Original Article

Protein profiling and analysis of drug sensitive and multidrug resistant isolates of *Mycobacterium tuberculosis* by native polyacrylamide gel electrophoresis and mass spectrometry

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ABSTRACT

Introduction: Tuberculosis (TB) remains a deadly infectious disease despite all the efforts to reduce its incidence. Spread of multidrug resistant TB has seriously undermined the efforts to control the disease globally. In this study protein expression profile of MDR and sensitive isolates of MTB were analyzed and compared in order to identify proteins, which could be used in prevention, diagnosis and treatment. **Methods:** A sensitive and MDR isolate of *Mycobacterium tuberculosis* (MTB) were cultured on Middlebrook 7H9 medium and the whole cell lysates were subjected to native polyacrylamide gel electrophoresis (NPAGE) for protein expression profiling. Protein bands present in the MDR cell lysate that were not detected in the sensitive cell lysate were sent for identification by Matrix-assisted laser desorption/ionization time-of-flightmass spectrometry (MALDI-TOF-MS). **Results:** Comparison of the protein expression profiles showed 6 bands that were not detected in the sensitive isolates. MTB Structural Annotation database search of the mass spectrometry results identified these bands as Rv3597c, Rv0379,Rv3614c, Rv0475, Rv0462, andRv0147and global transcriptional regulation, involvement in cell wall and cell processes and intermediary metabolism and respiration were the functions attributed to these proteins. **Conclusion:** Our results highlighted the complexities of linking protein expression to MDR phenotype as none of the proteins identified could be linked directly to drug resistance. The proteins identified in the present study were mostly those essential for survival or virulence of the bacteria, and could be used for diagnosis or as candidate vaccine, but with a better understanding of the function of these proteins their association with the MTB resistance to antibiotics might become clear.

KEYWORDS: MALDI-TOF-mass spectrometry, native PAGE, multidrug resistant, Mycobacterium tuberculosis.

INTRODUCTION

Mycobacterium tuberculosis (MTB) causes a chronic infection, which has remained a serious global health problem despite all efforts [1]. Although bacillus Calmette-Guérin vaccine (BCG), protects children against severe forms of tuberculosis (TB), but protection in adults is variable and the emergence of multidrug resistant isolates (MDR), defined as resistance to at least two first line anti-TB drugs isoniazid and rifampicin is a major impediment for TB control programs worldwide [1-2]. In 2014, approximately 9.6 million people developed TB and 1.5 million died from the disease [3-4]. It has been estimated that in developed countries the cost of standard TB treatment per patient amounts to approximately $2000 increasing up to as much as $50,000 and taking 3 times as longin a MDR case [5].The global number of new MDR-TB cases reported by World Health Organization in 2014 has been 480,000 accounting for 3.3% of all new TB cases reaching as high as 20% among those with previous treatment history [3].The high cost in human lives, the economic burden of treating MDR-TB and the capacity of MTB to develop resistance clearly

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demonstrate the urgent need for development of more effective diagnostic tools, drugs and vaccines[3-5].

Recently proteomic studies have attracted a great deal of attention as proteins functionally link genotype to phenotype and are good targets for drug and vaccine development [6].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)using separated proteins by one- or two-dimensional polyacrylamide gel electrophoresis (PAGE)has been used extensively to identify, quantify and compare isolates showing different phenotypes[6-7].MALDI-TOF-MS has become a widely used technique for analysis of proteins and peptides due to its operational simplicity, robustness and high sensitivity and atypical analysis consists of i- separation of protein(s)in a mixture by PAGE, ii- visualization of separated protein(s), iii- excision and in-gel digestion of protein(s), iv- analysis of the proteolytically digested peptides by MALDI-MS resulting in a map of peptide mass [7]. The use of this technique has provided the means for comparison of differentially expressed bacterial proteins under various culture conditions and by strains with different genotypes, thus supplying a more comprehensive view of the life of a pathogen[8].However, despite the advances in the field of MTB proteomics there are few reported literature dealing with differential expression of proteins in multidrug resistant bacteria compared to susceptible cells[9].In this study protein expression profile of MDR and sensitive isolates of MTB were analyzed and compared in order to identify proteins, which could be used in prevention, diagnosis and treatment.

MATERIALS and METHODS

Chemicals and strains

All the chemicals used were from Sigma, USA and the *M. tuberculosis* sensitive isolate no.1522 susceptible to all antibiotics and MDR strain no. 352 resistant to isoniazid and rifampicin were from TB-Bank collection of Pasteur Institute of Iran.

