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2D DIGE proteomic analysis of multidrug resistant and susceptible clinical *Mycobacterium tuberculosis* isolates

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ABSTRACT

Tuberculosis (TB) is the leading cause of infectious disease related mortality worldwide. Infection of *Mycobacterium tuberculosis* (Mtb) leads to nearly 3 million deaths every year due to tuberculosis. Rifampicin and Isoniazid (RH) are the key drugs to being used for the treatment of tuberculosis. Reports in recent years indicate that the increasing emergence of resistance to these drugs. The resistance to these drugs severely affects options for effective treatment. The current vaccine for tuberculosis has variable protective efficacy and there is no commercially available serodiagnostic test for this disease with acceptable sensitivity and specificity for routine laboratory use, especially in case of multidrug resistance. In order to develop a new diagnostic tool for detection of Mtb, multidrug resistant Mtb as well and improve the tuberculosis vaccine, it is necessary to indentify novel antigenic candidates, especially in identification of multidrug resistant associated protein antigens. Here, we present a 2-D gel-based proteomic survey of the changes in RH resistant Mtb. The proteins extracted from RH resistant and susceptible Mtb clinical isolates were analyzed by two-dimensional differential in gel electrophoresis (2D-DIGE). Protein intensities of 41 spots were found to be regulated in RH resistant isolates. A total of 28 proteins were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Twelve proteins of interest are NADH-dependent enoyl-[acyl-carrier-protein] reductase, 60 kDa chaperonin 2, Chaperone protein DnaK, 3-oxoacyl-(Acyl-carrier-protein0 reductase, Probable acetyl-CoA acyltransferase FadA2, two Acetyl/propionyl-CoA carboxylase, alpha subunit, Universal stress protein Rv1636/MT1672, Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, Glutamine synthetase 1 and two uncharacterized proteins (Rv2557 and Rv1505c).

Keywords: 2-D Differential in gel electrophoresis – 2D-DIGE; Mycobacterial proteomics; Mycobacteria, Tuberculosis; Multidrug resistance.

1. Introduction

Tuberculosis (TB) is the leading cause of infectious disease related mortality worldwide. Infection of *Mycobacterium tuberculosis* leads to nearly 3 million deaths every year due to tuberculosis. Multi-drug resistant strains, emergence of HIV-TB co-infection have increased the severity of tuberculosis epidemic. Multidrug resistance of tuberculosis leads to a serious threat in global tuberculosis control and treatment of patients seems to be impossible using currently available drugs. Multidrug resistant tuberculosis, associated with high death rates of 50-80%, spans a relatively short time from diagnosis to death [1]. Delayed detection of drug resistance could lead to an ineffective drug therapy and this could be one of the major factors contributing to multidrug resistant tuberculosis outbreaks. The emergence of multidrug resistant tuberculosis has increased interest in the understanding the mechanism of drug resistance in *M. tuberculosis* and the development of new therapeutics, diagnostics and vaccines.

The development of effective vaccines and rapid, simple, cheap test for the diagnosis is critical in the control and prevention of tuberculosis. The prevention of tuberculosis could be performed since 1921 by vaccination with Bacillus Calmette Guerin (BCG) vaccince. However, its efficacy continues to be debated. The basis of two meta-analyses of BCG vaccination demonstrated that the vaccine is efficient in preventing severe and life-threatening forms of tuberculosis in children [2]. Recently, several vaccine candidates including subunit vaccines have been developed and have entered clinical trials, and still more are in the pipeline of discovery [3-5].

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The lack of powerful diagnostic procedure, especially diagnosis of multidrug resistant Mtb, leads to the difficulty in the control of this disease. Traditional tests based on the isolation and antibiotic sensitivity for diagnosis are time-consuming [6]. Several novel methods have been investigated and developed in recent years for diagnosis of tuberculosis such as nucleic acid amplification test, T-cell based assay, interferon gamma release assay and enzyme-linked immunospot assay [7-13]. However these methods require expensive equipments, a well-equipped and professional laboratory, highly trained personnel, and still time consuming. The utilization of these methods is limited in the developing countries where having a high proportion of *M. tuberculosis* infection. Enzyme-linked immunoabsorbent assay (ELISA), a simple, high throughput and inexpensive method, could be used for detection of *M. tuberculosis* infection [14].

