Original Article

Differential protein expression in *Mycobacterium tuberculosis* susceptible and multidrug resistant isolates

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ABSTRACT

Introduction: Infections by multidrug resistant *Mycobacterium tuberculosis* (MDR-TB) is a major public health challenge. Secretion of proteins by *M. tuberculosis* plays an important role in the pathogenesis of the bacterium. We compared the protein profiles of susceptible *M. tuberculosis* and MDR-TB isolates using proteomic analyses, namely two dimensional gel electrophoresis (2DE) and mass spectrometry (MS). **Methods:** The bacilli were cultured on Middlebrook 7H9 medium and bacterial colonies were mechanically disrupted and proteins were extracted by ammonium sulfate. The 2DE and MS analyses were performed using Ettan IP Gphor 3 isoelecteric system and Autoflex II TOF/TOF, respectively. **Results:** Our study showed that in comparison to the sensitive strains, 27 proteins were over-expressed in the MDR isolates and these proteins were mainly involved in the cellular metabolism, cell wall and membrane structures and bacterial respiration. Bactoferritin (Rv1876) has been shown to play a role in antibacterial resistance. Increased intensity of Rv2031c, a heat shock protein (Alpha-crystallin/HspX), was also observed in the whole cell lysate of the MDR-TB. This protein is a marker of the latent TB and has been proposed as a target for vaccine development. **Conclusion:** Our results identified proteins that are overexpressed in the resistant *M. tuberculosis* which could be used as antibacterial targets or vaccine candidates.

KEYWORDS: Multidrug resistant *Mycobacterium tuberculosis* (MDR-TB), tuberculosis proteins, protein candidate, Mycobacteria antigen.

INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is present in all regions of the world as stated in WHO Global Tuberculosis Report 2014 which includes data compiled from 202 countries and territories [1]. This year's report shows even higher global totals for new TB cases and deaths in 2013 than previous years, reflecting the increased and improved use of national data in this regard. As parts of the post 2015 global TB strategy, the early diagnosis is emphasized and the inclusion of drug susceptibility testing (DST) is considered as a universal standard for patient care for both the new and the previously-treated patients. Detection of TB without investigation for drug resistance can lead to ineffective treatments.
Inappropriate treatment regimens and poor patient compliance have led to the appearance of multi drug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis* strains which make the treatment of TB costly, lengthy and difficult [2]. By definition, Multidrug resistant *Mycobacterium tuberculosis* (MDR-TB), is resistant to at least isoniazid and rifampin. The treatment of infections caused by MDR-TB is a major concern for TB control programs worldwide because it requires prolonged use of multiple second-line anti-TB drugs which are more expensive and toxic than the first-line drugs. TB death toll is very high although it could be prevented with a timely diagnosis and correct treatments. Over 95% of TB deaths occur in low- and middle-income countries where it is among the top three causes of mortality for women aged 15 to 44. The rate of new cases has been falling worldwide for about a decade and there has been a 45% reduction in TB mortality rate since 1990. Moreover, 56 million people have been successfully treated between 1995 and 2012 [1]. Therefore, rapid detection of drug resistance and therapeutic options according to the resistance profiles are important elements for controlling MTB infections.
In the present study, two dimensional gel electrophoresis (2DE) and mass spectrometry (MS) along with relative protein expression abundance calculations were used to compare the

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membrane protein expression profiles of susceptible and MDR-TB strains. The recent discovery of a true outer membrane in the cell wall of MTB has unraveled the previously-known host-bacterial interactions and consequently has made the antimicrobial susceptibility more complicated [3]. The aim of this study was to find proteins that could further explain the different phenotypes of the two strains, especially their distinct abilities which could be used to develop new drugs against MTB.

MATERIALS and METHODS

Bacterial strains

Sensitive MTB-1126 and MDR-TB isolates were obtained from TB-Bank of Pasteur Institute of Iran and were cultured on Middlebrook 7H9 medium at 37° C for 4 weeks. MDR-TB was defined as TB caused by strains resistant to at least isoniazid and rifampicin (Sigma, USA) and was determined by minimum concentration method (MIC) at a concentration of 0.2, 40, 2.0, and 4.0 µg/ml [4-6].

