Comparative proteomic analysis of sequential isolates of *Mycobacterium tuberculosis* from a patient with pulmonary tuberculosis turning from drug sensitive to multidrug resistant

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Background & objectives: Tuberculosis is a major health problem in India, and the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* (*Mtb*) has further complicated the situation. Though several studies characterizing drug sensitive and drug resistant strains are available in literature, almost all studies are done on unrelated strains. Therefore, the objective of this study was to compare the proteomic data of four sequential isolates of *Mtb* from a single patient who developed MDR-TB during the course of anti-tuberculosis therapy (ATT).

Methods: In this study, using two-dimensional (2D) gel electrophoresis and MALDI-TOF mass spectrometry, we compared and analyzed the cell lysate proteins of *Mtb* sequential clinical isolates from a patient undergoing anti-TB treatment. The mRNA expression levels of selected identified proteins were determined by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: The genotypes of all four isolates remained homologous, indicating no re-infection. The initial isolate (before treatment) was sensitive to all first-line drugs, but the consecutive isolates were found to be resistant to isoniazid (INH) and rifampicin (RIF) and developed mutations in the *katG*, *inhA* and *rpoB*. The intensities of 27 protein spots were found to be consistently overexpressed in INH and RIF resistant isolates. The most prominent and overexpressed proteins found during the development of drug resistance were GarA (Rv1827), wag31 (Rv2145c), Rv1437 and Rv2970c.

Interpretation & conclusions: This preliminary proteomic study provides an insight about the proteins that are upregulated during drug resistance development. These upregulated proteins, identified here, could prove useful as immunodiagnostic and possibly drug resistant markers in future. However, more studies are required to confirm these findings.

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Tuberculosis (TB) is a global emergency with an estimated nine million new cases and more than 1.5 million deaths occurring annually[1]. The situation has worsened after AIDS epidemic and with the emergence of multidrug resistant (MDR) forms of the causative agent *Mycobacterium tuberculosis* (*Mtb*). These drug resistant strains are more infectious by virtue of their high transmissibility in the population. Therefore, identification of the reliable diagnostic, prognostic and drug resistance markers is an urgent research priority. Various *in vitro* and *in vivo* studies have identified chromosomal mutations as determinants of drug resistance[2-4]. For example, mutation (s) in *rpoB* allele confers rifampicin (RIF) resistance (RIF[r]) in 90-95 per cent isolates[2], while isoniazid (INH)-resistance (INH[r]) is attributed to mutation (s) in one or more alleles *viz., katG, inhA, ahpC* and *ndh*. However, in about 20 per cent of INH[r] isolates, none of these known mutations are found, suggesting the possibility of unknown mutations or mechanisms[2,3]. On the other hand, the genetic mutations may not necessarily correlate with phenotypic resistance; further suggesting that other factors such as drug impermeability, drug-efflux pumps, formation of survivable "persister cells" under drug pressure and several other host factors could be involved in the outcome of treatment[2].

Hence, for unraveling the mechanism(s) of drug resistance, understanding the mode of action of anti-TB drugs is very crucial. Many studies have elucidated the mode of action of various anti-TB drugs using genetic analysis, mRNA expression and DNA microarray analysis[4,5]. Several groups have also explored the proteome of *Mtb* and provided comprehensive details about the subcellular localization and confirmed the genomic annotation[6-9]. In these studies two-dimensional (2D) gel electrophoresis followed by mass spectrometry (MS) identification of the differentially regulated proteins substantially helped in identifying the complex pathways and their regulatory enzymes. These studies also elucidated modes of action of various drugs and discovered new antigens that could be potential candidates for developing vaccines and diagnostics[6,7,9,10]. However, only a few studies are available which show differential expression of specific proteins in the drug resistant but not in drug susceptible cells[7–9,11]. Further, in all these studies, either the non-pathogenic mycobacteria or laboratory collections of drug sensitive and drug resistant strains of *Mtb* from different patients have been used. In the present study, protein profile of sequentially collected

four clinical isolates of *Mtb* was analyzed using 2D gel electrophoresis and the differentially expressed proteins were identified by MALDI-TOF-MS analysis. All isolates were from the same patient, who developed MDR-TB during the course of chemotherapy.

Material & Methods

The study was conducted between January 2006 and June 2010 at the TB Laboratory, Division of Clinical Microbiology and Molecular Medicine, Department of Laboratory Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi, India. This study was approved by the Institutional Ethics Committee of AIIMS and written informed consent was obtained from the patient. The patient was being treated at the designated microscopy and DOTS (directly observed treatment-short course) centres of Shahpurjat, New Delhi. This patient (22 yr old male) was diagnosed as having pulmonary TB on the basis of clinical and radiological findings and sputum smear microscopy. He was prescribed with anti-TB treatment (ATT) under the DOTS programme. The thrice a week treatment regimen comprised isoniazid, rifampicin, pyrazinamide (PZA) and ethambutol (EMB) (category I treatment) in intensive phase for two months followed by four month treatment with two (isoniazid and rifampicin) drugs regimen. Pre-treatment sputum specimen was used for isolation of *Mycobacterium* sp. by BACTEC MGIT-960 (Becton Dickinson, Sparks, MD, USA), which was positive. The isolate was identified as *Mtb* by conventional phenotypic and in-house PCR method[12]. This culture was labelled as isolate A, and was subjected to 16sRNA gene sequencing. The patient though took full six months course of treatment but became irregular in taking drugs after initial improvement in his clinical symptoms. After three months of cessation of treatment (6+3=9 month[13], his condition again deteriorated and his sputum culture was again positive for *Mtb*. We labelled this second culture as isolate B. He was re-treated with isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin (SM) (category II regimen). Within two months his clinical condition improved but he again defaulted. After an asymptomatic period of about four months his symptoms reappeared. His sputum was again culture positive and this culture was labelled as isolate C. The patient was again prescribed with the same treatment for 12 months after counselling but he stopped treatment after six months. His condition further deteriorated and he died of multisystem failure. The fourth sample was received just before his death

and the isolate from this sputum sample was labelled as isolate D.

All the four clinical isolates (A, B, C & D) were identified as *Mtb* using standard protocols[12,13]. The anti-mycobacterial drug susceptibility testing was performed on all the isolates by both BACTEC™ MGIT-960 (Becton Dickinson, Sparks, MD, USA) and proportional method using Middlebrook 7H10 (Difco, USA) agar plates containing first-line anti-TB drugs (SM 2.0 μg/ml, INH 0.2 μg/ml, RIF 1.0 μg/ml, EMB 6.0 μg/ml)[13,14]. All four isolates were also genotyped by spoligotyping and identified using SITVIT-WEB database[15]. The *rpoB, inhA* and *katG* gene targets were sequenced using the primers as described elsewhere[13].

Preparation of mycobacterial whole cell lysate: All *Mtb* isolates were grown without shaking in Middlebrook 7H9 medium supplemented with 0.2 per cent (v/v) glycerol, 10 per cent oleic acid, albumin-dextrose and catalase (OADC, Difco, USA) at 37°C for two weeks. Whole cell lysate was prepared according to protocol of Sharma *et al*[11]. Cells were washed three times with normal saline and then suspended in sonication buffer [50 mM tris-HCl containing 10 mM $MgCl_2$, 0.1% sodium azide, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1mM ethylene glycol tetra acetic acid (EGTA); *p*H 7.4] at a concentration of 1g wet cell mass per 5ml, and then broken by intermittent sonication for 15 min at 4°C using sonicator (Sonics & Materials Inc, USA). The homogenate was centrifuged at 12,000×g for 20 min at 4°C. The pellets were discarded and supernatant was stored at -70°C until further use.

Protein precipitation with sodium dodecyl sulphate (SDS)-trichloroacetic acid (TCA)-acetone: The cell lysates were treated with 1 per cent SDS and then subjected to TCA-acetone precipitation procedure[9]. The protein pellet was suspended in appropriate volume of two-dimensional rehydration buffer (Bio-Rad, USA), and the protein concentration was estimated using the Bradford method[16].

Two-dimensional gel electrophoresis: Isoelectric focusing (IEF) was done using the in-gel rehydration method (Bio-Rad, USA).