Diagnosis of drug resistant tuberculosis plays an important role in the effective treatment. Early detection of drug resistance is one of the priorities of tuberculosis control. It allows initiation of the appropriate treatment and surveillance of drug resistance. Conventional phenotypic method for detection of drug resistance is based on detection of growth of *M. tuberculosis* in the presence of the antibiotics or indicator [2, 15-16]. However this method is time-consuming, therefore a new approach have been established for detection of drug resistance based on the analysis of DNA. This method has several advantages [2]. However, not all molecular mechanisms of drug resistance are known. Furthermore, drug resistance is due to mutation of several positions of target genes. Molecular characterization of rifampicin- and isoniazid resistant *M. tuberculosis* strains was performed and showed several mutation sites in the *rpoB* and *catG* gene. [17-19]. This method becomes more difficult in detection of multidrug resistance. Discovery of drug resistance consistently associated proteins of *M. tuberculosis*, especially multidrug resistance, could provide new biomarkers for detection using simple methods such as ELISA, lateral flow immunochromatographic assay.

Proteomics is a powerful tool for stuying the protein composition of complex biological samples. The proteomic approach along with *in vitro* and *in vivo* assessment of vaccine candidates can be very valuable in identifying new potential candidates in order to expand the antigenic repertoire for the development of effective novel vaccines against tuberculosis. The global study of the protein profile of resistant and susceptible strains by proteomic approach could help in further revealing of resistance mechanisms and determining multidrug resistance-associated biomarkers. The obtained findings support to develop newer drugs, development of vaccine and rapid diagnosis tool for multidrug resistance tuberculosis.

Investigations of protein expression profiles of *Mycobacterium tuberculosis* under various growth conditions, genetic backgrounds, geographic distribution have been performed [20-26]. Recently, researches on comparative analysis of drug resistant and suscetiple *Mycobacterium tuberculosis* by 2-DE combined with MS have been reported to reveal proteins associted with resistance [27-29].

Some studies in identification of biomarkers for serodiagnosis of drug resistant *M. tuberculosis* were performed by proteomic approach. Immunoproteome analysis of serum from patients infected with drug resistant or drug susceptible *M. tuberculosis* strains and heathy control identified three proteins as possible candidate biomarkers for use in the serodiagnosis of drug resistant tuberculosis infections [30]. Similarly, a proteome-scale identification of novel antigenic proteins in *M. tuberculosis* showed total of 249 proteins reacting significantly with the serum samples from patients in comparison with healthy cases [31].

The aim of this study was the comparison of the protein profiles of RH resistant and susceptible clinical *M. tuberculosis* isolates on the 2-D DIGE gel to identify RH resistant associated proteins.

2. Material and Methods

Mycobacterial growth

The RH resistant and sensitive *M. tuberculosis* clinical isolates (n = 3 per group to minimize genetic background differences) were obtained from National Institute of Hygiene and Epidemiology. Bacteria were grown in Middlebrook 7H12 broth at 37°C for four weeks (10^7-10^8 cfu/ml).

Sample preparation for proteomics

Mycobacterial cell extract was prepared according to modified protocol of as described [28]. Briefly, cells were washed three times with phosphate saline buffer (1X PBS buffer, pH 7.4) and then suspended in extraction buffer containing 8 M urea, 2 M thiourea, 2% CHAPS (Sigma-Aldrich, USA). The cell suspension was broken by intermittent sonication (15 seconds ON, 15 seconds OFF) for 4 min on ice at 80% energy using sonicator (Sonics & Materials Inc, USA). Subsequently, the lysate was clarified by centrifugation at 16,000 x g for 1 hour at 4°C. The supernatant was collected in a new eppendorf tube and protein concentration of the supernatant was determined using a Bradford assay kit (Sigma-Aldrich, USA). Sample aliquots were stored at -80°C for later use.

Two-dimensional differential gel electrophoresis (2-D DIGE)

Protein extracts (n = 3 per group) and a pooled internal standard containing aliquots of all isolate samples were labeled prior to electrophoresis with CyDyes according to the manufacture' recommendation (GE Healthcare). Briefly, 50 µg aliquots of each protein sample derived from each group was minimally labeled with 400 pmol of either Cy3 or Cy5 and the Cy2 dye was used for the labeling of the internal standard sample. The mixtures were incubated on ice for 30 min in dark followed by adding of 1 µl of 10 mM lysine for

quenching.

