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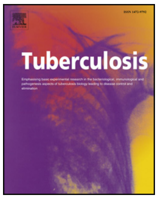

Immunological Aspects

Immunoscreening of the *M. tuberculosis* F15/LAM4/KZN secretome library against TB patients′ sera identifies unique active- and latent-TB specific biomarkers

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ABSTRACT

Tuberculosis (TB) protein biomarkers are urgently needed for the development of point-of-care diagnostics, new drugs and vaccines. *Mycobacterium tuberculosis* extracellular and secreted proteins play an important role in host-pathogen interactions. Antibodies produced against *M. tuberculosis* proteins before the onset of clinical symptoms can be used in proteomic studies to identify their target proteins. In this study, *M. tuberculosis* F15/LAM4/KZN strain phage secretome library was screened against immobilized polyclonal sera from active TB patients ($n = 20$), TST positive individuals ($n = 15$) and *M. tuberculosis* uninfected individuals ($n = 20$) to select and identify proteins recognized by patients′ antibodies. DNA sequence analysis from randomly selected latent TB and active TB specific phage clones revealed 118 and 96 ORFs, respectively. Proteins essential for growth, virulence and metabolic pathways were identified using different TB databases. The identified active TB specific biomarkers included five proteins, namely, TrpG, Alr, TreY, BfrA and EspR, with no human homologs, whilst latent TB specific biomarkers included NarG, PonA1, PonA2 and HspR. Future studies will assess potential applications of identified protein biomarkers as TB drug or vaccine candidates/targets and diagnostic markers with the ability to discriminate LTBI from active TB.

1. Introduction

Nearly 2 million deaths and more than 10.4 million cases of tuberculosis (TB) were reported globally in 2016 [1]. Approximately half of the people exposed to TB develop clinical symptoms within a year [2]. Individuals with latent TB infection (LTBI) have a 10% chance of developing active TB (ATB) during their lifetime [3]. Approximately, one-third of the world's population is latently infected with TB [1]. Co-infection with HIV increases the risk of progression to ATB by up to 10% per year, making TB the leading cause of death from an opportunistic infection in HIV/AIDS patients [3,4]. Therefore, LTBI plays a critical role in the current TB pandemic and is a constant source of new cases of TB disease especially among HIV/AIDS patients [2].

During infection, *Mycobacterium tuberculosis* secretes proteins into the surrounding host environment, to play an important role in host-pathogen interactions. Antibodies against mycobacterial proteins (antigens) are produced before the onset of clinical symptoms [5–8]. These antibodies target all immunologically relevant pathogen specific antigens [9] and may shed some light on the *M. tuberculosis* protein expression profiles during infection. However, the antibody response differs widely among TB patients [10] and this may be due to the disease progression stage during sampling, the relative specific antigen dominancy and the *M. tuberculosis* strain antigen composition [11,12]. Nevertheless, these antibodies can be used in proteomic studies to identify their cognate *M. tuberculosis* antigens or epitopes [13]. This could lead to the discovery of disease specific protein biomarkers that can be used as TB drugs and vaccine candidates or diagnostic or prognostic biomarkers. The significance of the discovery of disease state-specific biomarkers lies in their potential use to develop tools that can differentiate ATB from LTBI and possibly, monitor TB disease progression from LTBI to ATB, especially in high risk groups such as HIV infected patients.

Phage display is a powerful *in vitro* selection technology that can be used to specifically extract proteins with novel and desired properties

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from large protein libraries [14]. In the present study, a *M. tuberculosis* F15/LAM4/KZN phage secretome library [15] was interrogated by immunoscreening against patients′ sera to identify immunogenic LTBI and ATB specific proteins recognized by their antibodies. Sera from LTBI and ATB patients were used for isolation by affinity binding of phage particles displaying TB disease state-specific peptides. Therefore, the current study was aimed at the identification of novel, easily accessible disease-specific *M. tuberculosis* secreted biomarkers that can be used to distinguish ATB from LTBI.

2. Materials and methods

2.1. Patient recruitment and specimen collection

The study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (Ref. BE236/13). Participants were considered for inclusion if they were ≥18 years of age and willing to undergo an HIV test. The study participants were patients attending clinic with suspected respiratory disease that included active pulmonary TB, as well as latent TB and other illness. Fifty-five study participants were recruited with informed consent from the Centre for the AIDS Programme of Research in South Africa (CAPRISA) eThekwini Clinic, KwaZulu-Natal. All participants included in the study were HIV negative and not on TB treatment. HIV positive individuals were excluded from the study due to their poor immunological response to TB infection. The GeneXpert/MTB/RIF assay was used as a TB confirmatory test and GeneXpert positive participants were classified as ATB group ($n = 20$). GeneXpert negative participants were subjected to the tuberculin skin test (TST) for the detection of LTBIs. Subjects with an induration of ≥10 mm were TST positive individuals ($n = 15$), and TST negative ($n = 20$) as *M. tuberculosis* uninfected (MTB-uninfected) subjects. The TST positive individuals will be interchangeably referred to as LTB infected individuals.

Peripheral blood (5 mL) was collected using the closed blood collection method into BD Vacutainer serum tubes (red top, with clot activator and gel). The specimen was immediately mixed by inverting 6 to 10 times and stored at 4 °C before processing. After centrifugation at $1500 \times g$ for 10 min, serum was stored in aliquots at −80 °C until use.