2D gels were analysed using PDQuest Advanced software (version 8.0) (Bio-Rad, USA). After acquisition, the images were analyzed using step-wise spot detection and spot matching followed by differential expression analysis. The quantity of each spot was normalized by total valid spot intensity. The expression differences for all four mycobacterial isolates were compared using the same software. Images for sensitive and resistant isolates were manually checked for artifactual spots, merged spots and missed spots, and spots with more isolate-specific variability were omitted in the downstream processing. Equal amount of protein was loaded in all gels and experiments were repeated three times with three independent biological replicates.

In-gel digestion of protein spots with trypsin: Protein spots of interest were excised from the coomassie brilliant blue R250 stained 2D gels using spot picker Investigator ProPic (Genomic Solutions Ltd., Huntingdon, UK). Digestion of proteins and spotting of peptides on matrix assisted LASER desorption/ionization-time of flight (MALDI-TOF) target plate was carried out using protein digester investigator ProPrep (Genomic Solutions, Huntingdon, UK).

For protein digestion, method of Shevchenko *et al*[17] was followed with slight modifications. In brief, the gel plugs were de-stained and dehydrated by washing three times (10 min) with 25 mM NH_4HCO_3-50 per cent acetonitrile (ACN) (1:1v/v) solution and treated with freshly prepared 10 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 for 45 min at 56°C. After incubation, DTT was replaced with freshly prepared 55 mM iodoacetamide for 30 min and then dehydrated with 100 per cent ACN. The dried gel pieces were incubated for 12 h at 37 °C with 25 mM NH_4HCO_3 containing 0.02 μg/μl of mass spectrometry grade trypsin (Promega, USA). The resulting peptides were extracted twice from the gel pieces, using peptide extraction buffer [1:1v/v mixture of 70% ACN and 0.1% trifluoroacetic acid (TFA)].

Mass spectrometric analysis: Mass spectrometry (MS) was carried out as described earlier[9]. The digested samples were desalted and concentrated on C-18 ZipTips (Millipore, USA) using the manufacturer's protocol before mass spectrometric analysis. ZipTips were eluted on MTP 384 target plate with 2 μl of α-cyano-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich, USA) saturated solution dissolved in 50 per cent ACN and 0.2 per cent TFA. Mass spectra of digested proteins were acquired using Autoflex II TOF/TOF 50 (Bruker GmbH, Leipzig, Germany) in positive reflectron mode. AnchorChip target plate was placed in sample inlet of the instrument, controlled by flexControl 2.4 software (Bruker, Germany). The instrument was equipped with a 337 nm nitrogen LASER, delayed extraction

electronics, and a 50Hz digitizer and percentage of LASER energy was maintained at 30-40 per cent. The pulse energy was 105 μJ and pulse duration was 1.3 nano sec. Final mass spectra were produced by averaging 1500-2500 LASER shots taken at different positions within each spot. The spectra were acquired in positive reflection mode in the mass range of 500-3000 m/z. Calibration was performed using peptide calibration standard II (Bruker, Germany). The proteolytic masses obtained, were processed through FlexAnalysis v.2.4 programme (Bruker, Germany) for peak detection. Initially, the spectra acquired were processed for baseline subtraction with 80 per cent baseline flatness followed by smoothening with threshold of signal-to-noise ratio (S/N) >5. The contaminant m/z peaks originating from human keratin, trypsin auto-digestion and matrix were removed from the spectra to generate the peptide mass list for the database search. Finally, the proteolytic masses obtained were evaluated using MASCOT, a peptide mass fingerprinting (PMF) tool (Matrix Sciences, UK). Peak detection in MALDI spectra and peak lists were submitted to the UniProtKB/Swiss-Prot database using the MASCOT search engine (*http://www.matrixscience.com*) to identify the proteins from the annotated *Mtb* chromosome (strain H37Rv, EMBL/GenBank/DDBJ entry AL123456). Peptide mass tolerance was set in range of 50-100 ppm, with carbamidomethyl-cystein set as fixed modification, oxidation of methionine as variable modification and only 0 or 1 missed cleavage site was allowed. Further, matched precursor ions of identified proteins were selected for subsequent fragmentation using post source decay (PSD) for MS/MS. Lift_ATT method was performed in flex control software; parent peak mass spectrum was acquired by hitting LASER for 400-550 shots followed by acquisition of fragments of selected precursor ions for the same number of shots. Both parent and fragment spectra were pooled to generate MS/MS spectrum of a particular peptide. MS/MS spectrum was submitted to database using MASCOT wizard (Matrix Sciences, UK). The same parameters were used for MS/MS search in addition to the fragment mass tolerance from 0.5 to 2.0 Da.

Glycogen estimation: Logarithmic and stationary phase growth of *Mtb* sensitive (isolate A) and MDR (isolates B,C,D) isolates were collected by centrifugation (3 min at 5000 x *g* and at 4°C), and the pellet (15-20 mg wet weight) was re-suspended in 0.25M Na_2CO_3 and incubated at 95°C for 4 h. The glycogen content was estimated by following the procedure of Schulze *et al*[18].

Isolation of Mtb total RNA and real-time quantitative PCR (qRT-PCR): *Mtb* H37Rv was grown in Middlebrook 7H9 broth containing 10 per cent OADC, and was treated with INH (0.1μg/ml), RIF (1.0μg/ml), EMB (5μg/ml) and INH (0.1μg/ml) +RIF (1.0μg/ml). Total RNA was isolated using a TRI reagent (Sigma, USA) following manufacturer's instructions. To analyze mRNA expression, cDNA was synthesized from 1 μg of total RNA by using Superscript III (Invitrogen Life Technologies, USA) and random primers (Invitrogen, Life Technologies, USA), followed by amplification of the gene(s) by gene-specific primers, using master mix SYBR green (Applied Biological Materials Inc., Canada). The expression is represented in fold increase. 16sRNA was used as internal control for mRNA expression analysis of *ahpC*, *Rv1827*, *pknA*, *pknB*, *pknG* and *wag31*.

Each reaction was repeated thrice with three independent RNA samples in a smart cycler Cepheid machine (Cepheid, USA). RT-PCR conditions were as follows: an initial denaturation step of 10 min, followed by 40 amplification cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. Melting curve analysis was carried out to confirm the specificity of the amplified product. After baseline corrections and determination of threshold settings, calculation and statistical analyses were carried out using the $2^{-\Delta\Delta C_T}$ Method[19]. The results are shown as fold increase in expression profile.

Results

Drug resistance pattern and mutations in katG, inhA and rpoB genes of the isolates: The four sequential culture isolates were identified as *Mtb* by conventional phenotypic and in-house PCR method. Genotyping was done by spoligotyping and the results confirmed that all the isolates belonged to the Central Asian Strain Delhi (CAS1_Delhi, ST26) genotype. The drug susceptibility test results showed that the initial isolate (isolate A) was sensitive to all the four first-line drugs (SM, INH, RIF and EMB), but the consecutive isolates (isolates B, C and D) became resistant to three drugs; INH, RIF and EMB. The minimum inhibitory concentration (MIC) of isolate B increased as compared to isolate A against INH, RIF and EMB. However, it was still sensitive to kanamycin. The isolates C and D became resistant not only to INH, RIF and EMB but also to kanamycin. The sequencing of the *rpoB* (RIF[r]), *katG* and *inhA* (INH[r]) regions revealed mutated alleles associated with resistance to the respective drugs[13]. Morphologically the resistant isolates were stunted, thicker and cocco-bacillary in shape.

