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Comparing mRNA expression and protein abundance in MDR *Mycobacterium tuberculosis*: Novel protein candidates, Rv0443, Rv0379 and Rv0147 as TB potential diagnostic or therapeutic targets

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ABSTRACT

Tuberculosis (TB) is a sizable public health threat in the world. This study was conducted to determine the differential protein composition between susceptible and MDRTB strains. Tuberculosis proteins were extracted by Triton™ X-114 and ammonium sulfate. Two-dimensional gel electrophoresis protein spots were selected for identification by mass spectrometry and mRNA expression levels were measured by real- time PCR.

2DE-Western blot and T cell epitope prediction for identified proteins were made by the IEDB server. The result shows at least six protein spots (Rv0147, Rv3597c, Rv0379, Rv3699, Rv1392 and Rv0443) were differentially expressed in MDRTB isolates. However, difference in mRNA gene expression was not found in the six mRNA genes.

2DE-Western blot procedures indicated strong reaction against MDRTB proteins corresponds to 13, 16 and 55 kDa areas that might be used as new diagnostic tools. In conclusion, these MDRTB proteins identified in this study could be reliable TB diagnostic candidates or therapeutic targets.

1. Introduction

Tuberculosis (TB) is still a crucial health problem, one of the top ten causes of death in the world. World Health organization End TB strategy by 2030 is a 90 % reduction in the TB mortality rate and an 80 % reduction in the global TB incidence rate. It is estimated that 3.4 % of new TB cases worldwide and 8 % of cases classified as already treated have multi drug resistant TB or rifampin- resistant tuberculosis. Therefore, MDRTB continues to be a public health threat. In spite of increases in TB notifications, there are still wide gaps between the number of new cases declared and the calculated incident cases in 2018. These gaps may be caused by a variety of integration including underreporting or underdiagnoses of recognized cases with TB [1]. Although various new TB diagnostics have been developed and appear strong, priorities include early, accurate and potent TB diagnostic test for use at the point of care, new therapeutic targets and novel vaccine candidates to lower the risk of active TB disease [2]. Thus, there are critical needs to identify biomarkers that are required for the design of more effective vaccine or specific *Mycobacterium tuberculosis* therapeutics. Many proteomics studies have been conducted to identify proteins in *M. tuberculosis*

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strains and their subcellular localizations including cytoplasmic membrane, cell wall, and culture filtrate fractions [3–7]. These studies clearly display how the proteomics procedures complement genomics by characterizing differentially expressed genes [8]. Furthermore, profiling of the expressed proteins in the membrane /cell wall fraction will provide information about the host-pathogen interaction. These proteins are various membrane surface transporters and enzymes involved in bacterial host response and biosynthetic processes. They may potentially lead to the identification of potential therapeutic targets or new vaccines [9,10]. The present study is organized in two stages, Firstly; we compared the differences in mRNA expression levels and performed proteomic analysis of susceptible and multi-drug resistant tuberculosis (MDRTB) strains. Secondly, in order to *identify differentially* expressed protein candidates to represent reputed biomarker as a therapeutic target or diagnosis of TB, we used a proteome approach and Immune Epitope Database (IEDB), combining mass spectrometry, 2DE-western blotting procedures and T cell epitope prediction tools.

2. Materials and methods

2.1. Mycobacterium protein extraction

Susceptible and MDRTB isolates (MTB-1140 and MTB-1503) were obtained from the Mycobacterial Culture Collection, Pasteur Institute -Tehran. The strains were cultured in 7H9 broth at 37 ℃ for at least 45 days. Bacterial culture was centrifuged (5000 rpm for 10 min) and the pellet was washed with sterile phosphate-buffered saline, PBS pH 7.4, containing 10 % Glycerol, 12.5 mM Sucrose, µg/mL DNAse 1, 10 mM DTT, 0.5 %, Triton™ X-114 1 mM PMSF, and 20 mM EDTA. *M. tuberculosis* cells are lysed by being subjected to short and intense treatments with sonication using a cell sonicator on ice. Proteins were precipitated by ammonium sulfate and the resuspended precipitate fractions were subjected to dialysis against saline pH 7.4 [11,12]. The measurement of total protein concentration was performed by Bradford's assay [13].

2.2. Gel electrophoresis

One-*dimensional* gel electrophoresis of TB proteins was carried out with the Bio-Rad system (Mini-PROTEAN® Tetra Cell, Bio-Rad, CA) based on the Laemmli method [14]. Two-*dimensional* polyacrylamide gel electrophoresis (2D-*PAGE*) was conducted by the GE Healthcare system (EttanIPGphor 3 IEF system). Passive rehydration in the isoelectric focusing (IEF) tray was used for focusing process. IEF buffer consisted of 5 M urea, 1.5 M Thiourea, 3 % CHAPS, 70 mM Dithiothreitol (DTT), 50 mM Tris Hcl pH 7.4, 0.2 % ampholyte 3–10, and 2 mM Tributylphosphine. The following voltage was used for passive rehydration: 500 V for 1 h, 1000 V for 1 h, 8000 V for 3 h, and 8000 V for 20KVh, running conditions in 20 °C, current 50 µA per strip. IPG strip was incubated for 15 min in equilibration buffer (4%SDS, 6 M urea, 50 mM Tris Hcl pH 8.8, 30 % glycerol) with 130 mM DTT and then soaked in 135 mM IAA (Idoactamide). The second dimension was performed on 12 % SDS-PAGE electrophoresis at 5 mA per gel for 2 h and finally gels were fixed and stained with Coomassie Brilliant Blue R-250 as described previously [5,15,16]. The 2DE results were analyzed by the Melanie software version 7.0 (Geneva Bioinformatics, GenBio, SA). To estimate the size of proteins resolved by gel electrophoresis, the Thermo Scientific unstained protein molecular weight markers, consisted of a mixture of seven purified proteins ranging from 14.4 KDa to 116.0 KDa were used as protein ladders (https://www.thermofisher.com/order/catalog/product/26610#/26610). In order to identify the relevant peptides (proteins) by Mass Spectrometry, differentially expressed protein spots in MDRTB isolates compared to susceptible TB strains were excised from the 2DE gels and remitted to the Biology Department at York University, UK