2.2. Panning of M. tuberculosis F15/LAM4/KZN phage secretome library

A phage library displaying *M. tuberculosis* F15/LAM4/KZN secretome was constructed previously [15] using the pDJ01 phagemid vector [16]. Serum samples for each group of study participants were pooled, diluted 1:100 in phosphate-buffered saline (PBS) (pH 7.2) and used to coat the ELISA plate wells at 4 °C overnight. The secretome phage library was exposed to the immobilized serum samples. The concentration of phage particles at the beginning of each of 3 rounds for both LTB and ATB panning was 1×10^{11} pfu. In order to eliminate non-specific immunogenic peptides, the library was pre-incubated with immobilized sera from MTB-uninfected participants. Selective targeting of disease state specific phage displayed protein peptides was achieved by exposing the TB-free pre-absorbed library to LTB sera before the selection on ATB sera to identify ATB specific proteins. LTB proteins were selected by exposing the TB-free pre-absorbed library first to ATB sera and then to LTB sera. The binding phages were eluted with 0.1 M TEA (triethanolamine) for 10 min at room temperature. The eluate was neutralized with 1 M Tris buffer (pH 7), and amplified by infecting exponentially growing *E. coli* TG1 cells. Amplified phages were subjected to two further rounds of selective screening to enrich for clones that are specifically recognized by the patients' sera. After three successive rounds of interrogation against TB patients′ sera (Fig. 1), affinity binding and immunogenic LTB and ATB specific *M. tuberculosis* phage displayed protein peptides were enriched. For LTB selection, the amount of recovered phage particles for the three successive rounds was 1.8×10^3, 3.2×10^4 and 2.4×10^5 pfu respectively. The ATB recovered phage particles for three successive rounds was 2.2×10^3, 5.2×10^4 and 3.9×10^5 pfu respectively. Enrichment was calculated as the ratio of number of eluted phages in the third round of panning relative to the first round of panning.

2.3. DNA sequence analysis

The inserts of randomly selected clones were sequenced using the primer pDJ01R02 (5′-CCGGAAACGTCACCAATGAA-3′) and pDJF03 (5′-ATGTTGCTGTTGATTCTTCA-3′). The DNA sequences were analysed using the CLCBio Workbench 2.0. BLAST analysis of the nucleotide sequences against the *M. tuberculosis* database was performed in order to retrieve encoded open reading frames (ORFs) and complete protein sequences.

2.4. Functional categories and gene ontologies

The ORFs functional category information for annotated genes was retrieved from the TubercuList database (http://tuberculist.epfl.ch/) [17]. Protein sequences of unknown genes that are not documented on TubercuList database were categorized as unknown functional category. For further assignment of possible function, gene ontology (GO) features of identified LTB and ATB proteins were obtained using Universal Protein Resource Knowledgebase (UniProtKB) database (http://www.uniprot.org/) [18]. The Web Gene Ontology Annotation Plotting (WEGO) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) was used to analyze the GO categories [19].

2.5. Metabolic pathways and specialty proteins identification

The Pathosystems Resource Integration Centre (PATRIC) [https://www.patricbrc.org] and KEGG (Kyoto Encyclopedia of Genes and Genomes) [http://www.genome.ad.jp/kegg] databases were used to identify metabolic pathway proteins [20]. PATRIC specialty protein list was used to identify proteins essential for *M. tuberculosis* growth, virulence and survival during host infection [21].

3. Results and discussion

3.1. Demographic and clinical characteristics of participants

The characteristics of the treatment naïve participants are summarised in Table 1. Of the 55 participants, 67% were males. Their mean age was 39.18 years. Twenty patients tested positive for active TB with the GeneXpert, whilst 15 were TST positive and classified as LTBI patients. The 20 *M. tuberculosis* uninfected individuals were TST negative.

3.2. Identification of disease state specific proteins

After three successive rounds of immunoscreening against sera from LTBI ($n = 15$) and ATB patients ($n = 20$), affinity binding and immunogenic LTB and ATB specific *M. tuberculosis* phage displayed protein peptides were enriched by 133-fold and 177-fold, respectively. At the last round of panning, clones were selected randomly, and 157 and 127 phage DNA were sequenced for LTBI and ATB, respectively. DNA sequence analysis revealed 125 LTBI and 100 ATB distinct sequences. The BLAST search against *M. tuberculosis* databases identified 118 LTBI and 96 ATB encoded open reading frames (ORFs).

The immunodominant antigens recognized by *M. tuberculosis* infected humans are mostly secreted proteins, including well-studied antigens such as MPT64, the 6-kDa early secreted antigenic target (ESAT6), the 10-kDa culture filtrate protein (CFP10), and the antigen 85 (Ag85) complex [22,23]. However, it should be noted that ESAT6, CFP10, TB10.4 and other proteins are secreted via the type VII ESX pathway. The phage display method used in the current study had not been shown to facilitate successful display of proteins secreted via the

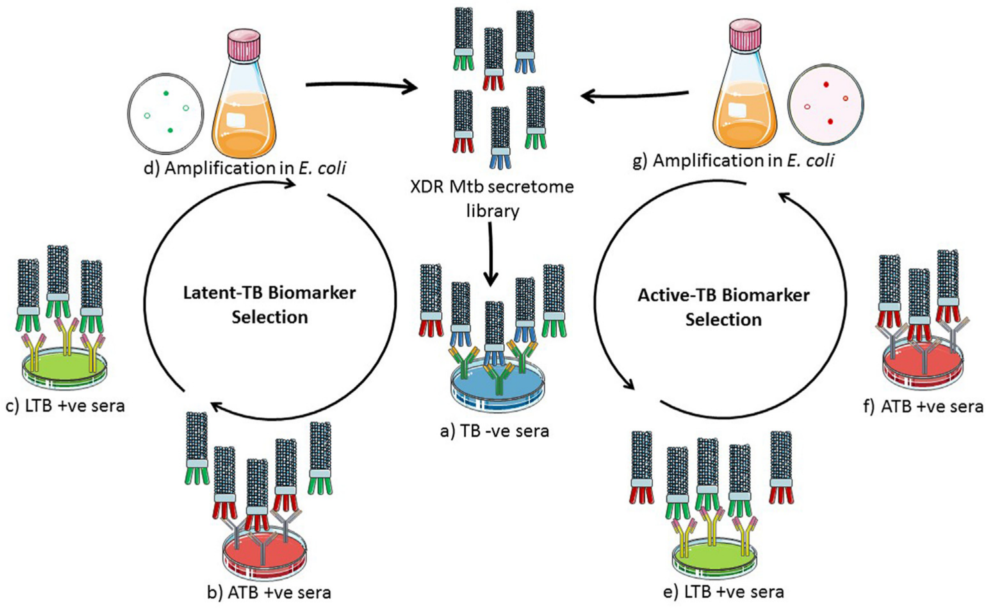

Fig. 1. Overview of steps involved in *M. tuberculosis* phage secretome library immunoscreening against clinical serum samples. For selection of LTB specific biomarkers, the library was initially exposed to: a) control TB –ve sera from MTB-uninfected participants to pre-absorb and remove phage particles displaying "ubiquitous" protein peptides. b) The unbound phages were transferred to a well coated with ATB + ve sera from ATB participants to remove protein peptides recognized by patients' antibodies. c) Final selection was performed against LTB + ve sera from LTB infected participants. Selection of ATB specific biomarkers began with a) TB –ve sera, followed by screening against e) LTB + ve sera from LTB infected patients and final screening f) against ATB + ve sera from ATB patients.