For 2-DE, two labeled samples (Cy3 and Cy5, each 50 µg) and the corresponding internal standard (Cy2, 50 µg) were mixed in rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 40 mM DTT, 0.6% Biolytes, pH 3-10 and trace of Bromophenol blue). Isoelectric focusing (IEF) was carried out using the method of in-gel rehydration [32]. Immobilized pH gradient strips of pH 4-7 and length 18 cm (Bio-rad, Hercules, CA, USA) were rehydrated overnight at 20°C with 150 µg labeled protein mixture. Strips were then focused on an IEF unit PROTEAN i12 IEF Cell (Bio-rad, Hercules, CA, USA) at 20°C using the following program: 250 V for 30 min in rapid mode, 10000 V for 2 h in gradual mode, 10000 V in rapid mode until 43 kVh reached and finally hold at 1000 V. The current limit was set at 50 µA per strip. After first dimension electrophoresis, IPG strips were equilibrated in buffer A (6 M urea, 0.375 M Tris, pH 8.8, 4% SDS, 20% glycerol and 1% DTT) for 15 min followed by equilibration in buffer B containing 2.5% iodoacetamide instead of DTT for further 15 min.

Separation of proteins on 12% SDS-polyacrylamide gels as the second dimension was performed [32] in a vertical electrophoretic dual gel unit PROTEAN II Xi (Bio-rad, Hercules, CA, USA) at constant power of 2 W for 16 hours. Images of the three different channels were acquired using a PharosFX Plus laser scanner (Bio-rad) at excitation/emission wavelength of 488/530 nm (Cy2), 532/605 nm (Cy3), and 635/695 nm (Cy5).

Spot detection, quantitation and statistical analysis

After scanning, analysis of the 2-D gel images was performed with the PDQuest Advanced software package version 8.0.1 (Bio-rad, Hercules, CA, USA). Images were analyzed using stepwise spot detection. Spot volumes were calculated based on the area and pixel intensities of spots. These spot volumes were normalized by a two-step procedure. In the first step, interchannel and intergel differences (Cy3, Cy5 and Cy2) in the intensities were corrected by dividing the background corrected values of the spot volumes by median of all spots of the channel (Cy2, Cy3 and Cy5). Then, fluorescence intensities of each spot were calibrated with the internal standard (Cy2) by dividing the median normalized spot volumes of the Cy3- and Cy5-channels by their correcting spot value of the Cy2 channel. The mean relative spot volumes of the group were used to reveal resistant-dependent changes in protein spot volumes by determining the ratios of the mean relative spot volumes between the susceptible and resistant group. We predefined stringent selection criteria in order to minimize the number of false positive results and defined the change in the level of a protein spot as significant only when the following two criteria were fullfilled: (i) the change in the intensity ratio between two groups had to exceed a factor of 1.5; (ii) the p-value of the corresponding Student's t-test had to be lower than 0.05. Resultant composite images for susceptible and resistant isolates were manually checked for artifactual spots, merged spots and missed spots.

Matrix assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS)

In order to identify proteins from spots showing significant differences in intensities, spots of interest from colloidal coomassie brilliant blue stained gels of protein extracts pooled from all isolates were manually excised and subsequently subjected to mass spectrometry for identifications. Preparation of peptide extracts and MALDI-TOF mass spectrometric analysis on a Proteome Analyzer 4800 (Applied Biosystems, Darmstadt, Germany) were carried out as described previously [32]. Identification of proteins was based on peptide mass fingerprint (PMF) data confirmed by at least one protein specific peptide fragmentation pattern. Identification with the UniProtKB/ Swiss-Prot database for *Mycobacterium tuberculosis* using the MASCOT algorithm via the GPS Explorer software (Applied Biosystems) were considered to be statistically significant when the protein and peptide ion scores exceeded 49 and 16, respectively, which corresponds to $p<0.05$. Mass tolerances for precusor ions and for fragment ions were 50 ppm and 0.45 Da, respectively. Methionine oxidation was set as variable and carbamidomethylation as fixed modification.