2.3. Mass spectrometry

Mass spectrometry (MS) was carried out at the Department of Biology, York University, (www.york.ac.uk/biology). Relevant protein spots were cut out from the 2DE gel and digested after reduction with S-carbamidomethylation and iodoacetamide Spectral processing was conducted by Bruker flex Analysis software (ver. 3.3) [17]. MALDI/ MS results were filtered to collect only proteins with an expected score of 0.05 or lower by the Mascot software (Matrix Science Ltd, version 2.4. The abundance of proteins was estimated by the protein abundance index (PAI) [18].

2.4. MDRTB antibody preparation

Prior to the western blotting, affinity chromatography was used for the purification of polyclonal antibodies against MDRTB antigens. In order to obtain sera of MDRTB patients, blood samples were collected from 17 confirmed MDRTB patients who admitted to at the Masih Daneshvari Hospital, Tehran, Iran. All patients were negative for HIV infection. Consent from all individuals was obtained before recruiting into the study. The study was approved by the Ethical Research Committee at the Pasteur Institute of Iran, Tehran. The sepharose 4B affinity resin, (Sigma chemical,St.Louis, MO), was packed into a glass column (20 × 3 cm) and then the column is equilibrated with binding buffer. Finally, affinity purification of specific MDRTB antibodies was performed using MDRTB antigens covalently coupled to cyanogens-bromide activated sepharose 4B as described previously [19,20].

2.5. Two-dimensional electrophoresis and Western blotting

Identified proteins, which their protein spot profiles had been already obtained by two-dimensional electrophoresis were transferred onto 0.45 mm nitrocellulose membranes (Schleicher & Schuell Bioscience, GmbH, Germany) in a Bio-Rad Blot Cell unit (Bio-Rad, CA, US) with transfer buffer (25 mM Tris, 190 mM Glycine, 20 % methanol) at a constant current of 10 mA for 60 min. Transferred Proteins were stained using Ponceau staining buffer (0.2 % Ponceau, 5 % glacial acetic acid). After three washes with [TBST], Tris-buffered saline with Tween (20 mM Tris pH7.5, 0.1 % Tween 20 and150mM NaCl) the membrane was blocked in 3 % BSA in TBST at room temperature for 1 h. The membrane is then incubated with primary antibody, MDRTB Abs, for 2 h. The blot was then washed again with 50 mM Tris−HCl buffer pH 7.4 and developed with secondary antibody (HRP-conjugated anti-rabbit IgG). Membrane was then incubated with 3, 3′-diaminobenzidine-tetrahydrochloride-dihydrate detection substrate and reaction was stopped by distilled water. Two-*dimensional* western blot was conducted in duplicate and no variation was detected between results. [21,22].

2.6. T cell epitope prediction

Predictions of MHC class I epitopes were performed using the IEDB server (http://tools.iedb.org). A consensus method includes multiple machine learning techniques that involve algorithms with high performances was applied to this analysis. The smaller percentile rank/score of an epitope corresponds to higher binding affinity between antigen and MHC. [23,24].

Roche Diagnostics', Roche Applied Science, Germany). In brief, collected the Penzberg, Germany)

2.7. Purification of total RNA

Total RNA was isolated using High Pure RNA Isolation kit according to the manufacturer's instructions (Roche Diagnostics', Penzberg, Germany). Briefly, mycobacterial cultures (suitable for 1×10^9 cells) were collected by centrifugations at 2000×*g* for 5 min. The supernatant was

removed and the pellets were resuspended in 200 µl Tris 10 mM pH 8.0. The bacterial suspension was transferred to sterile tubes containing 4 µl Lysozyme (50 mg/mL), incubated for 10 min at 37 ℃ and then were added 400 µl Lysis Binding buffer and mix well. The samples were transferred to the upper reservoir of the High pure Filter Tube (max 700 µl), and then centrifuged at 8000×*g* for 15 s. Following re-inserting the Filter Tube, 10 µl of DNase was added into a sterile reaction tube. After pipetting the solution in the upper reservoir of the filter tube, it was incubated for 15 min at 25 ℃. After washing the samples, elution buffer (100 µl) was added and the filter tube centrifuged at 8000×*g* for 1 min. The purity of eluted RNA was estimated by spectrophotometer at 260/280 nm [25,26].

2.8. Reverse transcription (cDNA synthesis)

The synthesis of cDNA was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLVRT) and oligo (dT) primer based on the manufacturer's instructions (RT PCR kit, Qiagen, GmbH Hilden, Germany). Briefly, five µg of total RNA and 50µM oligo (dT) primer were used to prepare solution 1. Solution 1 was mixed gently, centrifuged and s incubated at 70 ℃ for 5 min and then chilled on ice. Solution 2 was prepared using first-strand buffer (4 µl), dNTPs (1 µl), RNase (0.5 µl), and M-MLV (1 µl). The both solutions were mixed gently, centrifuged and incubated for 60 min at 42 ℃. Finally, the reaction was incubated at 70 ℃ for 5 min. The synthesized cDNA was confirmed by PCR amplification of the *rrs* gene (16 s rRNA). It was detected on 2% agarose gel electrophoresis [27].