Table 1
The summary of study participants′ gender, mean age and disease status.

Status	Male	Female	Mean age	Enrolled
GeneXpert positive	16	4	36.5	20
TST-positive	9	6	37.6	15
MTB-uninfected/TST negative	12	8	43.45	20
Overall	**37**	**18**	**39.18**	**55**

non-classical pathway as the surrogate *E. coli* used was not expected to mimic the *M. tuberculosis* type VII secretion pathway [15]. This would limit the discovery of most type VII/ESX secretion substrates as similarly observed in the study by Liu et al. who noted the lack of ESX-1 pathway antigens [24].

Nevertheless, some of the well-known antigenic proteins including Apa (Rv1860), CPF2 (Rv2376c), MPT51 (Rv3803c), MPT53 (Rv2878c), MPT63 (Rv1926c), MPT64 (Rv1980c), MPT70 (Rv2875), Ag85B (Rv1886c) and TB22.2 (Rv3036c) were identified in the present study. These include the abundantly secreted *M. tuberculosis* proteins found on the culture filtrate such as the Ag85 complex family that comprised of three proteins Ag85A, Ag85B, and Ag85C [25]. Ag85 complex proteins are involved in the final step of cell wall assembly, required for the maintenance of the bacterial cell envelope integrity and stimulate a strong humoral- and cell-mediated immune response [26]. The abundance of serum antibodies generated against the Ag85 complex in TB patients provides further support that the Ag85 complex could also function as a promising diagnostic marker [27]. In this study, we identified Ag85B and the closely related MPT51 which are responsible for the high affinity of mycobacteria to the host tissue cells [28]. The other identified major secreted immunogenic proteins (MPT63, MPT64, MPT70, TB22.2, MPT53, Apa and CFP-2) are known mycobacterial adhesins and proteins that contribute to the intracellular survival of *M. tuberculosis* [29]. Interestingly, similar proteins (Ag85B, Apa, MPT51, MPT53, MPT63, MPT64 and CFP2) were identified in exosomes isolated from human serum samples of TB patients [29]. Therefore, the identified proteins could serve as good candidates for development of antigen detection assay for TB diagnosis.

The ORFs were allocated functional categories according to the TubercuList database annotation [17]. The LTBI and ATB selected proteins were distributed to ten different TubercuList functional categories (Fig. 2). The majority of LTBI (43) and ATB (28) proteins were associated with the cell wall and cell processes. These were mainly surface membrane and transmembrane proteins involved in transportation of molecules across the cell wall and facilitate attachment to host target cells. The second most prevalent group of proteins were the conserved hypothetical proteins of unknown functions of which 22 were unique to the LTBI and 16 were unique to the ATB. Twenty-three proteins were observed to be common between the LTBI and ATB lists (Table 2). These included hypothetical proteins and immunogenic proteins such as Ag85B, MPT63, MPT64 and two PE/PPE family proteins, PE20 (Rv1806) and PE_PGRS54 (Rv3508).

3.3. *Gene ontology analysis*

Gene Ontology functional categories of 80 LTBI and 60 ATB proteins GO annotation were retrieved from UniProtKB database. WEGO analysis assigned the proteins to three functional categories; Cellular Component (CC), Molecular Function (MF), and Biological Process (BP), with a total of 31 GO terms (Fig. 3). Proteins involved in multiple activities or processes are assigned by WEGO to one or more associated GO terms and categories. Notably, 15 of 95 LTBI and 12 of 72 ATB proteins with either unknown function/s or unrecognized by Gene Ontology enrichment were not assigned to any functional categories.

Amongst the ATB proteins, the main CC terms comprised cell (42 proteins), cell part (41 proteins) and membrane (29 proteins). In the BP ontology, top terms included cellular process (27 proteins), metabolic process (26 proteins) and response to stimulus (11 proteins). The majority of MF category terms involved catalytic activity (31 proteins) and binding (24 proteins). The enzymes involved in catalytic activity were classified as oxidoreductase, transferase, hydrolase, lyase, ligase and isomerase.

For the LTBI specific proteins, the main CC terms included cell (60 proteins), cell part (59 proteins) and membrane (49 proteins). The main BP ontology terms comprised metabolic process (37 proteins), cellular process (31 proteins) and growth (13 proteins). The main MF terms included catalytic activity (43 proteins) and binding (26 proteins). The enzymes involved in catalytic activity were classified as oxidoreductase, transferase, hydrolase, lyase and ligase.

3.3.1. *Functional categories unique to ATB and LTBI*

3.3.1.1. ATB functional categories. The 3 GO terms unique to ATB included 2 MF terms (antioxidant and transcriptional regulator) and 1 BP term (immune system process). The antioxidant associated proteins were Rv2878c (MPT53) and Rv2429 (AhpD). MPT53 is a soluble

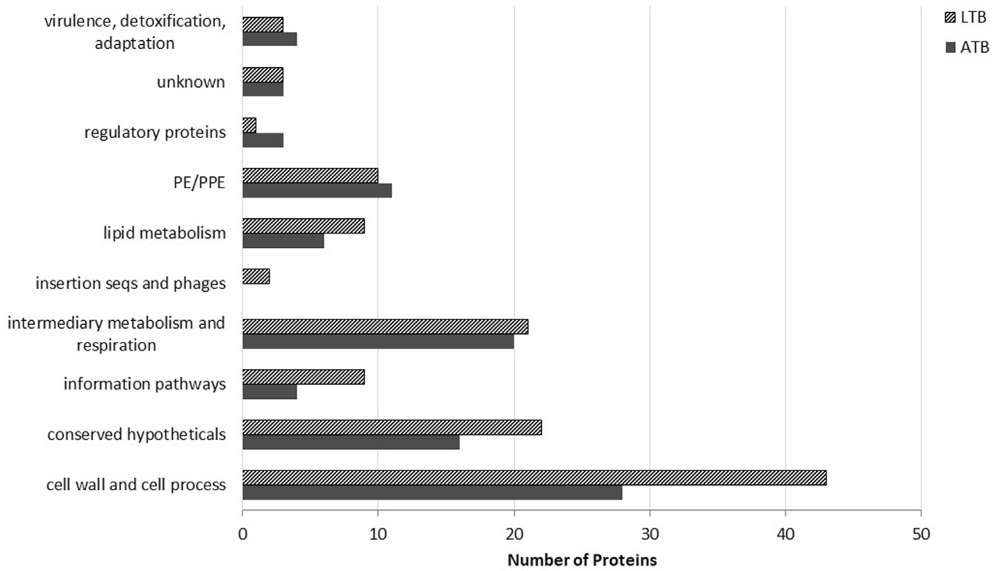

Fig. 2. TubercuList functional categories of proteins identified during selection for ATB and LTB biomarkers.