Differentially expressed proteins in drug sensitive and resistant isolates: The cell lysate proteins of four *Mtb* isolates were analyzed by 2D gel electrophoresis, which showed 430 protein spots in isolates A and 495, 556 and 395 spots in isolates B, C and D, respectively (Figs 1 & 2). Quantitative analysis of 2D gel spots was carried out using PDQuest software which revealed 27 spots upregulated in MDR isolates (Table I). The spots showing more than 2-fold upregulation were further identified by MALDI-TOF/TOF MS (Table II). To rule out possibility of any artifact, proteins showing equal intensity were taken as internal control (represented as square in Fig. 1). Upregulated proteins were functionally classified according to TubercuList web server which showed that most of the identified proteins belonged to the functional group 0, 1, 2, 3, 5, 7 and 9; corresponding to virulence, detoxification and adaptation (18.5%), lipid metabolism (11.11%), information pathway (14.81%), cell wall and cell process (3.7%), insertion sequence and phages (18.51%), and intermediary metabolism and information (29.62%); respectively (Table III). The magnified regions of upregulated proteins are shown in Fig. 3. Of the 27 upregulated proteins, eight were hypothetical protein (Rv2004c), probable glutamyl-tRNA (GLN) amidotransferase A gatA (Rv3011c), possible phosphoserine aminotransferase SerC (Rv0884c), probable lipase/esterase LipN (Rv2970c), probable phosphoglycerate kinase Pgk (Rv1437), conserved hypothetical protein with FHA domain, GarA (Rv1827), bacterioferritin (Rv1876) and conserved hypothetical protein (Rv0543) and were not found in 2D-PAGE database system accessible at *http://www.mpiib-berlin.mpg.de/2D-PAGE*, whereas three proteins probable iron-regulated aconitate hydratase Acn (Rv1475c), probable chaperone protein DnaK (Rv0350) and 60 kDa chaperonin 2 groEL2 (Rv0440), were found in two spots. Six proteins (gatA, serC, fbd, garA, Rv2204c and Rv0543c) in isolate B, 10 proteins (Rv685c, Rv3457c, Rv1479, Rv2970c, Rv1437, qor, and two spots each of Rv1475c and groEL2 family) in isolate C, three proteins (fadB, fabG4 and rrf) in isolate D, three proteins (Rv3075c, Rv1436 and GroES) in isolates C as well as in D were found upregulated. Only five proteins were consistently upregulated in all the three resistant isolates and these were identified as, chaperonin protein dnaK HSP70 (spots 4 and 5), hypothetical protein (Rv2004, spot 8), antigen 84 (wag31, spot 19) and bfrA (spot 24) (Fig. 1).

Among the identified proteins, we were more interested in studying the possible role of Rv1827 (GarA)

and wag31 in drug resistance, since these proteins have been identified as physiological substrates for protein kinases G (pknG*)*. Our result revealed that GarA and wag31 were upregulated in the drug resistant isolates. We analyzed the mRNA expression of *Rv1827* and its cognate protein kinases, *pknG, pknB, pknA* and *wag31*.

Drug induced changes in mRNA expression of protein kinases: To verify our protein expression observations, we studied the mRNA expression profile to see the effect of the four drugs on the standard strain of *Mtb* (H37Rv) which was sensitive to all anti-TB drugs. For this, the mRNA from H37Rv strain was isolated before and after exposing it to INH (0.1 µg/ml), EMB (5.0 µg/ml), RIF (1.0 µg/ml) and INH+RIF (0.1 + 1.0 µg/ ml) for 6 h. Consistent with the proteomic data, seen in clinical isolates, *Rv1827* expression was upregulated in all the tested conditions. As expected, the upregulation was 6.82 fold when the *Mtb* standard strain (H37Rv) was exposed to INH and RIF together, but other tested genes had relatively diminished expression. While combining the EMB, the expression of *wag31* was higher and *pknA* and *pknG* expressions were highest (Fig. 4).

Glycogen storage: GarA, which is a glycogen regulatory protein, was found upregulated in our MDR isolates. It was found that as compared to sensitive isolates the glycogen accumulation in MDR isolates was higher. Consistent with GarA protein levels, the glycogen accumulation measured after seven days was 1.8, 2.0 and 2.1 folds higher in isolates B, C and D, respectively, as compared to sensitive isolate A. Interestingly, after 15 days the glycogen storage remained almost unchanged (Fig. 5).

Discussion

Emergence of drug resistance in *Mtb* has become a major concern for TB control programme managers and treating physicians. Though advances in genome sequencing methods have provided better opportunities to our understanding about functional genomics and proteomics of the *Mtb*, the knowledge about mechanism of drug resistance still remains limited only to the association of genetic polymorphism. Most often, the data from proteomic studies are used to understand host-pathogen interaction, virulence, drug resistance and drug tolerance[8,9,19-23]. Such studies have provided a comprehensive list of *Mtb* proteins that are found differentially regulated in laboratory maintained standard H37Rv strain exposed to drug pressure.
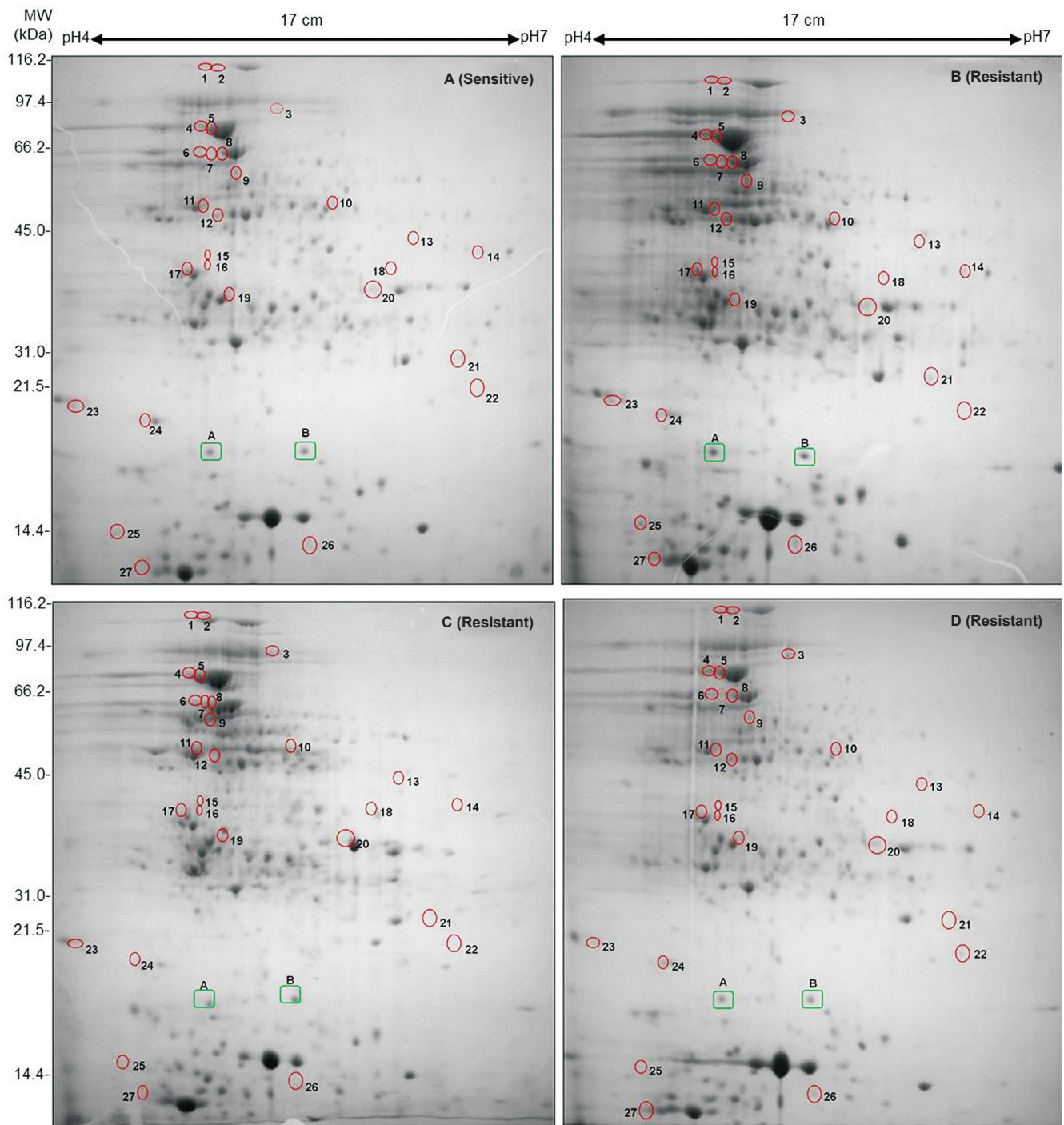
Fig. 1. 2D gel profiles of whole cell lysate proteins of *Mtb* clinical isolates collected sequentially from a single patient. The upregulated proteins are highlighted by circles. **(A)** First *Mtb* isolate (before treatment) sensitive to all 4 drugs, **(B)** Second isolate (during treatment) acquires MDR, **(C)** Third isolate (after 15 months of treatment) acquires drug resistance to yet another drug kanamycin and **(D)** Fourth MDR isolate after 27 months. Two Proteins, A (Rv1080c) and B (Rv2140c) marked in green rectangles, were selected for observing expression variation in all four samples and showed similar level of expression in all gels.