3. Results

Proteome reference map of Mycobacterium tuberculosis clinical isolates

In order to provide a baseline of the proteome of clinical *Mycobacterium tuberculosis* isolates and to define the functional classes of identified proteins, a proteome reference map was constructed. Total protein extracts from RH resistant and susceptible clinical *Mycobacterium tuberculosis* isolates were separated by 2-DE, visualized with a colloidal coomassie brilliant blue staining and 96 spots corresponding to the most abundant proteins were excised. After tryptic digestion, peptide mixtures were subjected to MALDI-TOF MS/MS for protein identification. A total of 79 proteins were identified (Supporting information Fig. S1 and Table S1). The majority of spots (91%) contained only one protein and 7 spots contained two proteins. Using the PANTHER (Protein Analysis Through Evolutionary Relationships at http://www.pantherdb.or) classification software, the identified proteins were assigned to biological processes (Fig. 1). Classification of identified proteins showed the most prominent categories belonging to metabolic process (47%), response to stimulus (9%), immune system process (9%).

Quantitative comparative proteome profiling of the susceptible and RH resistant isolates by 2-D DIGE

In order to identify changes in protein abundance in re-

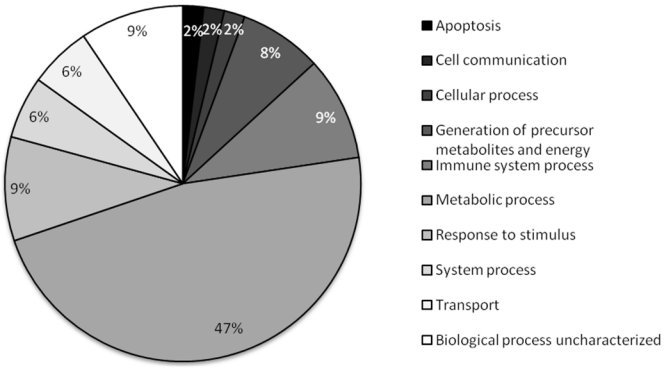

Figure 1. Functional classification of proteins of the clinical *Mycobacterium tuberculosis* reference map. Proteins were assigned to functional categories by uploading the results of the protein identification to the PANTHER classification software. The pie chart represents the distribution of identified proteins according to biological processes.

sistant isolates with high confidence, we employed the 2-D DIGE technology pioneered by Unlu et al. [33]. Representative examples of images of protein patterns generated with this sample set are shown in Fig. 2. A visual inspection of the images indicates that the protein patterns of the different groups looked similar. Quantification of the spot volumes with the PDQuest software package generated a spot set of a total of 458 spots, which were analyzed throughout all 2-D gels included in the experiment.

For quantitative analysis, the normalized spot volumes of the different groups of isolates were used to reveal multidrug resistant-dependent changes in the level of proteins by determining the ratios of mean relative spot volumes between the susceptible and RH resistant group. With a minimum of 1.5 fold change in level and a p-value of the corresponding Student's t-test lower than 0.05, a total of 41 protein spots was shown to display multidrug resistant-associated changes in intensity. The increase in spot volume in multidrug resistance was more pronounced than reduction (Fig. 3). The majority of those displayed increased intensity (23 protein spots – 56%) and a smaller proportion (18 protein spots – 44%) exhibited multidrug resistant-associated reduction of intensity (Supporting information Table S2).

Representative magnified regions of regulated proteins are showed in Fig. 4. These protein spots were excised from gels and subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

Identification of spots displaying RH resistant-associated changes in intensity

Analysis of the DIGE experiment indicated changed levels of proteins associated with RH resistance. In order to detect which proteins actually differ in amount, the corresponding protein spots were excised from gels. Spots of interest were digested with trypsin and subjected to analysis by MALDI-TOF MS. Twelve proteins of interest are NADH-dependent enoyl-[acyl-carrier-protein] reductase, 60 kDa chaperonin 2, Chaperone protein DnaK, 3-oxoacyl-(Acyl-carrier-protein0 reductase, Probable acetyl-CoA acyltransferase FadA2, two Acetyl/propionyl-CoA carboxylase, alpha subunit, Universal stress protein Rv1636/MT1672, Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, Glutamine synthetase 1 and two uncharacterized proteins (Rv2557/MT2634 and) (Table 1).