Table 2
List and description of 23 proteins common to ATB and LTB.

Rv	Name	Description
MT1330.1	*mt1330.1*	hypothetical protein MT1330.1
Rv0203	–	secreted protein with unknown function
Rv0327	*cyp135A1*	Cytochromes P450, a heme-thiolate monooxygenase Cyp135A1
Rv0630c	*recB*	exonuclease V (beta chain) RecB
Rv1009	*rpfB*	resuscitation-promoting factor RpfB.
Rv1029	*kdpA*	potassium-transporting ATPase a chain KdpA
Rv1133c	*metE*	Cobalamin-independent methionine synthase MetE
Rv1157c	–	Conserved secreted ala-, pro-rich protein
Rv1268	–	hypothetical outer membrane protein
Rv1269c	–	Conserved probable secreted protein
Rv1271c	–	Conserved hypothetical secreted protein
Rv1357c	–	Conserved hypothetical protein
Rv1703	–	Probable catechol-O-methyltransferase.
Rv1806	*pe20*	PE family protein PE20
Rv1886c	*fbpB*	Secreted antigen 85-B FbpB (85B)
Rv1926c	*mpt63*	Immunogenic 16 kDa protein Mpt63
Rv1980c	*mpt64*	Immunogenic protein Mpt64
Rv2152c	–	UDP-N-acetylmuramate-alanine ligase MurC
Rv2414c	–	Conserved hypothetical protein
Rv3157	*nuoM*	NADH dehydrogenase I (chain M) NuoK
Rv3218	–	Conserved protein. Function unknown
Rv3318	*sdhA*	succinate dehydrogenase flavoprotein subunit SdhA
Rv3508	*pe_pgrs54*	PE-PGRS family protein PE_PGRS54

secreted antigen reportedly recognized by TB patients′ humoral response [30] and AhpD is a reductase that is involved in oxidative stress response [31]. The transcription associated protein, Rv3058c is involved in transcriptional regulatory processes (TubercuList). The immune system process proteins comprised Rv2941 (fadD28), Rv3343c (PPE54), Rv1860 (Apa) and Rv1818c (PE_PGRS33) which are involved in negative regulation or modulation of host immune response. FadD28 is involved in fatty acid biosynthesis and together with PPE54, is involved in host phagosome maturation arrest [32]. Alanine and proline rich secreted protein, Apa facilitates the bacterial attachment to host cells [33]. The immunogenic PE-PGRS family protein, PE_PGRS33 facilitates cell surface interactions among mycobacteria and interactions of bacteria with macrophages [34,35].

3.3.1.2. LTBI functional categories. The 7 GO terms unique to LTBI included 1 CC term (organelle part), 4 MF terms (electron carrier, molecular transducer, structural molecule and translation regulator), and 2 BP terms (anatomical structure formation and developmental process). The organelle part protein was identified as Rv3459c (RpsK) that is essential for selection of protein biosynthesis (UniProtKB). The electron carrier activity proteins were Rv1161 (NarG) and Rv0688 encoding the putative ferredoxin reductase. NarG catalyses the consumption of nitrate in *M. tuberculosis* [36] while the putative ferredoxin reductase is involved in the transfer of electrons in various metabolic reactions (TubercuList). The molecular transducer protein (Rv3365c) is a conserved hypothetical protein proposed to be involved in cell-associated regulatory functions [37]. The translation regulator activity protein Rv2839c encoding InfB is an essential component for the initiation of protein synthesis (TubercuList). The developmental process protein (Rv0050) also known as PonA1 is involved in cell wall formation by synthesis of cross-linked peptidoglycan from the lipid intermediates [38].

3.3.2. Differentially expressed functional proteins

3.3.2.1. ATB differentially expressed proteins. The ATB differentially expressed proteins included 3 isomerases (Alr, TreY and TopA), the extracellular protein EspR (Rv3849) and the organelle proteins Rv1526c and PE_PGRS47 (Rv2741). Isomerase Alr is involved in peptidoglycan cross-linking through conversion of L-alanine to D-alanine which is a necessary precursor for peptidoglycan biosynthesis [39]. TreY is involved in starch and sucrose metabolism, specifically, trehalose biosynthesis. TopA is involved in DNA transcription and reportedly required for adaptation and survival of *M. tuberculosis* under stressful conditions. EspR is associated with cell wall functions and pathogenesis through regulation of multiple genes, including the *espACD* operon, which is a key ESX-1 component [40]. Rv1526c is a glycosyltransferase and is thought to be involved in cellular metabolism (TubercuList). The PE-PGRS family protein, PE_PGRS47 is required for growth and survival of *M. tuberculosis* during chronic TB and is also involved in evasion of innate and adaptive immunity [41].