(A)

Total proteins 495,
Specific protein 6

6
B

Total proteins 395,
Specific Protein 3

3
D

5

10
C

3

Total proteins 556,
Specific Protein 10

(B)

Proteins classification

Classification	No. of proteins
Regulatory proteins	1
Intermediary metabolism and respiration	8
Insertion seqs and phages	5
Cell wall and cell processes	1
Information pathways	4
Lipid metabolism	3
Virulence, detoxification, adaptation	5

No. of proteins

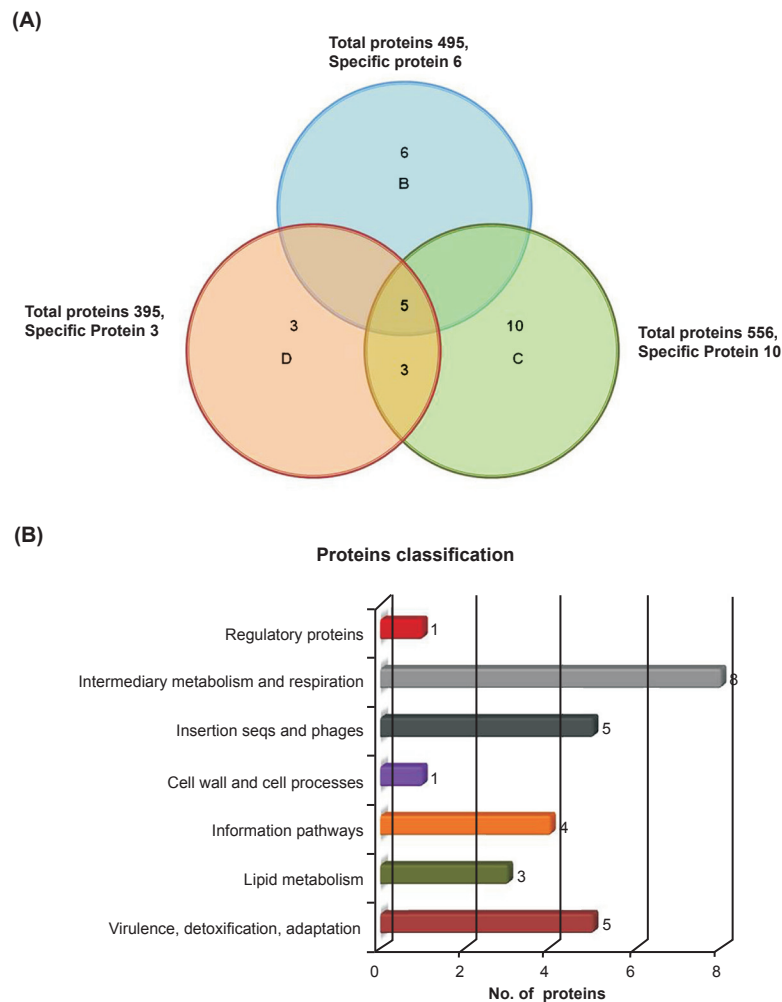**Fig. 2.** Upregulated proteins, their distributions, amount of overlap and functional classification in *Mtb* clinical isolates: **(A)** Upregulated proteins distribution and amount of overlap in clinical drug resistant isolates. **(B)** Functional classification of differentially expressed proteins according to the TubercuList Server.

However, in such studies protein (s) that could be modulated *in vivo* during or after acquiring *in vivo* drug resistance are missed out[22]. The ever-increasing evidences suggest that the expression of genes[22] and/or proteins[23] in clinical isolates is markedly different from the laboratory maintained H37Rv strain; suggesting that majority of these observations may not have direct impact in real life scenario.

In the present study, several proteins were identified that were upregulated in drug resistant isolates. Of the 27 upregulated proteins, five were upregulated in all sequential resistant isolates (B, C and D). Approximately half of these overexpressed proteins are reported as essential for *in vitro* growth of *Mtb*[21-24]. Although their functional role in drug resistance is elusive, validation

of these proteins as a biomarker of drug resistance will provide a scope for finding an effective candidate drug for MDR-TB. It also needs to be emphasized that identifying a protein as "upregulated" does not necessarily imply that it is a true determinant of drug resistance, because it is possible that some or all of these proteins are associated with adaptability of the *Mtb* to survive longer in the host system.

Among the upregulated proteins, rpoA (Rv3457c) was found 2.9 folds upregulated in the drug resistant isolates. In a similar study compensatory mutations in *rpoA* and *rpoC* (Rv0668) were identified particularly in more than 30 per cent of RIF-resistant strains and the authors proposed that mutation in these alleles could also be associated with MDR[24]. Though we have

Table I. Details of upregulated proteins identified by mass spectrometry in drug resistant isolates

Spot No.[†]	Proteins identified	Open reading frame (ORF) No.	Accession No.	Mascot score	Nominal mass (Da)	Isoelectric point (pI)	No. of peptides matched	Sequence coverage (%)	Densitometric ratio of protein upregulation between sensitive vs. resistant isolates			Protein classification according to Pasteur Institute of Genomics (TubercuList)
									B	C	D	
1	Aconitase hydratase*	Rv1475c	NP_215991	132	102728	4.95	18	21	1:1.0	1:4.36	1:1.71	7
2	Aconitase hydratase*	Rv1475c	NP_215991	126	102728	4.95	17	21	1:1.22	1:4.70	1:1.58	7
3	Probable fadB protein	Rv0860	NP_215375	171	76170	5.42	24	33	1:1.0	1:1.0	1:2.08	1
4	Chaperonin protein dnaK (HSP70)*	Rv0350	NP_214864	99	66790	4.85	11	22	1:6.88	1:7.88	1:4.76	0
5	Chaperonin protein dnaK (HSP70)*	Rv0350	NP_214864	165	66790	4.85	22	38	1:156.2	1:195.6	1:66.4	0
6	60kDa Chaperonin 2 (cpn60-2, groEL2)*	Rv0440	NP_214954	128	56692	4.85	16	29	1:1.22	1:4.70	1:1.58	0
7	60 kDa Chaperonin 2 (groEL2)*	Rv0440	NP_214954	231	56659	4.85	19	49	1:1.09	1:4.24	1:1.27	0
8	Hypothetical protein	Rv2004c	NP_216520	53	54959	5.89	10	29	1:54.21	1:132.28	1:49.82	5
9	Glutamyl tRNA (Gln) amidotransferase	Rv3011c	NP_217527	98	51787	4.91	9	27	1:2.26	1:1.13	1:0.93	2
10	Elongation factor Tu (EF-Tu)	Rv0685	NP_215199	248	43556	5.28	24	69	1:1.38	1:3.07	1:0.97	2
11	DNA directed RNA polymerase α-chain (rpoA)	Rv3457c	NP_217974	205	37740	4.64	16	43	1:1.7	1:2.98	1:1.0	2
12	Putative phosphoserine aminotransferase	Rv0884c	NP_215399	90	40266	4.77	7	28	1:2.21	1:0.70	1:1.75	7
13	Probable fabG4 protein	Rv0242c	NP_214756	207	46916	6.04	17	52	1:1.01	1:1.0	1:2.08	1
14	Probable moxR protein	Rv1479	YP_177816	103	40738	5.96	12	37	1:1.0	1:2.08	1:1.0	9
15	Probable lipase	Rv2970c	NP_217486	82	43685	6.33	6	30	1:1.19	1:2.28	1:1.0	7
16	Hypothetical protein	Rv1437	NP_217230	88	35519.79	5.14	10	41	1:1.91	1:2.82	1:1.28	7
17	Hypothetical protein	Rv3075c	NP_217591	98	33194	4.73	9	28	1:1.73	1:8.12	1:2.65	5
18	Glyceraldehyde-3-phosphate dehydrogenase	Rv1436	NP_215952	71	36105	5.19	6	17	1:1.0	1:20.61	1:7.0	7
19	Antigen 84 (wag31)*	Rv2145c	NP_216661	120	28260	4.8	8	43	1:12.0	1:7.0	1:3.0	3

Contd...