Sample preparation

The bacterial cells were gathered by centrifugation at 5000 rpm (45 min at 4° C) and the pellets were washed with Tris 50 mM twice and were resuspended in 50 mM Tris, 150 mM NaCl, 1 µg/ml DNase, 1 mM PMSF, 20 mM sucrose, 10 mM $MgCl_2$, 0.02 g sodium azide, 10% glycerol (pH~8.0). The pellets were sonicated for 1 h at 50 Hz (Bandelin, GM -220 Germany). The unbroken cell debris was put off by centrifugation at 5000 rpm (45 min at 4 °C). The proteins were precipitated by ammonium sulfate and incubated overnight at 4° C. After centrifugation at 13000 rpm for 45 min at -4° C, the proteins were suspended in 50 mM Tris (pH~8) and dialyzed exhaustively against 50 mM Tris (pH~8). The amounts of proteins were quantified using Bradford assay [7].

2DE

2DE was performed using the Ettan IPGphor 3 isoelecteric focusing system (GE Healthcare, Sweden). The gel strips (pH 4-7 and length 11 cm) were focused on an IEF unit according to the following program: 500V for 1 h, 1000 V for 1 h, 8000 V for 3 h, finally 8000 V for 20 KVh, at 20˚C and a current limit of 50 µA per strip. After IEF, the strips were equilibrated in equilibration buffer (15 min in 6M urea) , 20% SDS, 1.5 M Tris, 50% glycerol, 2% DTT, 2.5% idoacetamide (pH~8.8). The proteins were separated in second dimension on 12% SDS-PAGE in a vertical electrophoretic dual gel and were visualized by Coomassie (R-250) staining method [8-9].

MS and data analyses

Excised protein bands (1×1 mm) were sent to the Chemistry Department, York University, UK for MS. The purified peptides were applied to an Anchor Chip and were analyzed by MALDI-TOF Mass Analyzer (Bruker Daltronic Reflex III). Aliquot of each peptide mixture was carried out a ground steel MALDI (target plate). Fragmentation was committed in LIFT mode in absent of a collision gas. The primary calibration was performed for MS/MS spectra, which were subtracted and smoothed (cycles 4, width 0.15 m/z, Savitsky-Golay). For detection of monoisotopic peak, a SNAP averaging algorithm was used with a minimum S/N of 6. Analysis software (Bruker flex, version 3.3) was performed to spectral processing and peak list generation. By locally-running copy of the Mascot program (Matrix Science Ltd., version 2.4) through the Bruker Protein scape interface (version 2.1), the Tandem mass spectral data were submitted to database searching engine. The results were purified to accept peptides with just score of 0.05 or lower, allowing to acquire higher confidence identifications including Tandem spectral data in MASCOT <http://www.matrixscience.com/> searches and the data were assimilated with the MASCOT database for any sequence matches[10].

RESULTS

Our study identified 27 proteins that were upregulated or overexpressed in the MDR-TB strains compared to the sensitive isolates as summarized in Table 1. All the detected spots in the Coomassie Brilliant Blue stained 2DE gels were cut and applied for in gel digestion procedures, followed by MALDI-FOF/MS analysis. The analysis of two independent strains (sensitive and MDR-TB isolates), using image master, Melanie analysis software (version 6.0) revealed that the majority of these proteins were common to both strains. However, 27 different proteins were differentially expressed (upregulated/expressed proteins) in MDR compared to the sensitive isolates. The majority of the spots were found in the pH range of 4.0 to 6.5 with molecular weights ranging from 14.4 to more than 45.0 kDa. The identified proteins were short chain dehydrogenase/reductase (Rv3057c), peptidyl-prolyl-cis-trans (Rv0009), putative dioxygenase (Rv3161c), pre-protein translocase subunit SEcE2 (Rv0379), 2 cell wall proteins (Rv0379, Rv3614c), 3 information pathways proteins (Rv0009, Rv0685 , Rv2986c), 4 hypothetical proteins (Rv0443, Rv2114, Rv3311 and Rv0831), 2 secreted proteins (Rv3804c and Rv3614c), 2 heat shock proteins (Rv3418c and Rv2031c) and 14 proteins belonging to metabolism and respiration pathways. The Functions of identified MDR-TB proteins on the 2DE gels followed by analysis with MALDI-TOF MS are shown in Fig. 1. Fourteen proteins of intracellular MDR isolate (40.5%) belonged to the intermediary metabolism and the respiration category. Five proteins of the intracellular MDR isolate (16.7%) were conserved hypothetical proteins which were functionally characterized by the bioinformatics tools.