2.9. Real-time PCR assays

DNA sequences of MDR and susceptible TB isolates were used to design candidate primers to determine the optimal primer set. Therefore, a set of six designed primers which recognize six distinct sequences (metK, SecE2, aldehyde dehydrogenase,Lsr2 and two conserved proteins) on the target DNA are used to generate amplification products by that to permit primers to bind to these sequences that need for repeated cycles of thermal denaturation in real-time PCR assays (Table 2). Real-time PCR was carried out by a set of seven pairs of primers (Table 2) based on the selected M.tubeculosis genes (Rv0147, Rv3597c, Rv0379, Rv3699, Rv1392,Rv0443 and 16 srRNA) using the LightCycler® 96 Real-Time PCR System (Roche, Basel, Switzerland).

The expression level of each gene was normalized using the 16 s rRNA housekeeping gene and the relative level of each transcript, was acquired by the $2^{-\Delta Ct}$ procedure.

Real-time PCR reaction was performed in a 20 µl of final reaction volume containing 10 µl of PCR Master Mix-SYBR Green I (BIOFACT, South korea), 1 µl primer F (10 pmol/ µl), 1 µl primer R (10 pmol/ µl), 4 µl of cDNA template and 4 µl of distilled water. Amplifications were performed as follows: 95 °C initial denaturation for 15 min, followed by 40 cycles of 95 °C denaturation for 20 s, 55 °C annealed for 40 s and 72 °C extension for 30 s. Negative controls with double-distilled water were included for each real-time PCR assays [28–30].

3. Results

Susceptible and MDR *M. tuberculosis* protein contents were applied to one-dimensional gel electrophoresis and proteomic analysis comprising of two-dimensional gel electrophoresis (2DE), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and bioinformatics' tools.

3.1. One-dimensional gel electrophoresis

The Coomassie blue stain presented high-quality results with very obvious protein bands (Fig. 1). Several noticeable protein bands were present in susceptible and MDRTB isolates. There were at least four clear bands, ranging from 35 to 116 KDa in two isolates and 45 KDa band was well remarkable. As shown in Fig. 1, the banding pattern of the MDRTB isolates was distinct from that of sensitive TB strains. There were at least 3 differential protein bands with the molecular weights ranging from 45 KDa to 66 KDa in MDRTB isolates. Protein bands weighing less than 60KDa were sharp. In addition, there were at least three protein bands from 14 to less than 35 KDa in MDRTB isolates, approximately 13, 16 and 55 KDa, which were not seen on the profiles of susceptible-TB strains.

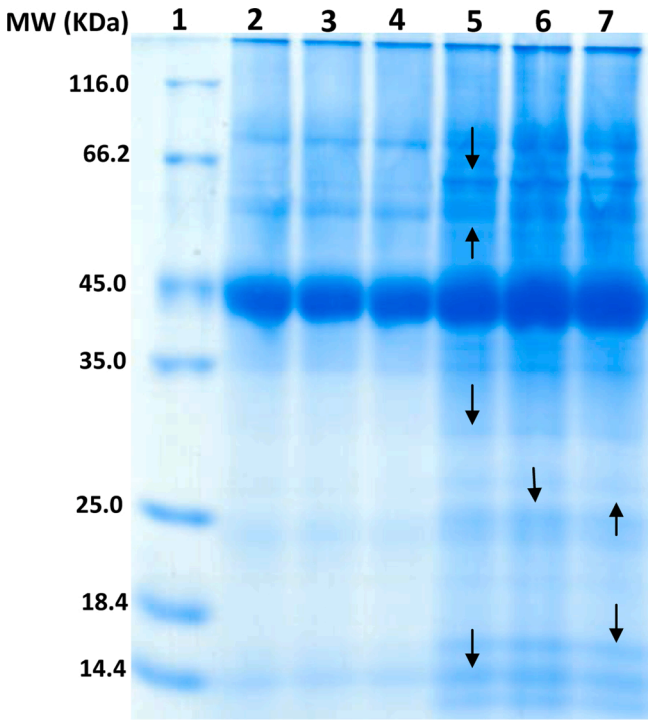

Fig. 1. Protein banding patterns to compare the susceptible and MDR-TB after SDS- polyacrylamide gel (10 %) electrophoresis and staining with coomassie blue. Lanes 2, 3 and 4: susceptible TB isolates, Lanes 5, 6 and 7 MDR-TB isolates, Lane1: Molecular mass markers (116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4 kDa). Arrows indicate proteins differentially expressed during growth of MDR-TB isolates (lanes 5, 6 and 7) Vs susceptible strains (lanes 2, 3 and 4).

3.2. Two-dimensional gel electrophoresis and Mass spectrometry analysis

The detected protein spots in the 2DE gels were subjected to in-gel digestion procedures and Matrix-assisted laser desorption / ionization (MALDI) mass spectrometry analysis. In total, 154 different protein spots were identified, with 73 proteins in the *M. tuberculosis* susceptible profiles, and 81 spots were only observed in the MDRTB isolates. Analysis of the protein spots using Melanie software (version 6.0) disclosed protein species with observed pI and MW values in the range from PH 3 to 10 and molecular weight 14.4 to 66.2 KDa, respectively (Fig. 2) while the majority of these proteins were common to both strains. Fig. 2 shows the low molecular mass proteins of MDRTB isolates include differentially regulated genes related to signal transduction pathways, protein transport, conserved hypothetical proteins and membrane or cell wall proteins. Moreover, analysis of the indicated protein spots revealed at least six different spot proteins that were solely expressed or upregulated in the MDRTB isolates compared to susceptible strains. The list of differentially identified proteins (spots) in MDR *M. tuberculosis* strains are shown in Table 1. Differentially identified proteins were classified based on the functional categories as described in TubercuList (http://genolist.pasteur.fr/TubercuList/) that hosted by Pasteur Institute.