Spot No.[†]	Proteins identified	Open reading frame (ORF) No.	Accession No.	Mascot score	Nominal mass (Da)	Isoelectric point (pI)	No. of peptides matched	Sequence coverage (%)	Densitometric ratio of protein upregulation between sensitive vs. resistant isolates			Protein classification according to Pasteur Institute of Genomics (TuberculList)
									B	C	D	
20	Probable quinone oxidoreductase (qor)	Rv1454c	NP_215970	80	34140	5.37	7	42	1:1.15	1:6.42	1:1.89	7
21	Antigen precursor (MPT51)	Rv3803c	YP_178017	64	31069	6.13	5	23	1:2.19	1:1.12	1:1.02	1
22	Ribosome recycling factor (RRF)	Rv2882c	NP_217398	108	20815	5.71	10	67	1:1.0	1:1.0	1:3.45	2
23	Hypothetical protein (GarA)	Rv1827	NP_216343	87	17240	4.29	5	45	1:7.66	1:1.37	1:1.16	5
24	bfrA (Bacterioferritin)	Rv1876	NP_216392	90	18443	4.5	12	82	1:9.27	1:37.5	1:38.58	7
25	Hypothetical protein	Rv2204c	NP_216720	103	12707	4.4	6	51	1:9.11	1:1.0	1:1.04	5
26	Hypothetical protein	Rv0543c	NP_215057	67	14743	5.2	5	38	1:3.03	1:1.01	1:1.53	5
27	10 kDa Chaperonin (cpn 10, groES protein)*	Rv3418c	NP_217935	162	10667	4.62	10	99	1:1.0	1:10.86	1:9.28	0

Cut-off limit ≥2.0 fold for overexpression of proteins. [†] Spot number of the protein as marked in Fig. 1. *Some proteins are having mobility difference.

Spot No.[†]	Peak mass (Da)	Protein identified	Rv No.	Nominal Mass (Da)	pI	Mascot score	Sequence peptide
1	829.4455	Aconitase hydratase	Rv1475c	102728	4.95	39	KSYQIYRL
	1132.6464	Aconitase hydratase		102728	4.95	32	RNGGILQYVLRN
	1170.5177	Aconitase hydratase		102728	4.95	34	RWGQGAFDDFKV
	1299.587	Aconitase hydratase		102728	4.95	37	RIDTPGEADYYRN
2	829.4455	Aconitase hydratase	Rv1475c	102728	4.95	37	KSYQIYRL
	1132.6464	Aconitase hydratase		102728	4.95	39	RNGGILQYVLRN
	1170.5177	Aconitase hydratase		102728	4.95	35	RWGQGAFDDFKV
	1299.587	Aconitase hydratase		102728	4.95	35	RIDTPGEADYYR.N
3	1530.73	Probable fadB protein	Rv0860	76170	5.42	49	KSGSSQPPLQDMIDRM
	1836.9363	Probable fadB protein		76170	5.42	40	KGVDFVIEAVFENQELKH
	2078.1801	Probable fadB protein		76170	5.42	37	KGSQLGLPEVTLGLLPGGGGVTRT
4	1568.0111	Chaperonin protein dnaK (hsp70)	Rv0350	66790	4.85	50	KLLGSFELTGIPPAPRG
	1743.8923	Chaperonin protein dnaK (hsp70)		66790	4.85	37	RATSGDNHLGGDDWDQRV
	2109.2639	Chaperonin protein dnaK (hsp70)		66790	4.85	40	RNGEVLVGQPAKNQAVTNVDRT
5	1062.611	Chaperone protein dnaK	Rv0350	66790	4.85	32	RTTPSIVAFARN
	1226.711	Chaperone protein dnaK		66790	4.85	33	KDAGQIAGLNVLRI
	1645.962	Chaperone protein dnaK		66790	4.85	37	RIVNEPTAAALAYGLDKG
	2613.426	Chaperone protein dnaK		66790	4.85	55	RSETFTTADDNQPSVQIQVYQGERE
6	914.6248	60kDa Chaperonin 2 (cpn60-2, groEL2)	Rv0440	56692	4.85	55	KGRNVVLEKK
	1223.7164	60kDa Chaperonin 2 (cpn60-2, groEL2)		56692	4.85	33	KTIAYDEEARRG
	1266.754	60kDa Chaperonin 2 (cpn60-2, groEL2)		56692	4.85	35	MAKTIAYDEEARR
	1580.0454	60kDa Chaperonin 2 (cpn60-2, groEL2)		56692	4.85	42	REGLRNVAAGANPLGLKR
7	1067.5167	60kDa Chaperonin 2 (cpn60-2, groEL2)	Rv0440	56692	4.85	34	KTIAYDEEARR
	1264.5909	60kDa Chaperonin 2		56692	4.85	36	KEIELEDPYEKI
	1529.7887	60kDa Chaperonin 2		56692	4.85	46	KWGAPTITNDGVSIAKE
	2075.0432	60kDa Chaperonin 2		56692	4.85	66	KTDDVAGDGTTTATVLAQALVRE
8	804.3472	Hypothetical protein	Rv2004c	54959	5.89	35	RERACIRE
	1434.7007	Hypothetical protein		54959	5.89	42	RIEHMVDEFVSGRE
	1796.9013	Hypothetical protein		54959	5.89	45	RIDDAAFLAMDLEFLGRK

Table II. MALDI-TOF/TOF (MS/MS) analysis of overexpressed proteins in MDR-TB clinical isolates

Contd...

Spot No.†	Peak mass (Da)	Protein identified	Rv No.	Nominal Mass (Da)	pI	Mascot score	Sequence peptide
9	1094.6241	Glutamyl tRNA (Gln amidotransferase subunit A (gatA)	Rv3011c	51787	4.91	43	RSPYDATLTARL
	1881.9284	gatA		51787	4.91	35	RYGLVACASSLDQGGPCART
	2017.1693	gatA		51787	4.91	36	RQPAALTATVGVKPTYGTVSRY
10	1413.8456	Ef-tu	Rv0685	43556	5.28	32	RQVGVPYILVALNKA
	1693.8359	Ef-tu		43556	5.28	40	RHYAHVDAPGHADYIKN
	2091.0286	Ef-tu		43556	5.28	42	KADAVDDEELLELVEMEVRE
11	1085.5899	DNA directed RNA polymerase α-chain (rpoA)	Rv3457c	37740	4.64	35	KLEVELVVERG
	1485.8157	DNA directed RNA polymerase α-chain (rpoA)		37740	4.64	27	RTLLSSIPGAAVTSIRI
	1611.8113	DNA directed RNA polymerase α-chain (rpoA)		37740	4.64	25	RIDGVLHEFTTVPGVKE
12	1352.6949	Putative phosphoserine aminotransferase		40266	4.77	34	RSLHLTYGEFSAKF
	1900.0249	Putative phosphoserine aminotransferase		40266	4.77	33	MADQLTPHLEIPTAIKPRD
	1928.0345	Putative phosphoserine aminotransferase		40266	4.77	38	RWVPDFLSLPIAVENSLKN
13	1237.69	Probable fabG4 protein	Rv0242c	46916	6.04	44	RQLGVPQPETLRR
	1393.7932	Probable fabG4 protein		46916	6.04	35	RQLGVPQPETLRRY
	1565.862	Probable fabG4 protein		46916	6.04	39	RAGEPPLTGSLLIGGAGRV
14	1425.9539	Probable moxR	Rv1479	40738	5.96	41	KRIIVGQDQLVERM
	1786.142	Probable moxR		40738	5.96	32	RIQFTPDLVPTDIIGTRI
	1983.2737	Probable moxR		40738	5.96	33	RDYVIPQDVIEVIPDVLRH
15	1550.7799	Probable lipase protein	Rv2970c	34146	4.83	34	RVVDLAIDGPAGPIGTRI
	1699.7705	Probable lipase protein		34146	4.83	37	RQHAVGADAIVVSVDYRL
	1740.8621	Probable lipase protein		34146	4.83	22	RIAVAGDSAGGTIAAVIAQRA
16	1315.7195	Phosphoglycerate kinase	Rv1437	42600	4.83	43	RGLLETYHDVLRL
	1420.7409	Phosphoglycerate kinase		42600	4.83	40	KGAFSVVGGGDSAAAVRA
	1683.9461	Phosphoglycerate kinase		42600	4.83	38	RAEGLTGGDILLLENIRF
17	1016.503	Hypothetical protein Rv3075c	Rv3075c	33194	4.73	25	KEFFAEFARD
	1322.619	Hypothetical protein Rv3075c		33194	4.73	60	RWFGDGNADWVRI
	1583.78	Hypothetical protein Rv3075c		33194	4.73	59	RDTGFGEDPATLAYARS