3.3.2.2. LTBI differentially expressed proteins. The LTBI differentially expressed proteins comprised SahH (Rv3779) associated with growth, the host intracellular part protein, SapM (Rv3310) and three cellular component's organization or biogenesis proteins DnaB (Rv0058), EmbC (Rv3793), and FadD17 (Rv3506). The S-adenosyl-L-homocysteine hydrolase (SahH) is involved in regulation of the intracellular concentration of adenosylhomocysteine and is essential for bacterial growth [42,43]. The acid phosphatase (SapM) plays an important role

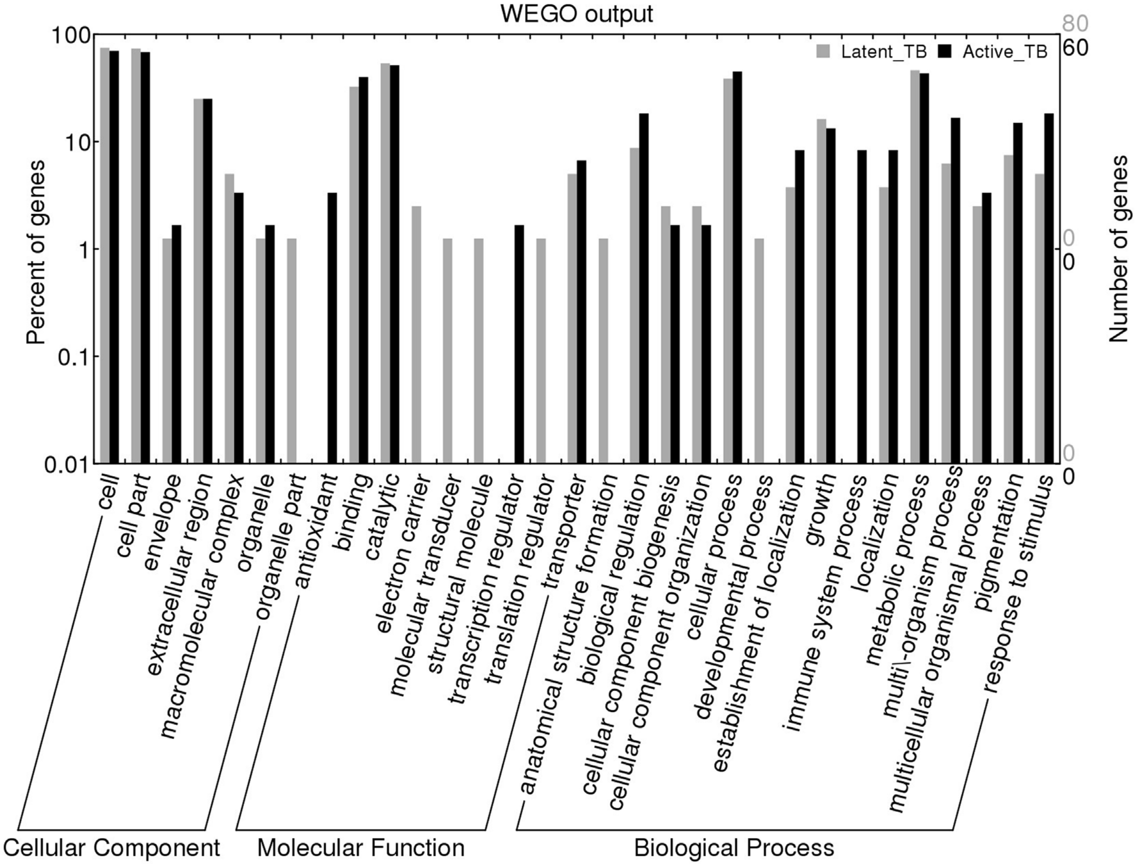

Fig. 3. Histogram of gene ontology classifications of ATB and LTB specific *M. tuberculosis* proteins recognized by sera of TB patients. The WEGO plot shows the three main GO categories: cellular component, molecular function and biological process. The right y-axis indicates the number of genes in a category. The left y-axis indicates the percentage of a specific category of genes in that main category. The WEGO plotting parameters were transformed into a log10 scale to represent both highly and lowly-enriched GO functions.

in blocking phagosome-lysosome fusion, thus participating in the intracellular survival of the pathogen [44]. The Arabinosyltransferase C (EmbC) is an integral membrane protein involved in the biosynthesis of the mycobacterial cell wall arabinan required for resistance to anti-TB drug ethambutol, hence, essential for *M. tuberculosis* growth [43,45] and survival. EmbC is a potential drug target and can be considered for use in TB diagnosis. FadD17 is required for salvaging cholesterol. The *M. tuberculosis* genome encodes 35 *fadD* genes which are suspected to be associated with fatty acid biosynthesis (TubercuList). When cholesterol is used by *M. tuberculosis* as a carbon source, up-regulated genes include *fadD17*, *fadD18*, *fadD19* and *fadD3* [46].

3.4. Metabolic pathway proteins

During LTBI and ATB, the expression of certain metabolic pathway genes is upregulated or downregulated [47]. The metabolic pathway genes encoded proteins (MPPs) are involved in catabolism and biosynthesis of molecules essential for growth of *M. tuberculosis.* Therefore these are attractive drug targets since inhibition of their functions may kill the pathogen. The *M. tuberculosis* PATRIC and KEGG metabolic pathway databases have 840 and 605 MPPs, respectively. The individual databases have some overlapping MPPs between databases and also database specific MPPs (Fig. 4). The databases were used to identify 14 ATB and 21 LTBI specific MPPs involved in *M. tuberculosis* metabolic processes (Table 3; Fig. 4).

Analysis of the generated protein lists revealed that different *M. tuberculosis* metabolic pathways were enriched. The pathways unique to ATB were amino acid biosynthesis, carbohydrate metabolism and iron metabolism, while cofactor and coenzyme biosynthesis, nitrogen metabolism and carbonic metabolism were unique LTBI enriched pathways. Among the complete list of metabolic pathways (Table 3), cell wall biosynthesis and lipid metabolism pathways were common to both ATB and LTBI.

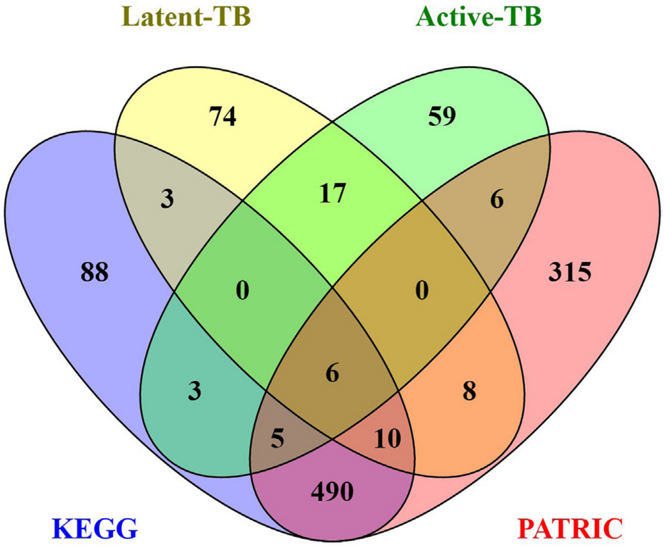

Fig. 4. Venn diagram showing metabolic pathway proteins from KEGG and PATRIC databases that overlap with the selected ATB and LTB proteins. There were 14 ATB and 21 LTB specific metabolic pathway proteins that were identified.

