Beijing		20
F11		10
F28		10
Unique		8
Other clustering strains		18
M. africanum		1
M. canettii		1
M. microti		1
M. bovis		1
M. bovis BCG		1
Non-tuberculous Mycobacteria		34
M. scrofulaceum		1
M. intracellulare		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		1
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 catarrhalis		1
Legionella pneumophila		1
Klebsiella pneumoniae	700623	1
Pseudomonas aeruginosa	27853	1
Burkholderia cepacia		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*, *Mycobacterim 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 trimethylammonium 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′, flanking the gene 90 bp upstream and 180 bp downstream of the gene. The amplicon size of the PCR product is 582 bp. The PCR master mix contained a final concentration of 1X *Taq* buffer (Roche Applied Science), 0.2 mM of each primer, 0.25 mM of dNTPs (Roche Applied Science), nuclease free water and 0.5 U *Taq* Polymerase (Roche Applied Science) in a reaction volume of 25 μl. Cycle conditions comprised of: initial 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*

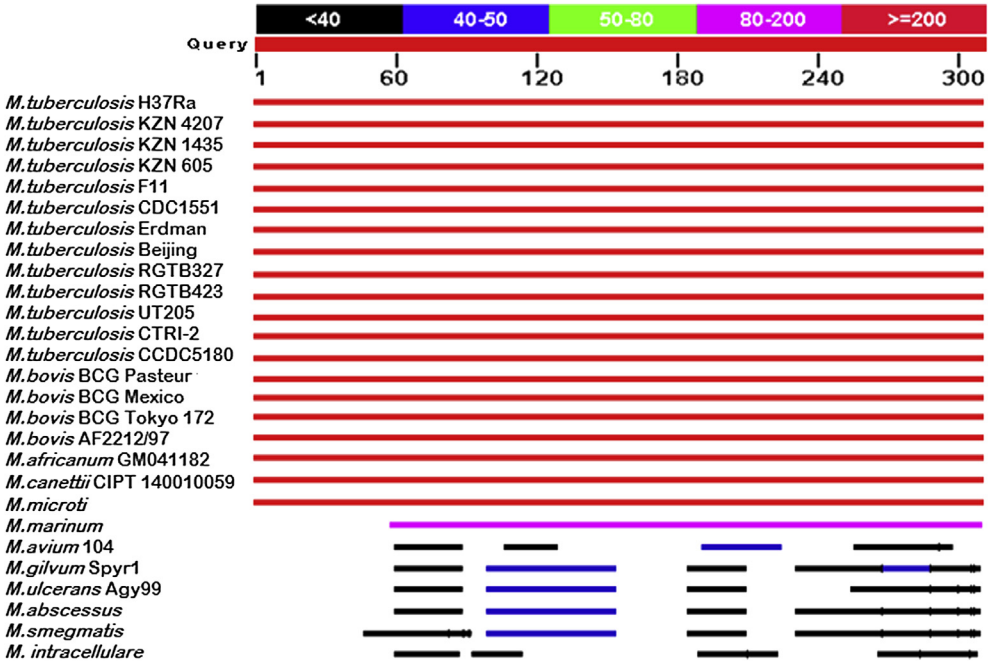

Figure 1. A pictorial representation of the BLAST analysis of the *mtp* gene of MTBC and NTM from the public genome databases. This was modified in the NCBI output diagram to show the organisms adjacent to a colour bar that represents the degree of homology of each organism's sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The query bar represents the input sequence of 312 base pairs of *M. tuberculosis* H37Rv that was 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.)

Table 2
Genomic BLAST analysis of MTBC, NTM, and respiratory organisms for the presence of the *mtp* gene. Publically available genomic databases were used to compare e-values and homology between *mtp* genes in various microbial species.

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. canettii	e^{-176}
M. microti	e^{-176}
M. marinum	$6e^{-15}$
M. paratuberculosis paratuberculosis K-10	0.087
Mycobacterium sp. MCS	0.087
Mycobacterium sp. KMS	0.087
Mycobacterium sp. JLS	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 Agy99	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. liflandii	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.