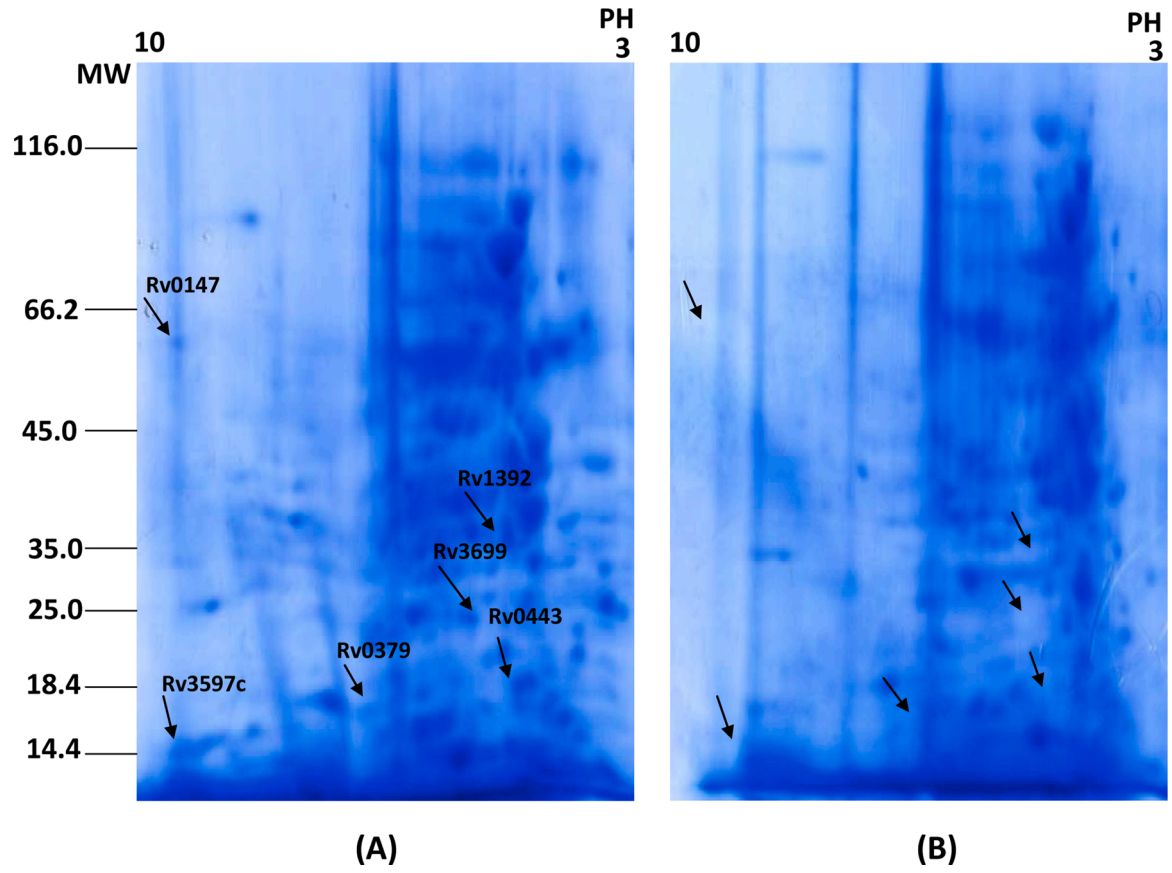

Fig. 2. Protein spots present in two dimentional electrophoresis (2DE) patterns of MDR **(A)** and Susceptible **(B)** *Mycobacterium tuberculosis* strains. Arrows indicates differentially expressed proteins (spots) in MDR-TB isolates compared to the sensitive TB.

Table 1
Multiple drug resistance *Mycobacterium tuberculosis* proteins identified by Mass spectrometry.

Protein (Rv)	Gene	PI	MW(KDa)	position	Protein description	Functional Category
Rv0147	*Rv0147*	9.21	55.11	membrane, cell wall	aldehyde dehydrogenase	conserved hypothetical
Rv3597c	Lsr2	10.08	12.09	extracellular cytoplasm	iron regulated H-Ns	Information pathways
Rv0379	secE2	7.98	16.68	cell wall, Plasma membrane	preprotein translocase subunit secE2	protein transport protein secE2
Rv3699	*Rv3699*	4.69	25.04	cell wall, cytoplasm	Methyltransferase	conserved hypothetical
Rv1392	metK	4.95	43.13	cell wall	s-adenosylmethionine synthase - OS	Involved in activated methyl cycle, ATP binding
Rv0443	*Rv0443*	4.60	13.27	cell wall	conserved protein	conserved hypothetical

Table 2
Primers for the selected *Mycobacterium tuberculosis* genes.

Rv no (identifier)	Gene/product	Primers (5′-3′)
Rv1392	metK	F-GGT AAG TCC GTG TTG CTG AA R-AAG GTT CGG ACA CTC GTC
Rv3699	conserved protein	F-GCC TTC ATT CAT GTC AAT CC R-CTT CTC GTC GTG ATC GTA T
Rv3597c	Lsr2	F-AAG TAA CCG TCA CCT TGG R-CAG TTT CGT GGC ATT CTT AG
Rv0379	SecE2	F-GGA TAG CGT CGA TGA CAT R-CTT GAT GCG GTA GGT GAT
Rv0443	conserved protein	F-GAG GAT GTG GCG AAG GTA R-ATG TAT TCC AGG GTC AGT TTA TC
Rv0147	aldehyde dehydrogenase	F-CAG AAG TTG ATG GAG GAC GAG AA R-GAT GTC AGC GAG GTA TGC
16 s rRNA	16 s rRNA	F-GCACCGGCCAACTACGTG R-GAACAACGCGACAAACCACC