Spot No.[†]	Peak mass (Da)	Protein identified	Rv No.	Nominal Mass (Da)	pI	Mascot score	Sequence peptide
	1648.036	Hypothetical protein Rv3075c		33194	4.73	43	KRLPNVPIVALVETARG
18	1085.7325	Glyceraldehyde-3-phosphate (G-3-P) dehydrogenase	Rv1436	36105	5.19	42	KAIGLVMPQLKG
	1134.7153	G-3-P dehydrogenase		36105	5.19	37	KVLDDEFGIVKG
	1384.8723	G-3-P dehydrogenase		36105	5.19	34	RAAALNIVPTSTGAAKA
	1085.7325	Glyceraldehyde-3-phosphate (G-3-P) dehydrogenase		36105	5.19	42	KAIGLVMPQLKG
19	1088.5844	ag84/wag31	Rv2145c	28260	4.8	22	RLIEENSDLRQ
	1171.6518	ag84/wag31		28260	4.8	31	RANAEQILGEARH
	1413.7358	ag84/wag31		28260	4.8	36	KHSEIMGTINQQRA
20	1011.5087	qor	Rv1454c	34140	5.37	43	RTGEEFSWRA
	1615.8965	qor		34140	5.37	40	KAEAIGVNFIDTYFRS
	2195.0742	qor		34140	5.37	34	KDAGADVVLDYPEDAWQFAGRV
21	1037.4687	Antigen precursor (MPT51)	Rv3803c	31069	6.13	34	RMFYNQYRS
	2044.93	Antigen precursor (MPT51)		31069	6.13	42	KWHDPWVHASLLAQNNTRV
	2132.9473	Antigen precursor (MPT51)		31069	6.13	45	KQWDTFLSAELPDWLAANRG
22	1655.8488	Ribosome recycling factor (RRF)	Rv2882c	20815	5.71	29	RNSDLGVNPTNDGALIRV
	1674.88	Ribosome recycling factor (RRF)		20815	5.71	37	KTTHQYVTQIDELVKH
	2146.0955	Ribosome recycling factor (RRF)		20815	5.71	38	KDLDKTTHQYVTQIDELVKH
23	1291.7384	Hypothetical protein (GarA)	Rv1827	17240	4.29	21	RFLLDQAITSAGRH
	1715.8809	Hypothetical protein (GarA)		17240	4.29	43	RHPDSDIFLDDVTVSRR
	1840.9915	Hypothetical protein (GarA)		17240	4.29	28	REPVDSAVLANGDEVQIGKF
24	1046.5234	Bfr (Bacterioferritin)	Rv1876	18443	4.5	40	MQGDPDVLRL
	1414.7907	Bfr (Bacterioferritin)		18443	4.5	37	RILLLDGLPNYQRI
	1935.8135	Bfr (Bacterioferritin)		18443	4.5	29	RAESFDEMRHAEEITDRI
25	1089.5305	Hypothetical protein Rv2204c	Rv2204c	12707	4.4	22	RYNLFFDDRT
	1281.7399	Hypothetical protein Rv2204c		12707	4.4	28	KTHGVILTEAAAAKA
	1198.665	Hypothetical protein Rv2204c		12707	4.4	35	RIAVQPGGCAGLRY
26	965.4423	Hypothetical protein Rv0543c	Rv0543c	14743	5.2	26	RDDAPYWAKY

Contd...

Spot No.[†]	Peak mass (Da)	Protein identified	Rv No.	Nominal Mass (Da)	pI	Mascot score	Sequence peptide
	1218.6172	Hypothetical protein Rv0543c		14743	5.2	25	MSVELTQEVSARL
	1595.7916	Hypothetical protein Rv0543c		14743	5.2	19	RLTSDLYGWLTTVARS
27	1034.4976	10kDa Chaperonin groES	Rv3418c	10798	4.62	40	RWDEDGEKRI
	1523.8874	10kDa Chaperonin groES		10798	4.62	21	KEKPQEGTVVAVGPGRW
	1776.0298	10kDa Chaperonin groES		10798	4.62	29	KRIPLDVAEGDTVIYSKY

[†]Spot number of the proteins marked as in Fig. 1.

not screened our resistant isolate for these mutations, this study supports our proteomic approach and identification of an upregulated rpoA protein in drug resistant isolates. Dussurget *et al*[25] reported that in *M. smegmatis*, IdeR negatively controls iron-uptake and expression of BfrA and BfrB. In our study, BfrA was upregulated in drug resistant isolates, suggesting a role of this protein in inducing resistance to INH. However, such conclusions could not be validated by deletion mutant of *bfrA* and *bfrB* in *Mtb*[22].

We found that the identified proteins spots (Rv1475c, dnaK, groEL2, groES and wag31) had different electrophoretic mobilities in resistant and sensitive isolates. Similar observations have been reported by Mattow *et al*[21] when the protein profile of intra-phagosomal *Mtb* H37Rv was analyzed. It has also been suggested that this shift is determined by the protein modifications rendered during sample preparation and growth conditions adopted by various laboratories[8]. However, this may be the unlikely factor in our study, as we strictly followed the same protocol throughout the study. Further, our analysis was stringent and we considered a particular protein to be "upregulated" only if the observation was consistent in three independent experiments. Even then, we were cautious not to conclude whether the changes in protein mobility necessarily reflect protein modification. Further studies are warranted to establish the role of post-translation modifications in these proteins during drug resistance.

Antigen 84 (Wag31 / Rv2145c) has been demonstrated to be involved in the regulation of cell morphology and pknG mediates the survival of *Mtb* inside the host macrophages, and genome-wide transcriptional analysis reveals that the *pknG* is upregulated during the exposure of INH[26]. Further, *pknG* is associated with the intrinsic resistance of *Mtb* to various anti-TB drugs[26]. Wag31 protein has been found to be overexpressed and involved in regulation of cell morphology. It also plays important role in survival of mycobacteria under oxidative stress[27] and provides optimal substrate for pknA and pknB. In addition, mRNA expression of wag31 was increased by 15.7 folds during the INH and RIF exposure. Earlier also, wag31 has been reported to be over expressed in the MDR isolates[8], supporting our hypothesis that wag31 plays an important role in drug resistance.

Of the five hypothetical proteins (Rv2004c, Rv1437, Rv3075c, Rv2204c and Rv1827) overexpressed in MDR isolates, three (Rv2004c, Rv2204c and Rv1437) could not be assigned to any function in survival or pathogenesis of the bacteria, though Rv3075c has been reported to be overexpressed in streptomycin resistant isolates[11]. In our study, Rv1827 (GarA) was found 7.6 folds upregulated in drug resistant isolates as compared to that in the susceptible isolate A. It has been identified as an optimal substrate for PknB and PknG. The protein is also reported to act as a phosphorylation-dependent molecular switch in mycobacterial signalling process mediated by protein kinases[28-30]. Further, GarA has been found to be predominantly expressed under the exponential growth phase and has been suggested as a regulatory model for glycogen degradation and glutamate metabolism[28-30]. To infer whether the protein overexpression of Rv1827 in drug resistant isolates facilitated increased glycogen accumulation, we quantified the glycogen content of drug resistant and sensitive isolates. The findings were consistent with this hypothesis and the glycogen content was relatively