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 *Paracoccidiodes 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. canettii*. 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

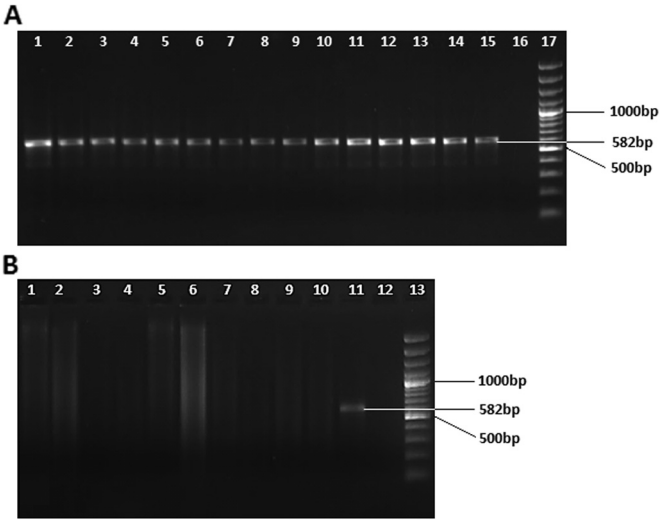

Figure 2. Detection of the *mtp* gene by PCR amplification in the MTBC and NTM strains. (A) MTBC: lane 1: F15/LAM4/KZN strain, lane 2: Beijing strain, lane 3: F11 strain, lane 4: F28 strain, lane 5: cluster 5, lane 6: cluster 6, lane 7: cluster 7, lane 8: cluster 8, lane 9: unique strain, lane 10: *M. bovis*, lane 11: *M. bovis* BCG, lane 12: *M. africanum*, lane 13: *M. canettii*, lane 14: *M. microti*, lane 15: H37Rv, lane 16: negative control (nuclease free water) and lane 17: Molecular weight marker (Fermentas). The amplicon size was 582 bp. (B) NTMs: lane 1: *M. fortuitum*, lane 2: *M. abscessus*, lane 3: *M. scrofulaceum*, lane 4: *M. intracellulare*, lane 5: *M. gordonae*, lane 6: *M. avium*, lane 7: *M. chelonae*, lane 8: *M. marinum*, lane 9: *M. smegmatis*, lane 10: *M. ulcerans*, lane 11: H37Rv, lane 12: negative control (nuclease free water) and lane 13: Molecular weight marker (Fermentas).

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 6.3% strand structure and no α-helix. *M. ulcerans* amino acid sequence had the