4. Discussion

Accurate quantitation of changes in protein expression remains one of the most important aspects of proteomics. Conventional 2-D PAGE with Coomassie Brilliant Blue or silver staining followed by image analysis is limited by the sensitivity and dynamic range of these detection methods [34,35]. 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) is a method to label proteins with CyDye fluors

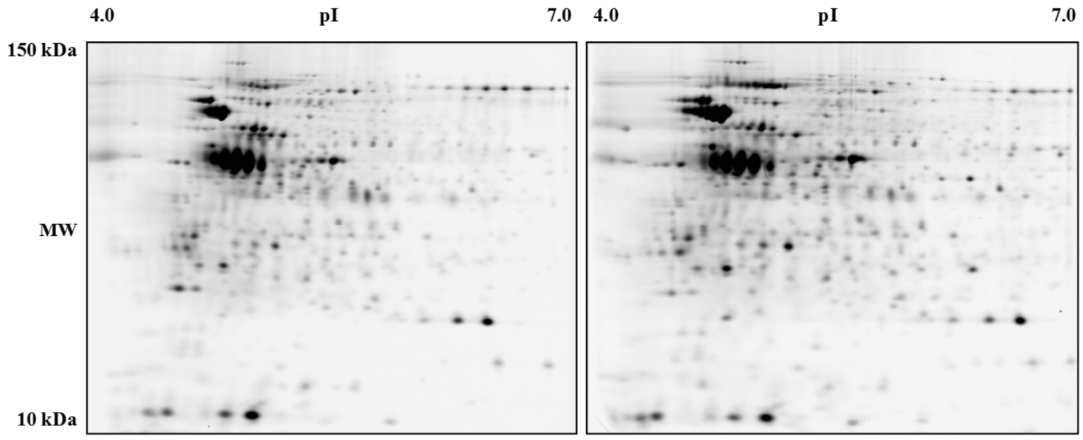

Figure 2. Protein patterns of susceptible and resistant clinical isolates of *Mycobaterium tuberculosis*. Protein samples were subjected to DIGE as described in Section 2. The images display representative examples of protein profiles of susceptible (A) and RH resistant isolates (B).

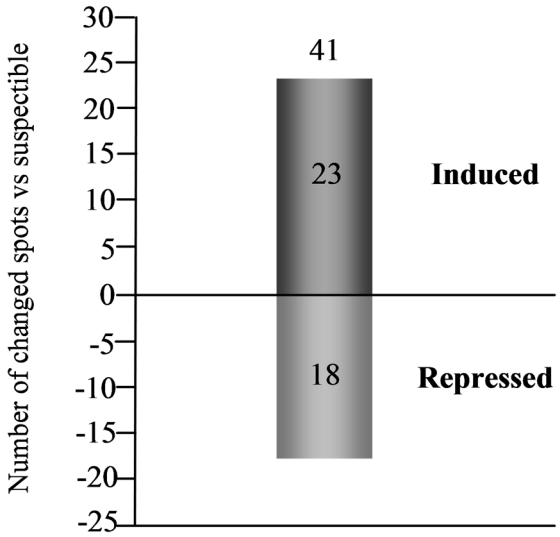

Figure 3. Bar graphs of protein spots displaying RH resistant-associated changes in intensity. Protein spot intensities were quantified and normalized to the internal standard using PDQuest software as above described. Protein display at least a 1.5 fold changes between the mean values of RH resistant isolate group compared to susceptible isolate group were considered as significant changed.

that are subsequently separated using 2-dimensional gel electrophoresis. This technology allows the separation of up to three different protein samples in the same 2-D gel because each of the three protein samples is labeled with one CyDye. The different labeled samples will be subjected to exactly the same 1st and 2nd dimension running conditions so the same protein labeled with a CyDye will migrate to the same position on the 2-D gel, this helps limit experimental variation. The multiplexing ability of DIGE experiment using an internal standard ensures correct co-identification of protein spots within the gel and accurate quantitation of expression differences. Thus, the technique is highly sensitive with a wide dynamic range for detection of proteins and compatible with state-of-the-art protein identification techniques using mass spectrometry. Comparative protein expression profiling is best performed with DIGE experiment [36,37].