Table III. Isolates specific proteins overexpression in MDR-TB isolates

S. No.	Spot No.	Proteins identified	Open reading frame (ORF) No.	Accession No.	Mascot score	Nominal mass (Da)	pI	No. of peptides matched	Sequence coverage (%)	Densitometric ratio of protein expression between sensitive vs. resistant isolates			Protein classification according to Pasteur Institute of Genomics (TubercuList)
										B	C	D	
Proteins overexpressed in MDR isolate (Isolate B)													
1	9	Glutamyl tRNA (Gln amidotransferase)	Rv3011c	NP_217527	98	51787	4.91	9	27	1:2.26	1:1.13	1:0.93	2
2	12	Putative phosphoserine aminotransferase	Rv0884c	NP_215399	90	40266	4.77	7	28	1:2.21	1:0.70	1:1.75	7
3	21	Antigen precursor (MPT51)	Rv3803c	YP_178017	64	31069	6.13	5	23	1:2.19	1:1.12	1:1.02	1
4	23	Hypothetical protein (GarA)	Rv1827	NP_216343	87	17240	4.29	5	45	1:7.66	1:1.37	1:1.16	5
5	25	Hypothetical protein	Rv2204c	NP_216720	103	12707	4.4	6	51	1:9.11	1:1.0	1:1.04	5
6	26	Hypothetical protein	Rv0543c	NP_215057	67	14743	5.2	5	38	1:3.03	1:1.01	1:1.53	5
Proteins overexpressed in MDR isolate (Isolate C)													
1	1	Aconitase hydratase	Rv1475c	NP_215991	132	102728	4.95	18	21	1:1.0	1:4.36	1:1.71	7
2	2	Aconitase hydratase	Rv1475c	NP_215991	126	102728	4.95	17	21	1:1.22	1:4.70	1:1.58	7
3	6	60kDa Chaperonin 2 (cpn60-2, groEL2)	Rv0440	NP_214954	128	56692	4.85	16	29	1:1.22	1:4.70	1:1.58	0
4	7	60 kDa Chaperonin 2 (groEL2)	Rv0440	NP_214954	231	56659	4.85	19	49	1:1.09	1:4.24	1:1.27	0
5	10	Elongation factor Tu (EF-Tu)	Rv0685	NP_215199	248	43556	5.28	24	69	1:1.38	1:3.07	1:0.97	2
6	11	DNA directed RNA polymerase α-chain (rpoA)	Rv3457c	NP_217974	205	37740	4.64	16	43	1:1.7	1:2.98	1:1.0	2

| S. No.	Spot No.	Proteins identified	Open reading frame (ORF) No.	Accession No.	Mascot score	Nominal mass (Da)	pI	No. of peptides matched	Sequence coverage (%)	Densitometric ratio of protein expression between sensitive vs. resistant isolates			Protein classification according to Pasteur Institute of Genomics (TubercuList)
										B	C	D	
7	14	Probable moxR protein	Rv1479	YP_177816	103	40738	5.96	12	37	1:1.0	1:2.08	1:1.0	9
8	15	Probable lipase	Rv2970c	NP_217486	82	43685	6.33	6	30	1:1.19	1:2.28	1:1.0	7
9	16	Hypothetical protein	Rv1437	NP_217230	88	35519.79	5.14	10	41	1:1.91	1:2.82	1:1.28	7
10	20	Probable quinone oxidoreductase (qor)	Rv1454c	NP_215970	80	34140	5.37	7	42	1:1.15	1:6.42	1:1.89	7
Proteins overexpressed in MDR isolate (Isolate D)													
1	3	Probable fadB protein	Rv0860	NP_215375	171	76170	5.42	24	33	1:1.0	1:1.0	1:2.08	1
2	13	Probable fabG4 protein	Rv0242c	NP_214756	207	46916	6.04	17	52	1:1.01	1:1.0	1:2.08	1
3	22	Ribosome recycling factor (RRF)	Rv2882c	NP_217398	108	20815	5.71	10	67	1:1.0	1:1.0	1:3.45	2
Proteins overexpressed only in MDR isolates (Isolates C and D)													
1	17	Hypothetical protein	Rv3075c	NP_217591	98	33194	4.73	9	28	1:1.73	1:8.12	1:2.65	5
2	18	Glyceraldehyde-3-phosphate	Rv1436	NP_215952	71	36105	5.19	6	17	1:1.0	1:20.61	1:7.0	7
3	27	10 kDa chaperonin (cpn 10, groES)	Rv3418c	NP_217935	162	10667	4.62	10	99	1:1.0	1:10.86	1:9.28	0
Proteins overexpressed only in all MDR isolates (Isolates B, C and D)													
1	4	Chaperonin protein dnaK (HSP70)	Rv0350	NP_214864	99	66790	4.85	11	22	1:6.88	1:7.88	1:4.76	0
2	5	Chaperonin protein dnaK (HSP70)	Rv0350	NP_214864	165	66790	4.85	22	38	1:156.2	1:195.6	1:66.4	0
3	8	Hypothetical protein	Rv2004c	NP_216520	53	54959	5.89	10	29	1:54.21	1:132.28	1:49.82	5
4	19	Antigen 84 (wag31)	Rv2145c	NP_216661	120	28260	4.8	8	43	1:12.0	1:7.0	1:3.0	3
5	24	bfrA (Bacterioferritin)	Rv1876	NP_216392	90	18443	4.5	12	82	1:9.27	1:37.5	1:38.58	7
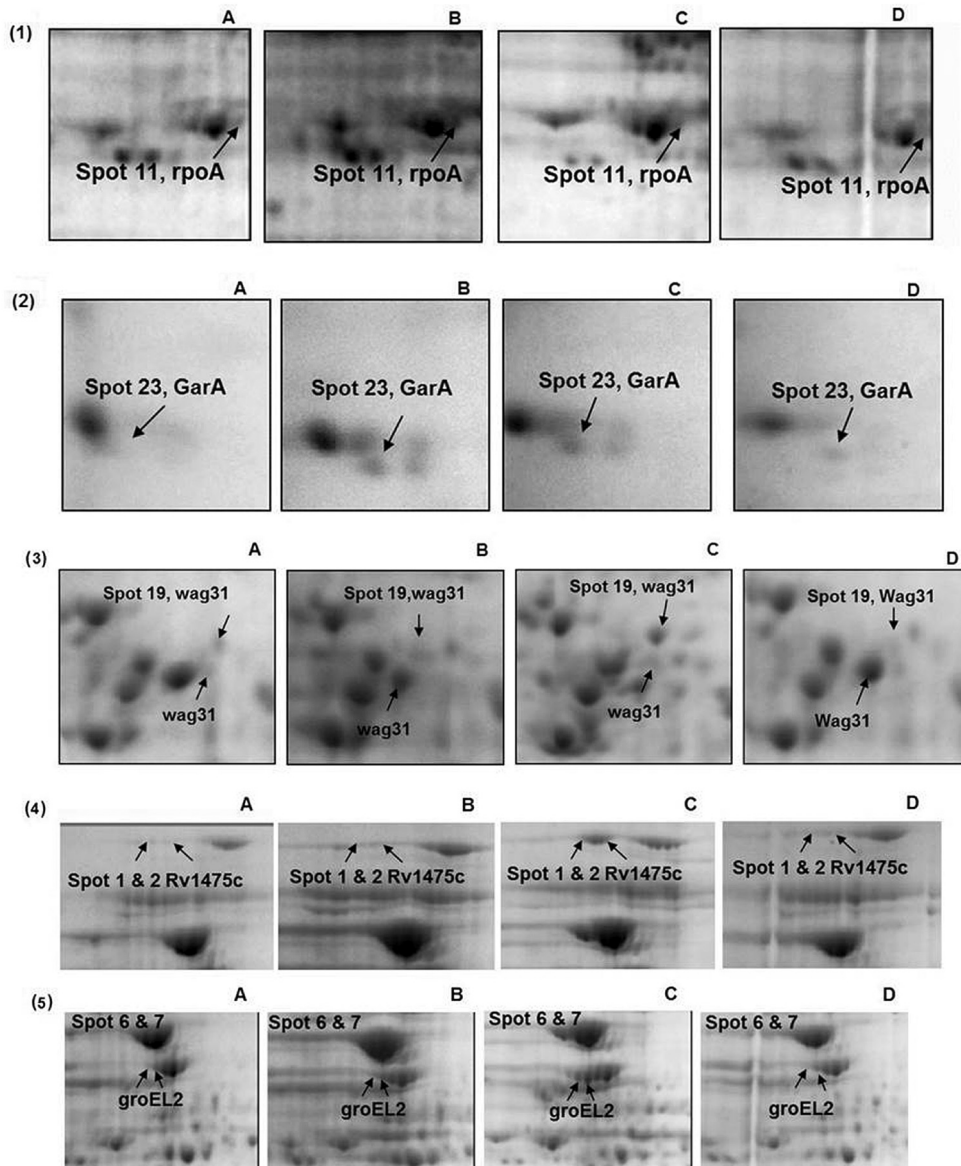
Fig. 3. Magnified region of overexpressed proteins present in MDR isolates. Zoomed in regions of 2D-gel images showing the overexpressed proteins in drug resistant isolates: (1) rpoA (2) GarA (3) wag31 (4) Rv1475c (5) groEL2.

more in resistant isolates (B, C and D) than the sensitive isolate A. However, the difference in accumulation was significant up to 7th day, but not after 15th day of growth, suggesting that the expression of Rv1827 might be an important marker of dormancy. While the precise role of glycogen storage in mycobacteria is not known, glycogen stores may serve as a reservoir of carbon and energy that can be mobilized by mycobacteria for survival during periods of carbon starvation. Such observations have been made in other bacteria such as *Vibrio cholerae* during transition stage between host and aquatic environments[31].