3.3. Reverse transcription and real-time PCR

The comparative RNA expression profile was determined for both MDR and susceptible *M. tuberculosis* isolates during the exponential growth phase. We evaluated mRNA levels for identified proteins (genes) during mid-log phase growth in broth culture. To identify differences in gene expression among MDR and sensitive TB strains, we used the real-time PCR assays with six determined spots/genes (Table 1). The mRNA levels were determined for the Rv0147, Rv3597c, Rv0379, Rv3699, Rv1392 and Rv0443in six multidrug-resistant TB and compared with RNA expression of proteins in susceptible TB isolates identified after MALDI TOFF mass spectrometry analysis. These six purified protein spots corresponded to: Aldehyde dehydrogenase (Rv0147), Iron-regulated H-NS like protein Lsr2 (Rv3597c), translocase SecE2 (Rv0379), methyltransferase (Rv3699), S-adenosylmethionine synthetas (Rv1392) and conserved hypothetical protein (Rv0443). Each of these MDRTB, RNA expression was distinct from the other sensitive mRNA levels (Table 1, Fig. 3). RNA expression of six genes showed there is no differential pattern of expression for Rv0147, Rv3597, Rv0379, Rv3699, Rv1392 and Rv0443 between MDRTB and sensitive isolates.

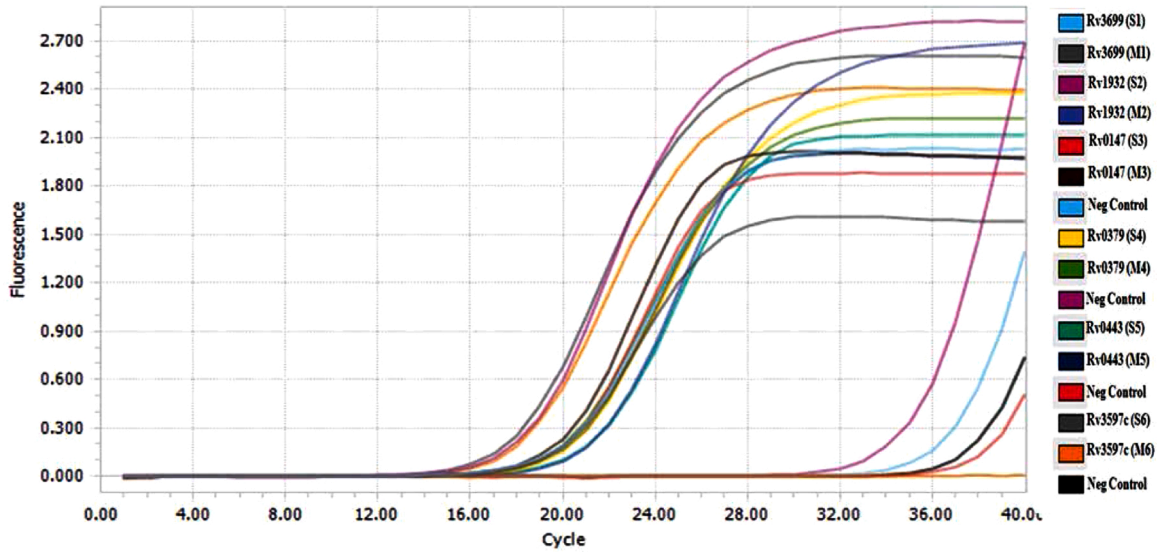

Fig. 3. Expression of each of the six mRNA, MDR (M1-M6) and Susceptible *Mycobacterium tuberculosis* strains (S1-S6) genes, during exponential growth in Middlebrook 7H9 broth as measured by quantitative real time RT-PCR.

Thus, quantitative PCR (RT-PCR) showing the same levels of RNAs expression in susceptible and multi drug resistant tuberculosis isolates in two culture conditions.

3.4. Two-dimensional gel electrophoresis-western blotting and T cell epitope prediction

In order to determine the specificity of identified proteins (Rv0147, Rv0443, Rv0379, Rv1392 and Rv3699) as some selective antigens with regard to the immune response to the Multidrug-*resistant TB* proteins, the antigenic spots which their protein spot patterns had been already obtained by two-dimensional electrophoresis, were recognized by western blotting procedures (Fig. 4). Firstly, affinity chromatography has been employed to purify multidrug-*resistant TB antibody using MDRTB proteins (antigens) coupled to cyanogen bromide-activated-sepharose* 4B resin in a glass Prepacked affinity columns and then protein spots (antigenic spots) on 2DE gel electrophoresis were analyzed by western blotting procedures versus multidrug-*resistant TB antibody*.

As shown in Fig. 4, although multidrug-*resistant TB antibodies* generated a robust reaction against MDRTB protein spots contains Rv3699, Rv3597c and Rv1392 in western blotting films, three identified protein spots Rv0147, Rv0379 and Rv0443 indicated the absence of antigen- antibody interaction. Most of the immune response to *Mycobacterium tuberculosis* involves cell immunity (CD4+ and CD8 + T cells). Both CD4+ and CD8 + T cells, once stimulated, secrete cytokines that generate an immune response. Moreover, Lysis or cytotoxicity of infected cells are also mediated by the CD8 + T cells [31]. Thus, on the path to TB elimination, effective T cell responses are essential. It was fundamental criteria for selection of identified protein candidates. In order to achieve this aim, western blotting results did not show any robust primary humoral immune response to MDRTB antigens on the 13, 16 and 55 KDa areas. Thus, this area regarded as cell mediated immune response regions followed by applying predictions and analysis of MHC class I peptide binding by using the Immune Epitopes Database (IEDB).