In the present study we have applied the DIGE technique coupled with mass spectrometry for the identification of changed proteins in RH resistant *M. tuberculosis* isolates compared to RH susceptible isolates. The two-dimensional electrophoresis protein patterns of RH resistant and susceptible groups of *M. tuberculosis* isolates were highly similar (approximately 91% homology). This observation was coincided with the previous research [27,28]. A total of 17 distinct proteins of all 41 regulated protein spots were identified by mass spectrometry.

Protein spots 1 and 2 were up-regulated in RH resistant *M. tuberculosis* isolates and identified as chaperone protein DnaK (heat shock 70 kDa protein) and 60 kDa chaperonin 2 (GroEL-2), respectively. Chaperones in particular heat shock protein play an essential role in the maintenance of living ability of the cell. They are proteins to assist nascent proteins to reach right fold, protect subunits from heat shock during the assembly of complexes, prevent protein aggregation or mediate targeted unfolding and disassembly. Up-regulation of these proteins in response to stress is a key factor in the health of the cell and life-span of an organism [38]. DnaK assists the folding of several proteins. Unfolded proteins are transferred to another chaperone, the barrel-shaped GroEL - a highly specialized folding machine. This complex forms a nano-cage in which a single protein chain is temporarily enclosed and allowed to fold while protected from external influences [39].

DnaK has characteristic peptide binding domain and ATPase domain, which indicate its role in active protein refolding and proper assembly. Functional categories of DnaK were related to virulence, detoxification and adaptation [40]. Based on reference map, the distribution of DnaK

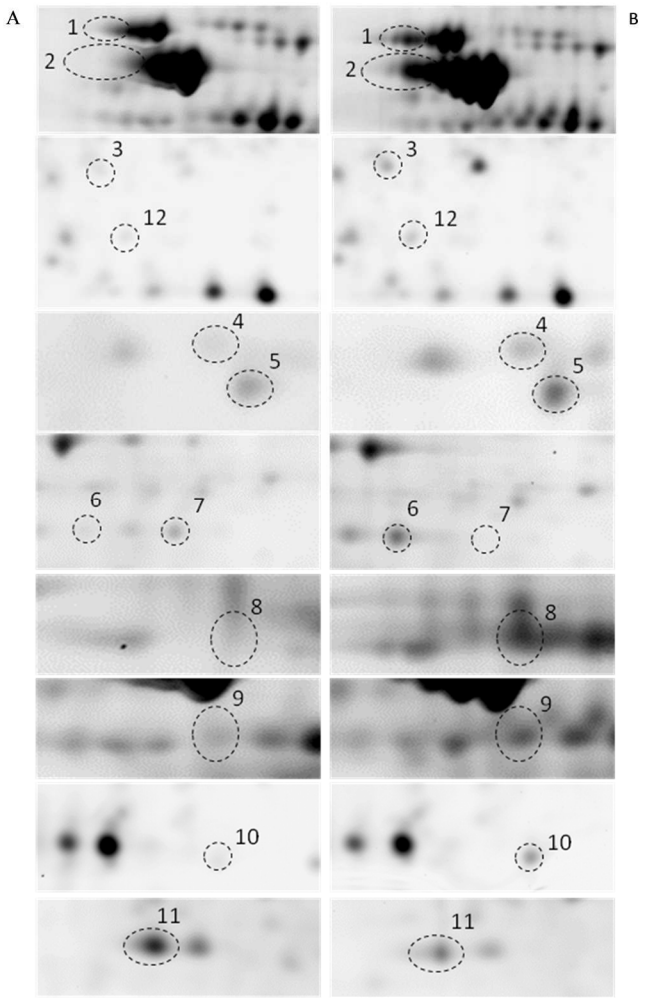

Figure 4. Representative magnified regions of 2-D gels showing the regulated proteins in comparison between the susceptible isolates (A) and RH resistant isolates (B).

Table 1. Identification of regulated proteins by MALDI-TOF mass spectrometry in RH resistant *Mycobacterium tuberculosis* isolates.