In our study, the identified proteins cannot be categorically classified as drug resistance-specific alterations, as this can also happen due to host immune response or stresses encountered by the individual strain *in vivo*. Most of these proteins are essential for the survival of mycobacteria in phagosome or as virulence factor (unpublished observation), but their role in drug resistance cannot be ruled out completely.

The 2D gel electrophoresis followed by MS-based proteomic analysis on sequential isolates showed approximately 500 proteins per gel. This resolution is
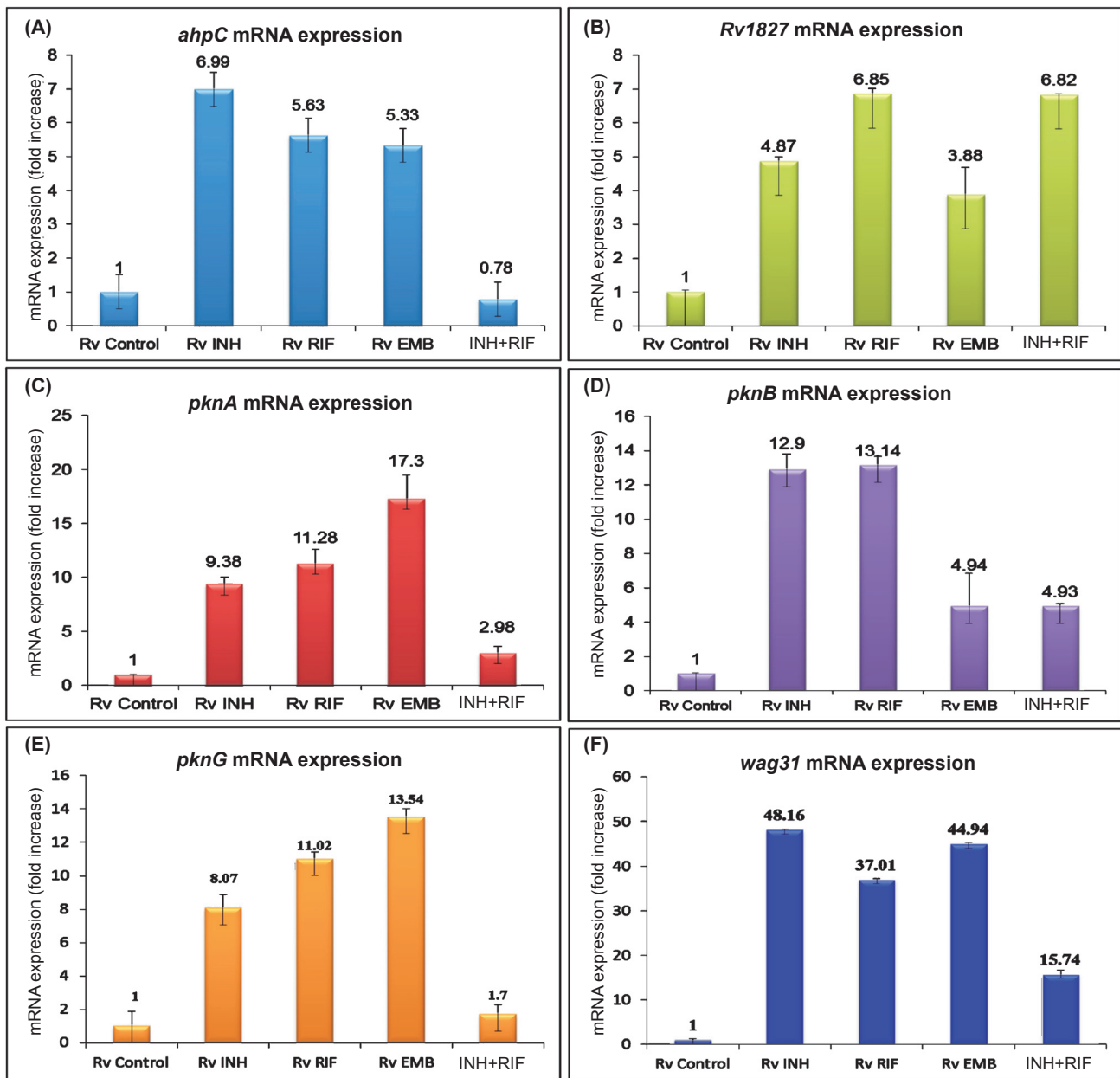
Rv-Mtb H37Rv; INH-isoniazid; RIF-rifampicin; EMB-ethambutol; IR-isoniazid+rifampicin

Fig. 4. Detection of mRNA expression in drug treated *Mtb* isolates. Isolates treated with isoniazid (INH, 0.1μg/ml), rifampicin (RIF, 1.0μg/ml), ethambutol (EMB, 5μg/ml) and INH (0.1μg/ml) +RIF (1.0μg/ml). The expression is represented in fold increase. The *16sRNA* was used as internal control for mRNA expression analysis. mRNA expression of **(A)** *ahpC*, **(B)** *Rv1827*, **(C)** *pknA*, **(D)** *pknB*, **(E)** *pknG* and **(F)** *wag31*. Calculation and statistical analysis carried out by using the $2^{-\Delta\Delta C_T}$ method and results presented in fold increase.

much less than expected, as there are 4000 predicted genes in *Mtb*. The poor sensitivity observed in the present study could be attributed to various reasons such as the extraction protocol; low resolution power of the coomassie brilliant blue stain used or due to the IEF-strips (*p*H 4-7). These strips resolve only the proteins having isoelectric point in the range of 4-7.

In conclusion, our study highlights the intricacies associated with sequential clinical isolates of *Mtb*, a rare opportunity for any laboratory, which is a natural phenomenon and cannot be generated artificially in the laboratory. The sequential isolation of four isolates from the same patient during the treatment period showed a phenomenon where a sensitive isolate turned
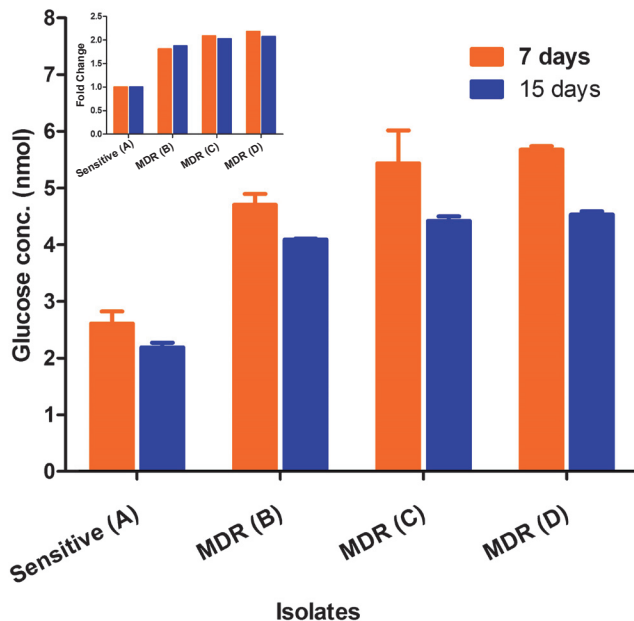
Fig. 5. Glycogen content of sensitive and MDR isolates of *Mtb*. Glycogen was extracted after 7 and 15 days and glycogen storage was determined by the estimation of glucose. The bars indicate glucose concentration of *Mtb* cells after 7 and 15 days, respectively. The inset shows the glycogen content in terms of fold increase after normalization in comparison to sensitive *vs*. MDR TB clinical isolates. Values are mean ± SD (n=3).

to a multidrug resistant isolate. It is possible that some of the upregulated proteins identified from MDR clinical isolates of *Mtb* in the present study may prove as potential biomarkers of drug resistance in future.

Acknowledgment

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