Investigation of the *Rv3065, Rv2942, Rv1258c, Rv1410c*, and *Rv2459* efflux pump genes expression among multidrug-resistant *Mycobacterium tuberculosis* clinical isolates

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**A B S T R A C T**

**Background:** Different resistance mechanisms for multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) have been reported. Although mutations in target genes are the main cause of drug resistance, efflux pumps (Eps) also play an important role in this process. Here, we investigated the overexpression of five putative EP genes plus gene mutations in MDR-TB clinical isolates.

**Methods:** A total of 27 *M. tuberculosis* (Mtb) clinical isolates including, 22 MDR and 5 sensitive isolates were analyzed. Minimum inhibitory concentrations (MIC) were determined in the absence and presence of efflux inhibitor. The expression level of 5 EP genes (*Rv3065, Rv2942, Rv1258c, Rv1410c, Rv2459*) was investigated by quantitative real time PCR (RT-qPCR). DNA sequencing of *rpoB, katG*, and *inhA* promoter was done.

**Results:** Among the 22 MDR-TB isolates, 13 (59.1%) showed significant overexpression (>4-fold) for at least one EP gene. The expression levels of 5 genes were significantly higher (P < 0.05) in MDR-TB isolates than sensitive isolates. The *Rv3065* (22.7%), and *Rv1410c* (18.2%) were found to be the most commonly overexpressed Eps. The observed MICs were as follows: RIF (2 to >128 μg/ml) and INH (2–32 μg/ml). After efflux pump inhibitor exposure, 10/22 (45.45%) isolates showed a decrease in MIC of INH, and 17/22 (77.27%) isolates showed a decrease in MIC of RIF. Of the isolates that overexpressed, 4 isolates lacked mutation in *inhA, rpoB*, and *katG* genes and 10 ones lacked mutation in *inhA* and *katG*.

**Conclusion:** The results showed that overexpression of EP genes in Mtb isolates, besides target gene mutations can contribute to the development of MDR phenotype.

1. Introduction

Tuberculosis (TB) is still a leading cause of mortality and morbidity worldwide, despite the availability of effective drugs and BCG vaccination [1, 2]. Based on the information provided by the World Health Organization (WHO), 7 million persons affected by *M. tuberculosis* (MtB) were reached with TB diagnosis and treatment in 2018 [3]. Today, the multidrug resistant (MDR) [rifampin (RIF) and isoniazid (INH) resistant] and extensively drug resistant (XDR) [MDR-TB additionally resistant to at least 1 of 3 second-line injectable drugs including capreomycin, kanamycin, and amikacin, and any fluoroquinolone] strains of TB are among the most important therapeutic challenges worldwide [4]. Besides, the latest report of WHO showed around 3.4% of new TB cases and 18% of previously treated cases were MDR, or RIF-resistant (RR-TB) and the average proportion of MDR-TB cases with XDR-TB was 6.2% that is widely considered to be a major threat to global health [5, 6, 7]. Different resistance mechanisms for MDR-TB and XDR-TB have been reported; mutation in target genes is the main basis of resistance to anti-TB drugs. For example, the *rpoB* gene is mutated in approximately 95% of RIF-resistant MtB strains, in more than 80% of INH-resistant MtB strains, resistance is related to mutations in *inhA*, and *katG* genes [8, 9] but, some of drug-resistant isolates have not these target gene mutations. So, mutation cannot always explain drug-resistance [10].

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Alternative resistance mechanisms associated with drug transport and cell wall permeability, such as efflux pumps (EPs), have also been reported [11]. The overexpression of EPs resulted in a decrease the intracellular levels of the antibiotics and prevent the drug to reach its cellular target, allowing the evolve of a subpopulation presenting high-level resistance [12]. Some EPs systems have narrow substrate specificity, while several extrude different types of drugs that are structurally unrelated and are found to be associated with the development of MDR-TB [13].

The resistance/nodulation/cell division (RND), ATP-binding cassette (ABC), small multidrug resistance (SMR) family, and the major facilitator structural unrelated and are found to be associated with the development of MDR-TB [13].

Some EPs systems have narrow substrate specificity, while several extrude different types of drugs that are structurally unrelated and are found to be associated with the development of MDR-TB [13].

This study aimed to investigate the overexpression profile of five putative EP genes including Rv2942 (mmpL7), Rv3065 (mmp), Rv1410c (P55), Rv2459 (jfa), Rv1258c (tap) plus mutations in target genes in clinical MDR-Mtb isolates in Iran.

2. Materials and methods

2.1. Ethical consideration and bacterial strains

This study was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1397.792).

In this study, a panel of 27 Mtb clinical isolates including 22 MDR isolates and, 5 sensitive isolates; and H37Rv ATCC 27294 as control strain were obtained from Ahvaz Regional TB Laboratory, Southwest of Iran. All MDR isolates were isolated from sputum and determined by the Xpert MTB/RIF assay and proportion method for INH and RIF in this laboratory.

2.2. Drug susceptibility testing

For confirmation of obtained MDR isolates, drug susceptibility testing (DST) was done for 22 MDR isolates on lowenstein-jensen (LJ) medium by proportional method according to the WHO guidelines [17] with critical concentrations of INH, 0.2 μg/mL, RIF, 40 μg/mL (Sigma Aldrich Co., USA). For DST, Mtb strain H37Rv (ATCC 27294) was used as a control. When compared to the control medium, drug susceptibility was defined as no or less than 1% growth on the antibiotic-containing LJ media.