Spot No.	Regulated (p-value)	Accession No.	Proteins identified	Mass (Da)	pI	Score	Ions score	Sequence coverage (%)	No. of peptides matched
1	1.75 ↑ (0.00376)	DNAK_MYCTU (Rv0350)	Chaperone protein DnaK	66831	4.59	478	377.9	36.32	15
2	2.24 ↑ (0.01671)	CH602_MYCTU (Rv0440)	60 kDa chaperonin 2	56727	4.56	359	225.3	48.89	17
3	1.67 ↑ (0.03186)	INHA_MYCTU (Rv1484)	Enoyl-[acyl-carrier-protein] reductase [NADH]	28528	6.02	157	123	33.09	7
4	1.56 ↑ (0.00822)	L7N5J4_MYCTU (Rv0243)	Probable acetyl-CoA acyltransferase FadA2 (3-ketoacyl-CoA thiolase) (Beta-ketothiolase)	46107	6.64	659	576.9	43.18	11
5	1.85 ↑ (0.00975)	O53665_MYCTU (Rv0242c)	3-oxoacyl-(Acyl-carrier-protein) reductase	46830	6.38	656	441.6	63.00	23
6	1.68 ↑ (0.00502)	L7N4G3_MYCTU (Rv3285)	Acetyl/propionyl-CoA carboxylase, alpha subunit	63783	5.51	590	375.5	58.83	26
7	1.82 ↓ (0.00830)	L7N4G3_MYCTU (Rv3285)	Acetyl/propionyl-CoA carboxylase, alpha subunit	63783	5.51	504	327.5	49.83	23
8	1.63 ↑ (0.03373)	ODP2_MYCTU (Rv2215)	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	57088	4.64	237	166.2	33.27	12
9	1.53 ↑ (0.02786)	GLNA1_MYCTU (Rv2220)	Glutamine synthetase 1	53570	4.84	257	173.6	41.42	13
10	1.73 ↑ (0.01660)	Y1636_MYCTU (Rv1636)	Universal stress protein Rv1636/MT1672	15312	5.62	225	152.2	80.14	8
11	1.69 ↓ (0.00021)	Y2557_MYCTU (Rv2557)	Uncharacterized protein	24295	4.54	296	175	72.32	14
12	1.54 ↑ (0.01525)	L0T720_MYCTU (Rv1505c)	Uncharacterized protein	24056	5.97	151	95.7	42.53	7

in the 2-D gel pattern tends to shift pH toward the acidic end of the IPG strip, suggesting that the identified spot was the result of posttranslational modifications (Fig. 3). This phenomena was observed with spot 2, identified as 60 kDa chaperonin 2 (GroEL-2) (Fig.3). GroEL-2 was identified in vitro as the most prominently phosphorylated proteins by PknJ [41] and this protein has been considered as the substrate of STRKs [42]. Therefore, the pI shift of this protein toward the acidic end may be due to phosphorylation. This protein was also known as heat shock protein 60, heat shock protein 65, 65 kDa antigen, cell wall protein A, antigen A [43]. As a chaperone, function of this protein was suggested to prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions. This protein was predicted to a specialised regulation in *M. tuberculosis* because the location of this gene in *M. tuberculosis* genome is away from classic GroEL-Cpn10 operon [28]. As an antigen the *M. tuberculosis* heat shock protein 60 plays also an important role in modulating immune response of T-cell and macrophage [44,45]. Furthermore, it is noteworth that an increase of this protein was also found at the lower spot on 2-D gel. This observation indicated that the identified spot was a proteolytic degradation product of original protein. Recent study demonstrated that proteolysis of this protein could provide a potential source of immunogenic peptides in human tuberculosis [46]. A DNA vaccine based on this protein was demonstrated to activate human immune response [3]. Therefore this protein is considered as a candidate antigen for development of a subunit vaccine against mycobacteria. Furthermore, these proteins have also been reported to be up-regulated in drug resistant *M. tuberculosis* strains [28,29], suggesting that overexpression of these proteins might be accompanied with the drug resistance of mycobacteria.

The *inhA* gene of *M. tuberculosis* has been proposed to encode the primary target of the antibiotic isoniazid (INH). Overexpression of InhA in mycobacteria results in a 20-fold increase in the minimum inhibitory concentration of INH [47,48]. Resistance to INH might the result from drug sequestration by this protein. Furthermore, NADH-dependent enoyl-[acyl-carrier-protein] reductase was related to mycolic acid biosynthesis, an essential component of cell wall [49]. In

this study we found that this enzyme was significantly induced in abundance in RH resistant isolates. Therefore, it is possible that the increase of this enzyme improve the integrity of cell wall, ensuring the survival of mycobacteria whenever exposing to INH drug.