Preparation of lysates

The susceptible and resistant isolate were cultured on Middlebrook 7H9 medium for 4 weeks at 37°Cand bacterial cells were harvested by centrifugation at 4000 rpm for 45 min at 4°C.The pellets were washed three times with 50mM Tris (pH~ 8.0) and approximately 2 g wet bacterial cell mass was suspended in15 ml sonication buffer containing 50 mM Tris, 150 mM NaCl, 1 µg/ml DNase, 1mM PMSF, 20 mM sucrose, 10 mM MgCl2, 0.02 g/ml sodium azide, 10%(v/v)glycerol. Cells were sonicated intermittently for 1h on ice at 50 HZ with 15 min on and 5 min off cycles using sonicator Bandelin, GM -220 (Germany). The unbroken cells and cell wall debris were removed by centrifugation at 5000 rpm for 45 min at 4°C.

Protein precipitation

The proteins were precipitated by addition of ammonium sulfate(70% w/v) and incubated overnight at4°C. After centrifugation at 13000 rpm for 45 min at – 4 °C, the pellets were suspended in 50 mM Tris (pH~ 8.0) and dialyzed extensively against 0.1 M ammonium bicarbonate (pH~ 8.0).After dialysis and quantification of total protein in the mixture by Bradford assay, the mixture was lyophilized for future use [10].

Protein profiling of MTB sensitive and MDR isolate by native PAGE

Protein profiles of the whole cell lysate of MTB sensitive and MDR isolate were obtained using native PAGE.Optimum percentage of acrylamide+bisacrylamide(%T) for high resolution separation of proteins in the whole cell lysates was determined empirically over a range of 15, 18, 20, 25 and 30 % T.

Lyophilized whole lysate of the isolates (3-4 µg/µl) was dissolved directly in 10 µl loading buffer containing 0.5 M Tris –HCl (pH~ 6.8), 60% glycerol (v/v) and 0.001 µg/ml bromophenol blue and loaded in each well of the gels. Electrophoresis was carried out at room temperature and gels were run at a constant current of 10 mA until the tracking dye entered the resolving gel, after which the current was increased to 15 mA till the dye front reached the end of the gel. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 [11].

Apparent molecular weight of separated proteins was calculated by plotting the relative mobility of a set of protein marker of known molecular weight (29, 66, 100, 132, 150 and 200 kDa) at different acrylamide + bisacrylamide concentration (%T) against logarithm of retardation coefficients (-logk) based on Ferguson plots[11].

Mass spectrometry and data analysis

Protein bands that were present in MDR whole cell lysate and were not detected in polyacrylamide gel of the sensitive MTB isolate were excised (1×1 mm) and were sent to University of York, Department of Biology, UK for Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The default calibration had been used for MS/MS spectra, which had been baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection had used a SNAP averaging algorithm with a minimum S/N of 6. Spectral processing and peak list generation had used Bruker flex Analysis software (version 3.3).

Tandem mass spectral data were submitted to database search using a locally-run copy of the Mascot program (Matrix Science, V. 2.4) through the Bruker Proteinscape interface (V. 2.1). The results were filtered to accept only peptides with an expected score of 0.05 or lower, allowing for higher confidence identifications by including tandem spectral data in MASCOT search and the data were compared with the MASCOT database for sequence matches.

Bioinformatics analysis of proteins expressed by the MDR isolate

Grand average of hydropathy (GRAVY) value of the MDR-specific proteins was calculated using www.gravy-calculator.de server. GRAVY represents the sum of hydrophobicity values of all amino acids in the protein divided by the number of its residues.

MTB Structural Annotation database was used for identification and assignment of protein function to the selected MDR-specific proteins [12]. 3D models of some of these proteins are also taken from this site.

Proteins transmembrane topology was determined using www.cbs.dtu.dk/services/TMHMM server which uses fasta format of primary amino acid sequence of a protein for prediction of transmembrane helices. The method can apparently distinguish soluble and membrane proteins with a specificity and sensitivity greater than 99% [13].

I-TASSER server for protein structure and function predictions was used for 3D modeling of proteins which did not have experimentally-determined structures deposited in the RCSB Protein Data Bank (PDB) [14].

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RESULTS

Discontinuous non-denaturing polyacrylamide gel electrophoresis was used for separation of proteins in the whole cell lysates of MTB sensitive and MDR isolates. Based on the optimization results the highest resolution separation of proteins was obtained at 20-25%T and 3.3% C (Fig.1) where T represented the total percentage concentration of acrylamide + bisacrylamide and % C was calculated from the formula %C= % bisacrylamide x 100/ percentage of T.