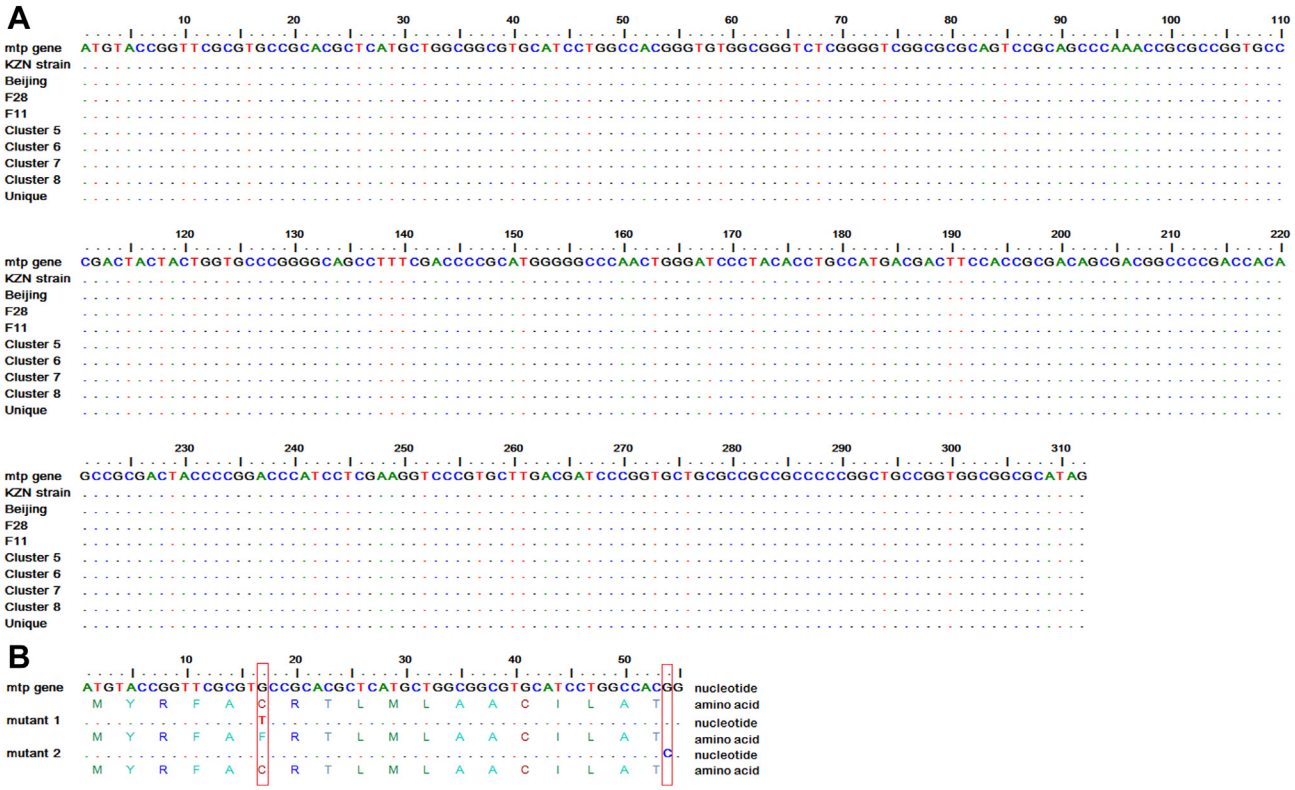

Figure 3. (A) Multiple sequence alignment of the *mtp* gene sequences of representative isolates in KwaZulu-Natal (KZN). The KZN, Beijing, F28, F11 and clusters 5–8 strains represent strain families dominant in KZN. The unique strain was isolated from a single patient in KZN. The alignment is shown as 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.

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*.

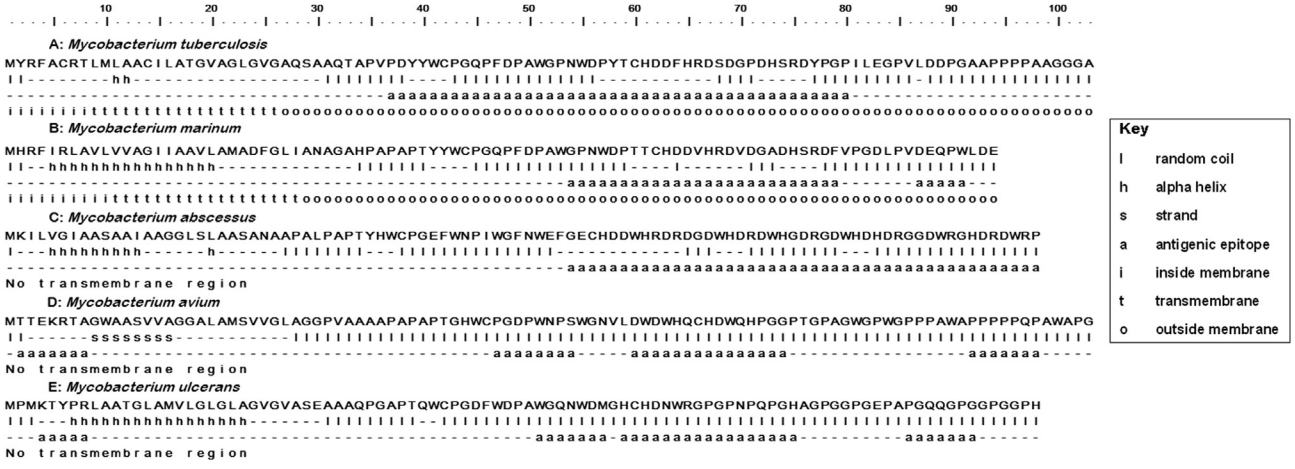

Figure 4. Bioinformatics analysis using Protein Predict and Abie Pro software of the *M. tuberculosis* pili (MTP) protein and the proteins of the NTMs that showed a partial homology upon BLAST analysis. (A) *M. tuberculosis* pilin protein has a transmembrane region (t), contains one antigenic epitope (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 pili 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 amplicon 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 NTM 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. ulcerans*, 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, pili 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 pili 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.

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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.

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