Another functional category of proteins that was significantly altered in abundance was related to the lipid metabolism. They were acetyl/propionyl-CoA carboxylase, alpha subunit, Probable acetyl-CoA acyltransferase FadA2, 3-oxoacyl-(Acyl-carrier-protein) reductase. Unlike NADH-dependent enoyl-[acyl-carrier-protein] reductase, no evidence indicated that there was direct interaction between these enzymes and INH adducts. However, it is possible that these enzymes might play a role in the construction of mycobacterial cell wall through lipid metabolism. Mycobacterial cell wall is known as the primary permeability barrier responsible for resistance to antibiotics [50]

The *dlaT* gene (Rv2215) and *glnA1* genes were encoding for dihydrolipoyllysine acetyltransferase (DlaT) component of pyruvate dehydrogenase complex and glutamine synthase, respectively. They were found to be up-regulated in RH resistant isolates. DlaT was required to convert pyruvate to acetyl-CoA, which is central to the intermediary metabolism and respiration of *M. tuberculosis*. DlaT enzyme is together with AceE and Lpd enzymes to construct the pyruvate dehydrogenase [51] and the lack of DlaT enzyme was leading to the lost of pyruvate deghydrogenase activity and reduction of *M. tuberculosis* viability in the host. Furthermore, there was evidence indicating that the mutation of the *M. tuberculosis dlaT* gene resulted in the impossibility of significant pathology in infected animal [52]. Similarly, GlnA1 enzyme, an enzyme of central importance in nitrogen metabolism, was demonstrated as essential factor in the pathogenicity of *M. tuberculosis* [53]. Therefore, these enzymes are considered as an essential for pathogenesis of *M. tuberculosis* and thus inactivation of DlaT and GlnA1 enzymes by some ways might be a potential approach for tuberculosis therapy. It will become a potential target for development of newer drugs in tuberculosis treatment. Besides its essential role in intermediary metabolism and respiration, DlaT enzyme also involves in antioxidant defense of *M. tuberculosis* a long with AhpC, AhpD and Lpd, [54]. The increase of antioxidant activity in this case seems to be compensation of the lack of the KatG catalase-peroxidase activity reduced in many isoniazid resistant strains. There was the existence of evidence indicating that antioxidant and glutamine synthetase activity of *M. tuberculosis* play an important role in suppressing host immune responses, suitable for pathogenesis. A modified BCG vaccine with reduction of these activities showed improvement in vaccine safety and effectiveness [55]

In the present study a universal stress protein (Rv1636/MT1672) was found to be up-regulated in resistant isolates. This protein was belonging to iron-regulated universal stress protein family and also considered as a member of family of *M. tuberculosis* hypothetical proteins including Rv2005c, Rv2623, Rv2026c, Rv1996, with unknown function [56]. However, in generally the universal stress protein (Usp) is a small cytoplasmic bacterial protein whose expression is enhanced when the cell is exposed to stress agents. Usp enhances the rate of cell survival during prolonged exposure to such conditions, and may provide a general stress response activity.

5. Concluding Remarks

This study is the first report applying 2-D DIGE technique for comparative analysis of RH resistant and susceptible clinical *M. tuberculosis* isolates. Total of 41 proteins spots were found to be regulated in RH resistant isolates. Identified proteins from these spots were functionally classified into virulence, adaptation and lipid metabolism. These differentially expressed proteins from RH resistant isolates might be considered as candidates for development of novel vaccine and rapid diagnosis tool for multidrug resistant tuberculosis.

6. Supplementary material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/168/0

Supplementary Fig. S1 - Proteome reference map of *Mycobacterium tuberculosis* clinical isolates. Total extract of proteins was separated by 2-DE DIGE gel. The most abundant spots were excised and subjected into MALDI-MS/MS for protein identification
Supplementary table S1 - Protein identification of spots on 2-D-DIGE gel by MALDI-MS for generating proteome reference map of *Mycobacterium tuberculosis* clinical isolates
Supplementary table S2 - Multiple DIGE data

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