Table 3

List of metabolic pathway proteins that were uniquely enriched during selection in identified ATB and LTB immunoscreening.

Pathway	Rv	Name	Product	Function
Active TB specific MPPs				
Amino acid biosynthesis	Rv0013	*trpG*	Anthranilate synthase, amidotransferase component	Essential for the establishment, maintenance of infection and survival of *M. tuberculosis* [40], no human homolog.
	Rv2537c	*aroD*	3-dehydroquinate dehydratase II	Required for protein synthesis
Carbohydrates Metabolism	Rv1563c	*treY*	maltooligosyl trehalose synthase	Key component of a variety of glycolipids required for growth and virulence of *M. tuberculosis* [41–43], no human homolog.
	Rv3264c	*manB*	D-alpha-D-mannose-1-phosphate guanylyltransferase	
Lipid Metabolism	Rv1130	*metE/prpD*	5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase	Expressed during infection [44] and required for intracellular survival [45].
	Rv2252		diacylglycerol kinase	
Cell wall biosynthesis	Rv3330	*dacB1*	D-alanyl-D-alanine carboxypeptidase penicillin-binding protein 5/6	Control of cell elongation and septum development via the regulation of the peptidoglycan cross-linking [46,47].
	Rv3423c	*alr*	Alanine racemase	No human homolog, necessary precursor for peptidoglycan biosynthesis [31].
other metabolic associated	Rv1876	*bfrA*	Bacterioferritin	Required for efficient utilization of stored iron under low iron conditions [49,50], no human homolog.
	Rv2070c	*cobK*		Involved in cobalamin (vitamin B12) biosynthesis
	Rv3834c	*serS*	Seryl-tRNA synthetase	Essential gene involved in translation mechanism [35,51].
	Rv0509	*hemA*		
	Rv1393c	–	monoxygenase	
Latent TB specific MPPs				
Alternative energy metabolism	Rv1161	*narG*	Respiratory nitrate reductase alpha chain	Associated with virulence and supports anaerobic growth on glycerol [52,53] and up-regulated in the present of nitrate during dormant stage [28].
	Rv3273	*mtCA3*	Na(+)-dependent bicarbonate transporter BicA/Carbonic anhydrase, beta class	Involved in utilization of alternative carbon sources such as carbon dioxide or carbonic acid in the absence of primary carbon sources [54].
cofactor and coenzyme biosynthesis	Rv3119	*moaE1*		
	Rv3324c	*moaC3*		Essential for the catalytic activity of key enzymes involved in metabolism of carbon, nitrogen and sulphur [55].
	Rv3601c	*panD*		Essential for virulence [56].
Lipid Metabolism	Rv0222	*echA1*	Enoyl-CoA hydratase	
	Rv0769		3-oxoacyl-[acyl-carrier protein] reductase	
	Rv1141c	*echA11*	Enoyl-CoA hydratase	
	Rv1142c	*echA10*	Enoyl-CoA hydratase	
	Rv2249c	*glpD1*	Glycerol-3-phosphate dehydrogenase	
	Rv2277c		Glycerophosphoryl diester phosphodiesterase	Required for *in vivo* growth [48].
	Rv3229c	*desA3*	linoleoyl-CoA desaturase; stearoyl-CoA 9-desaturase	Required for *in vivo* growth [48].
	Rv3280	*accD5*	Propionyl-CoA carboxylase beta chain	Key enzyme in the catabolic pathway of odd-chain fatty acids and essential for growth [51].
	Rv3506	*fadD17*	Long-chain fatty-acid-CoA ligase, Mycobacterial subgroup FadD17	
Cell wall biosynthesis	Rv0050	*ponA1*	Pyrroline-5-carboxylate reductase	Expressed during intracellular growth [57] and are involved in stationary-phase survival under non-replicating conditions [58].
	Rv3682	*ponA2*	Multimodular transpeptidase-transglycosylase	
	Rv1884c	*rpfC*	Resuscitation-promoting factor RpfC	Promote the resuscitation and growth of dormant, non-growing cell [28].
	Rv3793	*embC*	Integral membrane indolylacetylinositol arabinosyltransferase EmbC	Required for anti-TB drug ethambutol resistance, essential for growth and intracellular survival [37,48].
Other metabolism associated	Rv1547	*dnaE*	DNA polymerase III alpha subunit	
	Rv3248c	*sahH*	Adenosylhomocysteinase	Involved in regulation of the intracellular concentration of adenosylhomocysteine and it is essential for bacterial growth [34,35].
	Rv3309c	*upp*	Uracil phosphoribosyltransferase	
	Rv3310	*sapM*	Acid phosphatase	Block phagosome-lysosome fusion, required intracellular survival [36].

3.4.1. Pathways unique to Active-TB

3.4.1.1. Amino acid biosynthesis. Two pathways, the amino acid biosynthesis and carbohydrate metabolism, were unique to ATB. Two amino acid biosynthesis enzymes identified included TrpG (Rv0013) and AroD (Rv2537c). Anthranilate synthase (TrpG) catalyses the biosynthesis of tryptophan which is essential for the establishment, maintenance of infection and survival of *M. tuberculosis* [48]. TrpG is secreted to the extracellular environment and is an essential enzyme involved in tryptophan biosynthesis pathway which is conserved in mycobacterial species, while absent in humans [48]. Therefore, TrpG is an ideal anti-TB drug target [49] and meets the criteria for a potential diagnostic biomarker for antigen detection assay. Since a good diagnostic biomarker must be abundantly expressed by *in vivo* bacteria, be present in the extracellular surrounding environment, and able to resist degradation by the host cellular enzymes [50]; further studies will be required to evaluate *in vivo* expression levels of TrpG and devise a strategy to enhance its detection if expressed at low levels.