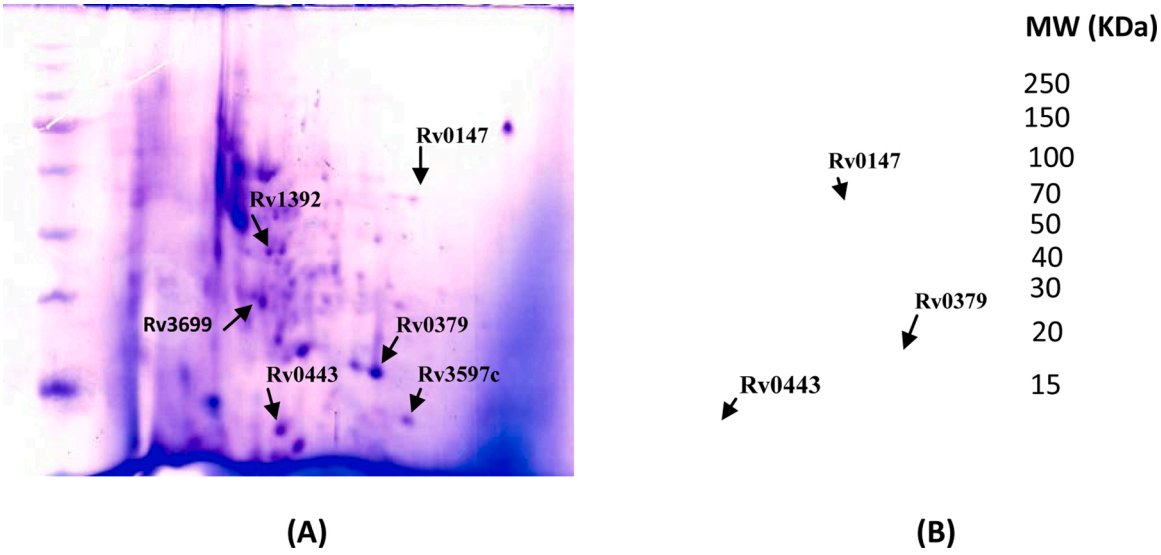

Fig. 4. The 2DE- Western blot patterns of purified protein fractions on the SDS Page gels against MDR-TB antibody. (A) Protein spots followed by two dimensional electrophoresis on the SDS polyacrylamid gel (B) Protein patterns on the western blotting film. The arrows 13, 16 and 55 KDa indicated non immunogenic proteins compared to the SDS Page gels.

The MHC I peptide binding predictions of these diagnostic target protein spots on the SDS page gels (Rv0147, Rv0443 and Rv0379) were performed by the Immune Epitope Database tools. The major purpose of immune epitope database analysis resource is to supply access to well documented and tested tools through the IEDB web portal (www.iedb.org). This concentrated interface allows users to select and edit easily comparisons between various prediction styles [32]. The predictive performance of the MHC I peptide binding based methods on the IEDB analysis resource data set is shown in Table 3. The IEDB-AR prediction result is given based on the IC50nM (nano Molar units) and the percentile degree. Generally, a lower score or number indicates a higher affinity. Accordingly, peptides with IC50 estimation $<$ 50 nM are regarded as high affinity, $<$500 nM intermediate and $<$5000 nM low affinity. Furthermore, in the IC50 evaluation for each protein, a percentile level is generated by comparing the peptides IC50 value versus those of a set of random proteins from SWISS-PORT proteomics server. A small recorded percentile grad indicates strong binding affinity between MHC and protein. In this study, we made binding predictions for three diagnostic markers (e.g. Rv0443, Rv0147and Rv0379) or probably therapeutic target protein spots (Table 3). The output scores of the predictive peptides were shown to be strong binding for each of the identified peptides, e.g. strong binders are defined as having percentile rank 0.08 $<$, 0.24 and 7.9 for identified peptides consisting of Rv0443, Rv0147 and Rv0379 respectively. However, the rank of predicted initial affinity of Rv0443 presumably leads to stronger binding affinity compared to the other two. Therefore we select highly conserved, experimentally confirmed MDRTB antigens, including Rv0147, Rv0379 and Rv0443 to design and introduce a novel multi-epitope subunit vaccine against tuberculosis.

4. Discussion

Diagnosis and successful treatment of people with TB or MDRTB prevents millions of deaths each year, but there are still sizable and continuous gaps in TB diagnosis and treatment. Urgent efforts are needed to improve the coverage and quality of diagnosis, treatment and immunity of people with TB or drug-resistant TB [2]. Closing the gaps in TB detection and treatment will require much higher coverage of TB -DST (drug susceptibility testing), rapid diagnostic tests, increasing treatment coverage for MDRTB, reducing TB underdiagnosis, new models of care that facilitate to follow-up in TB patients to achieve successful outcomes, new treatment regimens with higher efficacy or better safety, and the development of new TB vaccines. The purpose of this work is to examine the pattern of protein profiles and gene expression differences between MDR and susceptible *M. tuberculosis*. This could introduce *new* relevant protein biomarkers as TB or MDRTB diagnostic and therapeutic targets. Therefore, we used a proteomic approach combining two-dimensional electrophoresis, mass spectrometry and real-time quantitative PCR. The proteome of a cell or microorganism reflects its functional situation in reaction to physiological and environmental status. Proteomics can be used to complement genomic studies. According to the combined use of one and two-dimensional electrophoresis gels and the functional gene classification, identified proteins have been assigned putative functions. Fig. 1 depicts the distribution of the protein banding patterns to compare the susceptible and Multidrug-*resistant M. tuberculosis*. We supposed that differentially expressed MDRTB proteins, were characteristic of isolates and these protein patterns could be used in the identification of Multidrug-*resistant M. tuberculosis* strains. The prognosis indicates that the 13 and 55 kDa bands (Fig. 1, Lane 5) and 16 kDa band (Fig. 1, Lane 7) can be considered as diagnostic target proteins or potential novel marker for the early diagnosis of MDRTB strains. Moreover, the existence of two bands of about 18.4–25 kDa (Fig. 1, Lane 6) related to the MDRTB isolates may be regarded as new candidate diagnostic markers. Thus, MDRTB protein profiles as disclosed by Coomassie blue staining were possible to recognize specific protein bands that could serve as diagnostic marker for Multidrug-*resistant M. tuberculosis* strains. A comparative study of the proteome of Isoniazid-resistant and susceptible strains of *M. tuberculosis* demonstrated that the differentially expressed proteins from INH resistant strains might be used as potential immunodiagnostic antigens and

Table 3
MHC-I binding prediction results of Multidrug Resistant *Mycobacterium tuberculosis* protein isolates identified based on Ic50 value and percentile grad by Immune Epitope Database analysis resource /IEDB-AR (www.iedb.org).