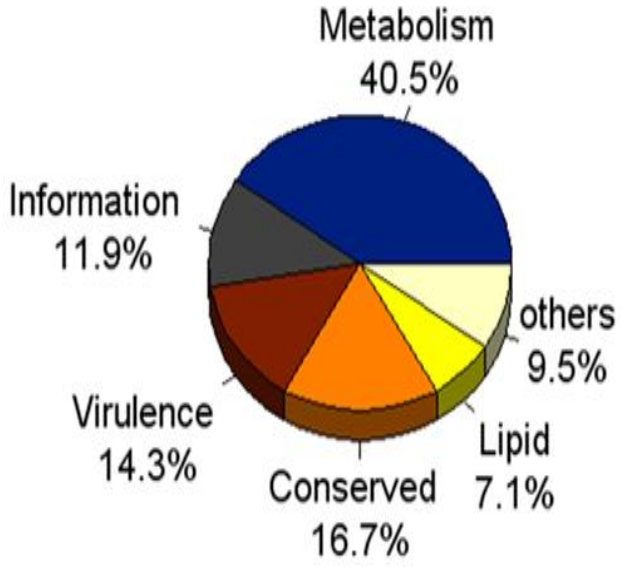


Fig. 1. Function of identified MDR-TB proteins on the 2DE gels followed by analysis with MALD-TOF MS.

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Table 1. Identified expressed proteins of MDR Tuberculosis isolates on the 2DE gels and MALDI-TOF mass spectrometry.

N0	Gene	Gene product	(MW)	(PI)	Protein length (aa)	Location (kb)	Score	Gene length (bp)	Putative function
1	Rv3418C	10 kDa chaperonin GroES	10.79	4.51	100	3836.99	470	303	Virulence, detoxification
2	Rv2971	oxidoreductase	30.51	4.70	282	3326.1	95	849	metabolism and respiration
3	Rv1308	ATP synthase alpha chain AtpA	59.48	5.03	549	1463.23	332	1650	metabolism and respiration
4	Rv0831C	Conserved protein	30.17	5.09	271	922.894	226	816	conserved hypothetical
5	Rv1876	Bacterioferritin	18.44	4.51	159	2125.34	220	480	metabolism and respiration
6	Rv0379	pre-protein translocase subunit SEcE2	16.68	6.98	135	455.977	136	216	cell wall cell processes
7	Rv1240	Malate dehydrogenase	34.35	4.65	329	1383.21	210	990	metabolism and respiration
8	Rv2114	Conserved protein	28.26	4.8	260	2373.83	254	624	conserved hypothetical
9	Rv0443	Conserved protein	13.27	4.60	128	532.396	173	516	conserved hypothetical
10	Rv3248C	Adenosylhomocysteinase	54.4	5.07	495	3628.16	86	1488	metabolism and respiration
11	Rv3804c	secreted antigen Ag85B	34.72	6.82	495	4265.64	120	1017	lipid metabolism
12	Rv2986C	35 kDa protein OS	29.24	5.71	270	3343.18	84	645	Information pathways
13	Rv3614c	ESX-1secretion-associated protein	19.87	3.93	184	4054.14	306	555	cell wall cell processes
14	Rv2215	dihydrolipoamide actyltransfrase	57.11	4.9	553	2481.97	181	1662	metabolism and respiration
15	Rv1392	S-adenosylmethionine synthetase	43.13	4.95	403	1566.82	238	1212	metabolism and respiration
16	Rv3028C	electron transfer flavoprotein subunit alpha	31.67	4.71	318	3387.07	414	957	metabolism and respiration
17	Rv1189	RNA polymerase SigI	24.72	5.39	226	1332.09	76	873	metabolism and respiration
18	Rv3311	Conserved protein	45.81	4.14	420	3698.12	142	1263	conserved hypothetical
19	Rv3057c	short chain dehydrogenase/reductase	30.67	5.27	293	3417.8	344	864	metabolism and respiration
20	Rv0462	Dihydrolipoyl dehydrogenase OS	49.43	5.53	464	552.614	589	1395	metabolism and respiration
21	Rv2031c	Heat shock protein HspX	16.22	4.75	144	2278.5	76	435	virulence, detoxification,
22	Rv0685	Elongation factor Tu	43.56	5.2	396	784.821	302	1191	information pathways
23	Rv3161c	Possible dioxygenase	42.50	5.00	382	3529.99	213	1149	metabolism and respiration
24	Rv0462	Dihydrolipoyl dehydrogenase OS	49.43	5.53	464	552.614	589	1395	metabolism, and respiration
25	Rv0009	Peptidyl-prolyl-cis-trans	19.23	6.2	182	12.468	198	549	information pathways
26	Rv3699	Conserved protein	25.04	4.69	233	4142.04	287	702	conserved hypothetical
27	Rv1392	S-adenosylmethionine synthetase	43.13	4.95	403	1566.82	238	1212	metabolism and respiration