The 3-dehydroquinase (AroD) catalyses the biosynthesis of aromatic amino acids such as tyrosine, tryptophan and phenylalanine [51]. Consequently, the co-selection of TrpG and AroD in the present study is indicative of actively growing *M. tuberculosis* that synthesizes precursors such as tryptophan and other aromatic amino acids that are required for protein synthesis. Interestingly, in the selected LTBI gene list no amino acid biosynthesis pathway associated proteins were identified. This is consistent with the report that the synthesis of certain *M. tuberculosis* proteins is reduced by more than 90% in order to shutdown most of the metabolic activities during dormancy [52]. This was supported by the down-regulation of tryptophan and methionine biosynthesis pathways during dormancy [47].

3.4.1.2. Carbohydrate metabolism. Two carbohydrate metabolism enzymes, ManB (Rv3264c) and TreY (Rv1563c) essential for growth [43] were identified from the ATB list. Of these, TreY (malto-oligosyltrehalose synthase) is involved in starch and sucrose metabolism, specifically, trehalose biosynthesis. Trehalose, a disaccharide, is a key component of a variety of glycolipids required for growth and virulence of *M. tuberculosis* [53–55]. Interestingly, trehalose is absent in mammals but produced by bacteria, plants, fungi and insects [53]. The trehalose biosynthesis pathway enzymes, products and by-products are abundantly available during the chronic TB disease stage [56], therefore, can serve as ATB diagnostic biomarkers.

3.4.1.3. Iron metabolism. Bacterioferritin A (BfrA; Rv1876), another ATB associated metabolic enzyme, is one of two iron storage proteins (BfrA and BfrB) encoded by the *M. tuberculosis* genome and is unique to bacteria [57,58]. BfrA is required for efficient utilization of stored iron under low iron conditions while BfrB (Rv3841) is required for storage of iron under iron excessive conditions and has been associated with the dormant phase (LTB) [58]. Since BfrA has no human homolog it may be a good ATB biomarker candidate.

3.4.2. Pathways unique to latent-TB

3.4.2.1. Alternative energy metabolism. The NarG (Rv1161) and MtCA3 (Rv3273) enzymes involved in alternative energy metabolism were identified in the LTBI list. Nitrate reductase (NarG), is a nitrogen metabolism enzyme, that catalyses the consumption of nitrate in *M. tuberculosis*. This enzyme belongs to the *narGHJI* operon that is associated with virulence and supports anaerobic growth on glycerol [59,60]. NarG is dependent on molybdopterin cofactor to perform its function, is conserved in mycobacteria [61] and up-regulated in the presence of nitrate during the dormant stage [36]. Therefore, since NarG is associated with LTBI, further investigations are required to determine potential application as drug target or diagnostic biomarker.

The *M. tuberculosis* genome contains at least three beta-class carbonic anhydrases, MtCA1 (Rv1284), MtCA2 (Rv3588c) and MtCA3 (Rv3273) [62]. The carbonic anhydrases are involved in utilization of alternative carbon sources such as carbon dioxide or carbonic acid in the absence of primary carbon sources [63]. In this study, we identified the transmembrane MtCA3 reportedly associated with pathogenesis and is a TB drug target [64].

3.4.2.2. Cofactor and coenzyme biosynthesis. The LTBI cofactor biosynthesis enzymes involved in the molybdopterin biosynthesis pathway include the molybdenum cofactor biosynthesis protein C (MoaC3) and molybdenum cofactor biosynthesis protein E (MoaE1), as well as aspartate alpha-decarboxylase (PanD) involved in the biosynthesis of the coenzyme, pantothenate. Molybdopterin cofactor is essential for the catalytic activity of key enzymes involved in metabolism of carbon, nitrogen and sulphur [65].

3.4.3. Comparable cell wall biosynthesis pathway

Peptidoglycan, the key component of *M. tuberculosis* cell wall, is made up of glycan chains and determines the bacterium cell shape. Glycan chains consist of two different sugars that are cross-linked via short peptide side chains. Peptidoglycan polymerization is mediated by enzymes such as bifunctional penicillin-binding proteins that can both polymerize glycan strands and cross-link peptides [45].

Amongst the ATB list, two enzymes associated with peptidoglycan synthesis, the penicillin-binding protein DacB1 (Rv3330) and the alanine racemase, Alr (Rv3423c), were identified. The eleven penicillin-binding proteins (PBPs) encoded by the *M. tuberculosis* genome can be divided into class A, B and C [66]. The membrane associated DacB1 (D-alanyl-ᴅ-alanine carboxypeptidase) is a member of class C penicillin-binding proteins that is required for the control of cell elongation and septum development via the regulation of the peptidoglycan cross-linking [66,67]. Alr is involved in peptidoglycan cross-linking through conversion of ʟ-alanine to ᴅ-alanine which is a necessary precursor for peptidoglycan biosynthesis [39]. Interestingly, DacB1 is known to be expressed by actively growing bacteria [68], while there is no known Alr homolog in humans making both proteins the potential ATB biomarkers for design of new drugs, vaccines or diagnostic assays.

The LTBI cell wall biosynthesis protein list included EmbC (Rv3793) and 2 bifunctional penicillin-binding protein enzymes, PonA1 (Rv0050) and PonA2 (Rv3682). Both PonA1 and PonA2 belong to class A penicillin-binding proteins [66] and are involved in cell wall formation by synthesis of cross-linked peptidoglycan from the lipid intermediates [38]. The *ponA1* and *ponA2* genes are reportedly expressed *in vivo* [69] and are involved in stationary-phase survival under non-replicating conditions [70]. Both PonA1 and PonA2 may well be involved in inhibition of resuscitation protein factors [71]. This inhibition is achieved by the reported increased expression of PonA1 during the dormant stage [72].

In the present study, two resuscitation protein factors (Rpf), RpfB (found in both LTBI and ATB) and RpfC (in LTBI only) were identified. The *M. tuberculosis* genome encodes five Rpfs (A, B, C, D and E) and their function is to promote the resuscitation and growth of dormant cells [36]. The selection of RpfB and RpfC suggests a possible attempt to stimulate growth of dormant *M. tuberculosis* cells, possibly triggered by host environmental factors. The selection of RpfB by both LTBI and ATB sera could be due to sero-conversion from LTBI to ATB state. Therefore, RpfB and RpfC are potential biomarkers for monitoring progress from LTBI to ATB in high risk groups such as HIV/AIDS patients. This will possibly permit initiation of TB treatment before the onset of clinical symptoms.