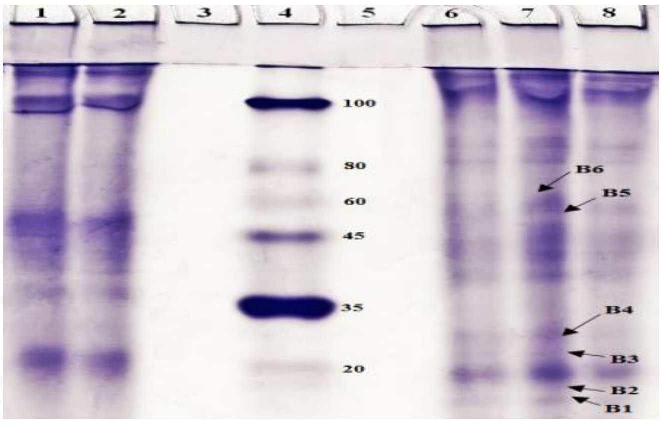

Fig. 1. Protein profiles of MTB sensitive and MDR isolate obtained using native PAGE. Lanes 1-2: sensitive isolate. Lanes 6-8: MDR isolate. Lane 4: Protein Marker. Bands indicated by arrow were excised and analyzed by mass spectrometry.

Comparison of the gel electrophoresis data for the sensitive and MDR isolates used in this study showed 6 protein bands (B1-B6) that were present in the MDR whole cell lysate but not detected in the lysate from the sensitive isolate (Fig. 1). Molecular weight of these proteins estimated from their relative mobility in the native gel were B1 ~ 13 kDa, B2 ~ 18 kDa, B3 ~ 21 kDa, B4 ~ 24 kDa, B5 ~ 52 kDa and B6 ~ 57 kDa respectively. However, the exact molecular weight of the identified proteins was lower in the MALDI-TOF-MS report, but the differences were not statistically significant (Table 1).

The highest isoelectric point (pI) was reported for protein B1 (10.08) representing the pH value at which the net charge of protein would be zero and the protein insoluble and the lowest pI of 3.93was for B3 (Table 1).

MTB Structural Annotation database search of the mass spectrometry results identified these bands as Rv3597c, Rv0379,Rv3614c, Rv0475, Rv0462, andRv0147proteins (Table 1).The assigned functional categories of these proteins as determined by the same database are also given in Table 1.

Of the 6 identified proteins Rv0379 (B2) and Rv0462 (B5) had positive Gravy value indicating hydrophobic nature of these proteins while the remaining polypeptides (B1= Rv3597c, B3 =Rv3614c, B4= Rv0475 andB6 = Rv0147 had negative Gravy values and considered hydrophilic (Table1).

Table 1. Proteins of MDR-MTB identified and characterized by mass spectrometry, MTB Structural Annotation database and GRAVY- calculator server.

PAGE band no.	Protein ID	description	Functional category	MW estimated	MW calculated	No. aa	PI	GRAVY Values
B1	Rv3597c	Iron-regulated H-NS-like protein	Global transcriptional regulator	13.0	12.09	112	10.08	-0.6589
B2	Rv0379	pre-protein translocase subunit	cell wall and cell processes	18.0	16.68	136	7.98	0.9014
B3	RV3614C	ESX-1secretion-associated protein	cell wall and cell processes	21.0	19.87	184	3.93	-0.384
B4	RV0475	Iron-regulated heparin binding protein	cell wall and cell processes	24.0	21.52	199	9.17	-0.5929
B5	Rv0462	LpdC, dihydrolipoamide dehydrogenase	intermediary metabolism and respiration	52.0	49.43	464	5.53	0.1036
B6	RV0147	aldehyde dehydrogenase	conserved hypothetical protein	57	55.11	506	9.21	-0.144

According to the data obtained using TMHHM server [13] only one of the identified proteins (B2=Rv0379) contained transmembrane loops.

Experimentally-determined structures of 4 of these proteins as deposited in RCSB Protein Data Bank (PDB) are shown in Fig. 2, which also includes the 3D structures ofB3 (Rv3614) and B4 (Rv0475) as predicted by I-TASSER server [14]. The selection criteria for the predicted 3D structure of the protein sequences submitted to the I-TASSER server was the calculated C-score.

Global transcriptional regulation, involvement in cell wall and cell processes and intermediary metabolism and respiration were the functions attributed to B1-B6 proteins by the MTB Structural Annotation database, but none of the proteins could be directly linked to antibiotic resistance (Table 1).

The low resolution of 1-D gels observed in this study could be the result of various factors such as the extraction protocol, the low sensitivity of coomassie brilliant blue stain used or the inability of this type of gel to effectively separate complex mixture of proteins.