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DISCUSSION

Our study of the protein profiles of MDR and the sensitive *M. tuberculosis* isolates showed that the majority of the proteins in the whole cell lysates were present in both groups and had similar relative abundance. However, the overexpressed protein in the pH range in this study were antigen 85 (Ag85), bacterioferitin, pre protein SEcE2, EsX-1 secretion associated protein, RNA polymerase SigI, short chain dehydrogenase/reductase, heat shock protein HspX, putative dioxygenase and peptidyl polyl cis-trans. Ag85 protein is responsible for the high tendency of mycobacteria for fibronectin, the great adhesive glycoprotein which facilitates the attachment of MTB to murine alveolar macrophages. The protein also helps to maintain the integrity of the cell wall by catalyzing the convey of mycolic acids to the cell wall's arabinogalactan and through the synthesis of cord factor [11]. Ag85A and Ag85B (Rv3804c) have been reported to be ideal vaccine candidates in a number of studies [2]. Bacterioferritin, bfrA, is involved in iron storage which is a necessary requirement for the growth of bacilli. However, its excess as a source of free iron is toxic and leads to the production of reactive oxygen species and consequently oxidative damage. Ferritin has been reported to show increased intensity under the absence of oxygen condition and nitric oxide deficiency but such activity has not been observed for bacterioferritin. The function of bacterioferritins may not be just limited to the iron uptake and they may be contributing to other metabolic activities which their mechanisms are still unclear [12]. These results showed the upregulation of bacterioferritin (Rv1876) in MDR-TB isolates. It is suggested that the exclusive expression of bacterioferritin in MDR-TB indicates its probable involvement in resistance to the first line of anti-mycobacterial drugs.

Although our findings show a significant improvement compared to these studies, there are some differences between the present and the earlier studies. The discrepancy between the observed and the predicted protein pI values is a common feature of proteomic analyses using 2DE gels. This is considered, amongst other things, to be due to the conformational differences, post-translational modifications and other processing events of proteins which may affect their migration. MS identification of mycobacterial proteins from 2D gels has only been applied so far to in vitro grown cultures where abundant amounts of proteins are available for analysis.

Peptidyl-prolyl cis-trans isomerase (Rv0009) also known as cyclophilin is iron-regulated and is necessary for protein folding and is thought to participate in processes such as signaling, cell surface recognition and chaperoning. Rv0009 is a major cellular target for the immunosuppressive drug cyclosporine [13]. Calcium dodecin (Rv0379) binds to calcium ions and plays a role in sequestering additional small ligands and is involved in protein transport [4].

Arockiasamy et al. have shown that the earliest interactions of MTB with macrophages result in a number of alterations in Ca+2 signaling events that are critical for the phagosomes maturation [14]. Malate dehydrogenase, (Rv1240) has been identified in the immunodominant membrane fractions or cell wall of MTB [15]. The elongation factor (EF-Tu) was identified at another spot with significantly different pI and higher molecular mass, suggesting that it was a proteolytic degradation product. The increase in EF-Tu expression may reflect either an overall increase in the protein synthesis related to the growth in the intracellular environment or may indicate some regulatory functions during the translational control of gene expression. A previous study by Wong et al. has demonstrated that EF-Tu is up-regulated when MTB is exposed to high iron conditions in vitro. EF-Tu (Rv0685) belongs to GTP-binding elongation factor family and has been predicted as a possible vaccine candidate [16].

Two of the upregulated proteins in this study were identified as molecular chaperones. These are a diverse set of proteins that mediate the correct folding, assembly, transport and degradation of other proteins and are induced under oxygen deficient conditions [17]. These proteins are considered as important players in the survival of mycobacteria in the presence of drugs and elucidation of their exact contribution to the bacterial drug resistance may help to develop new anti-mycobacterial therapeutics. In conclusion, here we focused on comprehensive comparison of proteins from sensitive and MDR-TB strains based on the high resolution 2DE patterns. This comparison is primarily aimed at the identification of proteins present in MDR-TB, but absent in susceptible *M. tuberculosis* species, are considered as valuable antigens for novel diagnostic, therapeutic and/or vaccination strategies against TB.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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