Gene (Identified proteins)	Allele	#	Start	End	Length	Peptide	Ic50	Percentile rank
	HLA-A*01: 01	1	48	55	8	DSAGKITY	16048.78	7.9
	HLA-A*01: 01	1	15	22	8	TSWEQAAA	21962.60	16
	HLA-A*01: 01	1	14	21	8	PISWEQAA	27073.13	28
Rv0379	HLA-A*01: 01	1	54	61	8	TYRIKLEV	27891.98	31
	HLA-A*01: 01	1	3	10	8	VYKVIDII	30538.48	42
	HLA-A*01: 01	1	11	18	8	GTSPTSWE	30853.65	44
	HLA-A*01: 01	1	2	9	8	SVYKVIDI	31317.46	46
	HLA-A*01: 01	1	58	65	8	KLEVSFKM	31775.51	49
	HLA-A*01: 01	1	61	68	8	VSFKMRPA	32013.62	50
	HLA-A*01: 01	1	52	59	8	KITYRIKL	32337.02	52
	HLA-A*01: 01	1	194	201	8	MTELVYRY	101.29	0.24
	HLA-A*01: 01	1	487	494	8	SSFIYPPY	2739.80	1.9
	HLA-A*01: 01	1	286	293	8	QTCVAPDY	3963.87	2.3
Rv0147	HLA-A*01: 01	1	110	117	8	TTSAEAKY	4264.33	2.4
	HLA-A*01: 01	1	192	199	8	HLMTELVY	6734.02	3.3
	HLA-A*01: 01	1	41	48	8	SDEKQTDV	11872.00	5.5
	HLA-A*01: 01	1	80	87	8	LMDENEDA	12321.00	5.7
	HLA-A*01: 01	1	201	208	8	YLDTEAIA	12528.97	5.8
	HLA-A*01: 01	1	484	491	8	PDLSSFIY	12578.14	5.8
	HLA-A*01: 01	1	40	47	8	VSDEKQTD	17194.08	8.8
Rv0443	HLA-A*01: 01	1	30	37	8	LTDQLACY	22.18	0.08
	HLA-A*01: 01	1	105	112	8	ADLLSGYY	517.59	0.66
	HLA-A*01: 01	1	104	111	8	PADLLSGY	5416.77	2.8
	HLA-A*01: 01	1	26	33	8	LTDGLTDQ	12110.85	5.6
	HLA-A*01: 01	1	31	38	8	TDQLACYR	13046.08	6.1
	HLA-A*01: 01	1	76	83	8	WVDRFGLD	13551.07	6.4
	HLA-A*01: 01	1	108	115	8	LSGYYHAV	16745.19	8.5
	HLA-A*01: 01	1	27	34	8	TDGLTDQL	21295.00	15
	HLA-A*01: 01	1	115	122	8	VHKLTLEY	21996.13	16
	HLA-A*01: 01	1	134	141	8	VVDTSWNP	22122.15	16

new drug target candidates against drug-resistant TB [15]. Similarly, Garbe et al. previously found the significantly upregulated 32 KDa protein band in *M. tuberculosis* isolates exposed to isoniazid and induced expression of the Ag85 complex [33]. They also determined a 27-KDa protein band that was likely identical to the 27-KDa polypeptide observed in the isoniazid mono-resistance experiments.

In Fig. 2 the Coomassie blue 2DE patterns of MDR and susceptible *M. tuberculosis* purified proteins were identified. The MALDI-TOF mass spectrometry analysis led to the identification of six structural protein (gene) candidates (Table 1). Moreover, the in-depth analysis of our data indicated that 3 proteins were consistently identified in MDRTB profiles as a non immunogenic protein spots (Fig. 4) and not in susceptible TB strains and these were: aldehyde dehydrogenase (Rv0147), calcium dodecin SecE2 (Rv0379) and conserved hypothetical protein (Rv0443). Proteomic analysis of the identified proteins suggested that these proteins were involved in cellular metabolism, protein transport, cell wall, cell processes, conserved hypothetical proteins or unknowns proteins. The purified protein, aldehyde dehydrogenase, Rv0147 is a membrane/cell wall protein with 55KDa and has a wide range of substrate specificity and plays several key physiological functions [34]. The *M. bovis* aldehyde dehydrogenase encoding gene was cloned and later found to be identical to the adhC of *M. tuberculosis* [35]. Its over-expression in *M. bovis* enabled its purification and full biochemical characterization. These studies suggested that *M. bovis* aldehyde dehydrogenase might be involved in the biosynthesis of the free lipids required for the formation of the mycobacterial cell envelope [36,37]. Confirmation of this theory would mean that this membrane protein might be an interesting target for the development of new anti tuberculosis drugs.

Ca^{2+} has been reported to have a significant role in phagocytosis of the pathogen through various receptor-mediated events [38]. Moreover, whole-genome sequencing demonstrated the presence of Ca^{2+} binding domains in *M. tuberculosis*. Ca^{2+} binding proteins have different domains such as helix-loop-helix, Greek key motifs of ßγ crystalline, EF-hand domain and so on [39–41]. The Ca^{2+} signaling pathway along with mitogen-activated protein kinase and IFNγ, is one of the important cycling pathways used by *M. tuberculosis* strains to avoid phagosome-lysosome fusion in host [42]. Several binding domains are available which effectively control Ca^{2+}concentration.