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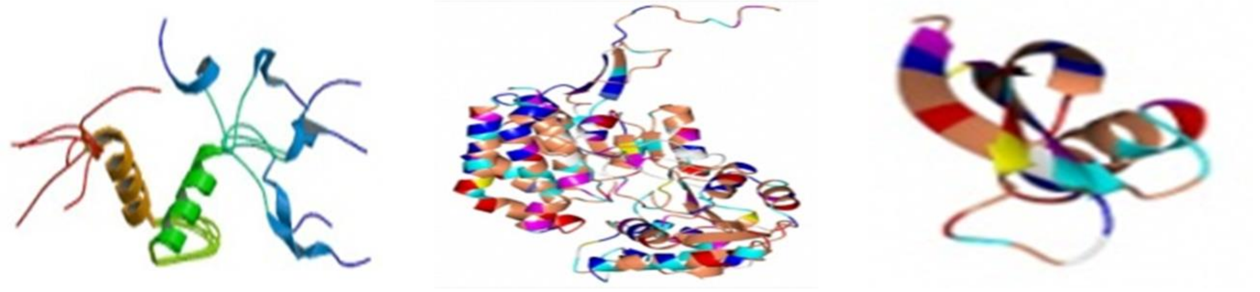

B1=Rv3597c **B6=Rv0147** **B2=Rv0379**

B5=Rv0462 **B4=RV0475** **B3=Rv3614c**

Fig. 2. Proteins of MDR-MTB identified and characterized by mass spectrometry, MTB Structural Annotation database and GRAVY- calculator server.

Discussion

Despite the progress made in the treatment of MTB, resistance to antibiotics regularly develops making management of this infectious disease problematic. Proteomic data obtained by comparative studies of MTB isolates under different culture conditions or various perturbed states has contributed to elucidation of many pathogenic and virulence aspect of the bacterium by identifying differentially expressed proteins.

Proteomic comparison of isogenic strains resistant to isoniside alone or combined with rifampicin has been reported [9, 15],but none of the proteins identified in this study matched those reported in the study conducted on MDR isolates in India [9], whereas similar to our study Nieto *et al*. comparing isogenic strains of Beijing genotype observed over expression of Rv0379 (B2) in isoniazid-resistant isolates [15].The linage of the isolates in this study was not determined, but the similarity in genotypes could not be ruled out. Rv0379 also known as calcium dodecin (Ca dodecin)is a calcium binding protein with about 70 amino acids and a molecular weight of approximately 8 kDa, which forms a dodecameric oligomer when binding a Ca ion [16]. The in vivo function of Ca dodecin is unknown, but it has been identified as an immunodominant antigen recognized by sera from TB patients with potential foruse in sero-diagnosis or as a candidate vaccine [17].

B1 (Rv3597c = Lsr2) on other hand is a histon-like protein that by binding to DNA molecules protects them from damage by reactive oxygen intermediaries, which is essential for MTB survival in host macrophages. Furthermore Lsr2 has been identified as a global transcriptional regulator up- or down-regulating various mycobacteria responses including those triggered by antibiotic treatment. This characteristic has led to the suggestion that this highly immune reactive protein might be involved in multidrug tolerance [18-19].

MTB uses various secretory pathways for protein transport across its complex cell wall including ESAT-6 system 1(ESX-1) or type VII secretion system, which is essential for mycobacterium entry and intracellular spread as well as escape from phagosomes. EspD (B3=Rv13164c) encoded by *espD* is part of *espA-espC-espD* gene cluster which is not linked to ESX-1 locus. However, despite the progress in characterization of this gene cluster and their products, the role played by EspD is yet to be fully elucidated [20, 21].

Heparin-binding hemagglutinin (B4= RV0475) a cell-wall associated protein, is a 198 amino acids long protein binding to heparan sulfate glycosaminoglycans on the surface of epithelial cell. This important adhesion of m plays an important role in extra pulmonary spread of the bacteria, as well as being a highly protective antigen which has been used as a diagnostic tool and a candidate vaccine [22-24].

The remaining two native page-separated proteins is in this study were identified as Rv0462 LpdC, dihydrolipoamide dehydrogenase (B5) and RV0147 aldehyde dehydrogenase (B6) respectively. LpdC is an enzyme in pyruvate dehydrogenase complex (PDH) helping MTB to withstand the effects of its host reactive nitrogen intermediaries. It has been shown that deletion of the *rv0462* (*lpdc*) gene significantly attenuates MTB *in vivo* suggesting that Lpd might be a good target for chemotherapy [25].

The relatively small MTB genome (~4000 genes) contains 10 genes encoding aldehyde dehydrogenase (ALDH) proteins, one of which is NAD+ dependent Rv0147. The function of this protein is unknown, but the major role of this family of proteins is elimination of toxic aldehydes and has been considered essential for survival in human host [26]. But whether this suggestion should be applied to individual genes in this group of proteins remains to be determined as complete or partial deletion of this gene in some clinical isolates has been reported [27].

Our results highlighted the complexities of linking protein expression to MDR phenotype as none of the proteins identified could be linked directly to drug resistance. The proteins identified in the present study were mostly those essential for survival or virulence of the bacteria and could be used for diagnosis or as candidate vaccine, but with a better understanding of the function of these proteins their association with the MTB resistance to antibiotics might become clear.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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