3.5. Virulence, essential and drug target proteins

Using PATRIC's specialty proteins dataset that includes virulence factors, drug targets, antibiotic resistance and essential proteins, 17 and 18 specific *M. tuberculosis* proteins unique to ATB and LTBI lists respectively, were identified. Five proteins (Rv1009, Rv1980c, Rv2152c, Rv1357c and Rv1886c) were common to “Active-TB”, “Latent-TB” and “Specialty” lists. Two of five are immunogenic proteins MPT64 and Ag85B [30]. The essential protein MurC (Rv2152c) is involved in the peptidoglycan biosynthesis pathway [43,73]. The other 2 proteins are the resuscitation-promoting factor RpfB (Rv1009) and the conserved hypothetical protein (Rv1357c).

3.5.1. *Virulence proteins*

Amongst the ATB speciality proteins were 11 virulence factors including BfrA and the cell surface protein PirG (Rv3810), both required for intracellular survival and replication [57,74]; the amino acid permease (Rv1979c) that modulates the host immune response [75]; and the transcriptional regulator EspR (Rv3849), a key component of *M. tuberculosis* type VII secretion system (TSS7), ESX-1 system. EspR regulates the transcription of *espACD-rv3613c-rv3612c* operon required for ESX-1 system responsible for the secretion of 6-kDa early secreted antigen target (ESAT6) and 10-kDA culture filtrate protein (CFP10) [76,77]. There were two PE/PPE family proteins, PE_PGRS33 (Rv1818c) that plays a role in adhesion to host cells [78] and PPE54 (Rv3343c), the member of MPTR (major polymorphic tandem repeats) subfamily which affects the phagosome [32]. Lastly, the signal recognition particle receptor FtsY (Rv2921c) is required for bacterial cell division [79].

The LTBI specialty proteins included 12 virulence factors including NarG, RpfC, DesA3, SapM, PanD and PonA2 discussed in the previous section. Other virulence proteins comprised PPE5 (Rv0304c), HspR (Rv0353), Pks6 (Rv0405), MmpL10 (Rv1183), Rv1184c and Rv2277c. Polyketide synthase (Pks6), PPE5 and Rv2277c are reportedly expressed during initial lung infection [80], and therefore, involved in establishment or invasion of the host by *M. tuberculosis* during infection. The transcriptional repressor (HspR) is required to minimize the host immune-surveillance that may result in efficient killing of the pathogen by suppressing the expression of heat-shock genes that encode the immunodominant heat-shock proteins such as Hsp70 [81].

DesA3 (Rv3229c) is a membrane-bound stearoyl coenzyme A (CoA) desaturase that is involved in biosynthesis of oleic acid, an essential component of mycobacterial membrane phospholipids and triglycerides [82], therefore, it is required for *in vivo* growth [43].

3.5.2. *Essential proteins*

Essential proteins of *M. tuberculosis* include those required for growth and survival during infection. Three ATB associated essential proteins, the TrpG, TopA (Rv3646c) and SerS were identified. TrpG and TopA are essential for the establishment, maintenance and survival of *M. tuberculosis* during infection [83,84]. SerS is involved in the translation mechanism and is, therefore, essential for growth [43]. The 2 LTB identified specific essential proteins included the ribosomal protein S11p RpsK (Rv3459c) and ribonuclease P protein component RnpA (Rv3923c). Interestingly, MmpL7 (Rv2942), an efflux pump conferring antibiotic resistance, was the only antibiotic resistance transmembrane transport protein [85] identified in this study.

3.5.3. *Drug targets*

Two potential drug targets identified within ATB list included the AroD (Rv2537c) and Alr (Rv3423c). Drugs targeting these proteins will inhibit protein synthesis [39,51] resulting in bacterial death. The 3 LTBI specific prospective drug targets SahH (Rv3248c) and EmbC (Rv3793); and the peptide deformylase Def (Rv0429c) were reported to be essential for *M. tuberculosis* growth [43,73].

3.6. *Study limitations*

There were several limitations in the study. These include the use of TST test, the exclusion of HIV positive participants and the random selection of enriched ATB and LTB phage clones. The disadvantage of using TST is the low specificity, since it is based on antigens shared with environmental mycobacteria and *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) [86]. Therefore, false positive reactions can occur in patients with prior BCG vaccination or those with exposure to environmental mycobacteria [87]. False negative tests can also be caused by immunosuppression (e.g. HIV infection), recent TB infection and concomitant bacterial infections. As a result, HIV positive participants were excluded in the study since it has been shown in previous studies that many immunogenic mycobacterial antigens that elicit antibody responses in HIV negative TB patients do not elicit the same magnitude of response in HIV positive TB patients [88]. Furthermore, TST cannot differentiate active disease from LTBI, requiring additional tests to establish the diagnosis [87]. In mitigation of the above limitation, GeneXpert was used to diagnose ATB. Moreover, the approach used to select the identified biomarkers was a negative selection process that involved ‘removal’ of non-specific proteins by pre-incubation with the appropriate serum sample. This was followed by a literature review on identified proteins to ascertain whether they were previously identified in any other study, as well as to verify if they were associated with the specific disease states.

Lastly, only the randomly selected clones were sequenced not all the enriched ATB and LTB phage clones could be analysed. Nevertheless, the disease state-specific biomarkers were identified and this will contribute towards the development of diagnostic tools that can differentiate ATB from LTB infection. Some of the biomarkers are potentially suitable for monitoring of disease progression from LTBI to ATB in high risk groups such as HIV/AIDS patients.

4. Conclusion

Phage display is one of the most powerful techniques used to identify proteins that bind to targets of interest. In this study, we identified protein biomarkers recognized by ATB and LTB patients′ antibodies. These included 4 ATB specific biomarkers (TrpG, TreY, Alr and BfrA) that have no human homologs and are essential for pathogenesis, and 5 LTB specific biomarkers (PonA1, PonA2, NarG, MoaE1 and HspR) associated with dormancy. The identified biomarkers will be exploited in future work to design subunit TB vaccines and produce recombinant proteins for the development of monoclonal antibodies. These antibodies can be used in designing diagnostic assays for antigen detection of ATB and LTB infection in high risk groups including HIV infected individuals.

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Appendix A. Supplementary data

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