A very rare and exclusive Ca^{2+} binding domain is described in *M. tuberculosis* gene Rv0379 which has 71 kDa protein and is thought to be involved in protein transportation as secE2 [43]. This protein is identified by its crystal structure as Ca^{2+} dodecine and it shows similarity to the copper-binding domain of the amyloid protein [44]. The protein, which is found nowhere else in the genome, has a wide structural range and various roles in the pathogenesis of *M. tuberculosis*. The true orthologue of the *M. tuberculosis* gene, Rv0443, shows the highest level of homology to Rv0442c/PPE10. PPE10 is one of the PPE protein family members nearly identical to hypothetical protein from *M. tuberculosis* [45]. Furthermore, another study revealed that one of the protein secretion systems of mycobacteria, ESX-5, is important in maintaining the structure of their cell envelope. The mycobacterial cell envelope (capsule) is composed of polysaccharides, proteins and glycolipid molecules and is thought to interact with the host immune system. In addition, PPE10; a protein secreted by ESX-5, as the major protein responsible for capsular integrity, attenuates virulence in the early stages of infection and is thought to play a role in immune evasion [46]. Similarly, our previous study showed that these identified proteins were differentially expressed between MDR and susceptible TB and could be considered as T cell activators and may be candidate antigens for the development of a novel TB vaccine or therapeutic strategy against tuberculosis [47].

To find out the differences in expression profile s between susceptible and MDRTB isolates, the two strains were cultured to log phase and the variations in gene expression for the selected proteins (genes) were confirmed by quantitative real-time PCR. The real-time PCR data showed that no difference in gene expression was found between the two strains for the six mRNA genes. Similar to our findings, in a study that describing the comparison of novel genes associated with drug resistance in *M. tuberculosis* and pan-sensitive strains by general analysis of mRNA gene expression, it was found that the gene expression profile between the pan-sensitive H37Rv and a clinical multidrug-resistant isolate of *M. tuberculosis* did not differ by microarray analysis, suggesting similar gene expressions between both isolates [48].

In other words, these results suggest that if a comparison is to be made between the two methods, the results may not necessarily be the same. The discrepancy between the results used to identify specific genes (mRNA gene expression profiles) and those obtained by proteomic analysis can be explained by the many processes between transcription and translation and protein stability as a major factor. The concentration of proteins in steady-state cell populations under different growth conditions can vary. Other factors are the lower rate of mRNA transcription compared to protein translation [6]. Normally, in both bacteria and eukaryotes, the cellular concentrations of proteins are compatible with the abundances of their corresponding mRNAs, but not vigorously [49]. Furthermore, elementary evidence shows that when orthologs are considered across very different species, abundances of proteins are more conserved than abundances of the corresponding mRNAs, suggesting that protein abundances may be evolutionarily preferred [7,50]. Thus, genomics has made notable steps concerning the understanding of biological processes at the overall DNA level. Nonetheless, the degree of gene expression, measured by the abundance of mRNA, is often a poor predictor of phenotype because it does not necessarily correspond to the level of protein profiles [3,51]. To evaluate six identified proteins in selecting appropriate T cell targets or corresponding immune response for MDRTB proteins (biomarkers), we also performed 2DE-western blotting and Tcell epitope prediction by the Immune Epitope database tools. As indicated in Fig. 4, the white areas (non-visible or negative bands) not covered by any protein band on the western blot film in the 13 (Rv0443), 16 (Rv0379) and 55 KDa (Rv0147) may be due to the absence of antigen-antibody interaction, which can be considered as robust non-immunogenic MDRTB protein spots (antigens). As it happens this was one of the most important criteria for selections of identified peptides consist of the Rv0147, Rv0443 and Rv0379, as a protein candidates or MDRTB biomarkers. For this purpose, we performed MHC class I peptide binding predictions and analyses of the molecular targets of the T-cell immune response using IEDB analysis tools for three non immunogenic identified peptides. Binding prediction methods facilitate the selection of potential epitopes. The tool compares the predicted affinity to that of a large set of randomly selected peptides and assigns a percentile rank; a lower percentile rank corresponds to a higher binding affinity [52]. We have selected all peptides with IC50 value less than 500 nM and lower percentile rank which are main strategies for selecting potential binders correlate with a threshold previously associated with immunogenicity [53]. Therefore, the results show that high binding affinity to predict immunogenicity of the three non immunogenic identified peptides, but affinity predicted performance for Rv0443 compared to the other two leads to stronger binding affinity and they could be considered as potential primary targets for TB vaccine development.

5. Conclusion

In summary, the present study has supported the fact that although no differences are found in the mRNA expression of MDR and susceptible *M. tuberculosis* strains, the proteome spectrum of the two isolates is different. Our study provided the opportunity to compare the proteomic data of the investigated MDR and susceptible TB strains with corresponding genomic data. Moreover, at least two of the three identified peptides, Rv0443 and Rv0147, are immunogenic proteins that have been described as major T-cell in stimulating interferon production. In addition, the conserved hypothetical protein Rv0443 showed significant homology with several other mycobacterial hypothetical proteins.

Further studies are required to determine the potential of the three identified peptides as protein candidates for TB vaccine. Taken together, the MDRTB specific proteins identified in this study are interesting candidates for useful vaccine design against TB. Alternatively, these identified proteins can be included in TB subunit vaccine and it may be formulated with the appropriate potent adjuvant in animal models and confirmed in a larger study.

Ethical statement

The study was approved by the Ethical Research Committee at the Pasteur Institute of Iran, Tehran and all procedures performed in studies involving human participants were in accordance with the ethical standards. Informed consent was obtained from all individual participants included in the study.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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