2.3. Minimal inhibitory concentration determination

Resazurin microtiter assay (REMA) was performed as described by Palomino et al [18] for 22 MDR-TB. Briefly, 100 μl 7H9–s medium was added in every well of sterile 96-well microtiter plates except perimeter wells where 250 μl D/w was dispensed to prevent evaporation during incubation. Two-fold serial dilutions of RIF or INH (ranging from 0.125 to 128 μg/ml) were prepared into the wells. 100 μl mycobacterial inoculum (diluted 1: 20 in 7H9–s) of turbidity resembling 0.1 MacFarland index were added to each well except perimeter wells containing D/w. Medium control (only medium without drug, and inoculum), growth control (medium without drug and but with inoculum) were also made in each plate. MICS were also determined in the presence of efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma, USA) (0.5 μg/ml). Then, plates were sealed and covered in a plastic bag and incubated at 37 °C for 7 days. After 7 days 30 μl of 0.02% resazurin sodium salt (Sigma, USA) solution was added to each well and again incubated overnight at 37 °C. A color change from blue to pink was considered as positive growth and MIC was considered as that prevented a color change The MIC was defined as the lowest antibiotic concentration that prevented a color change. Isolates with MICS of RIF ≤1 μg/mL and INH <0.25 μg/mL were considered as susceptible to RIF and INH, respectively [16, 19, 20].

2.4. Detection of mutations associated with resistance

DNA was extracted from MDR isolates using a DNA isolation kit (Bio Basic, Canada) for polymerase chain reaction (PCR) and sequencing. PCR sequencing and detection of mutations in the mentioned genes for 4 of 22 MDR-isolates were done as described previously [21], and in this study, we performed the sequencing for the remaining 18 isolates.

The primers used for sequencing are presented in Table 1. A final volume of 20 μl containing Taq buffer (5X), forward and reverse primers (10 pM), Taq DNA polymerase (1 U) (Ampliqon, Denmark), DNA template (50 ng), and dNTPs (100 μM) was used as a PCR reaction. The PCR protocol was as follows: 5 min at 94 °C, 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C (polB, mab-046A)/55 °C (katG) for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. PCR products were sequenced on both strands using Big Dye Terminator V.3.1 Cycle Sequencing kit in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Obtained sequences were aligned together using ClustalW ([https://www.genome.jhu.edu/tools-bin/clusdaw] software to determine the consensus sequences. Consensus sequences were subjected to nBLAST analysis ([http://blast.ncbi.nlm.nih.gov](http://blast.ncbi.nlm.nih.gov)) and compared with Mtb strain H37Rv.

2.5. Total RNA isolation and cDNA synthesis

Total bacterial RNA extraction was done on 22 MDR isolates, 5 sensitive isolates, and H37Rv strain grown in antibiotic-free LJ medium incubated at 37 °C for 3–4 weeks using High Pure RNA isolation kit (Roche, Germany) according to the manufacturer’s instructions. RNA quantification was carried out by NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). After treatment with DNAse I (Biocompare, China), the lack of DNA contamination was confirmed by PCR of the housekeeping polymerase A (polA) gene on RNA.

The cDNA synthesis was conducted with the PrimeScript® reagent cDNA Synthesis Kit (Takara, Japan) in accordance with the company instructions.

2.6. Gene expression

All of the EPs primers were designed in this study and are shown in Table 1. Quantitative real-time PCR (qPCR) was performed in ABI StepOnePlus thermocycler (Applied Biosystem, Scientific, Darmstadt, Germany) using the amplified cDNA from two replicates of each isolate (Biological replicates) in a reaction volume of 12.5 μl containing 6.25 μl of SYBR1 Green high ROX Master Mix (Ampliqon, UK), 0.25 μl of 10 μM of each forward and reverse primer (final concentration: 200 nM), 3 μl cDNA template (25 ng) and 2.75 distilled water (D.W). The assay conditions were 95 °C for 10 min, 40 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Also, for ensuring specific amplification the melting curves of each run were performed. All reactions were performed in duplicate (Experimental replicates), and the mean value was used to calculate the expression level of each investigated gene compared to the reference strain after normalization to the polA housekeeping gene. The relative expression levels were determined using ΔΔCt comparative method in StepOne™ Software v2.0.2. Also, the 2−ΔΔCt method was used for determination of the relative expression fold changes of mRNAs in comparison with the H37Rv reference strain [22]. Result equal to one indicates that the expression level of the gene is the same as the reference strain, expression levels above 1 were considered to be increased and an overexpression level of >4-fold is considered as the cut-off for distinguishing overexpressed samples [16].

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**Table 1.** Quantitative real-time PCR (qPCR) was performed in ABI StepOnePlus thermocycler in accordance with the company instructions.

| Gene | Primer sequence (5’–3’) | Melt curve (°C) |
|------|------------------------|-----------------|
| polA | F: 5′-GACGTTTCTGATTACATCTGAGT-3′<br>R: 5′-GTCCGGTCTAATGCTTGG-3′ | 75–85 |
| ΔpolA | F: 5′-TCTCGCCATTGATTTGATTCT-3′<br>R: 5′-GTTTCTGATTACATCTGAGT-3′ | 72–80 |

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2.7. Statistical analysis

The SPSS version 22 statistics software (IBM Corporation, Armonk, NY, USA) was used to analyze the descriptive data. The T-test was used to determine the significance. Furthermore, the significance association was set at P-value < 0.05.

3. Results

By performing DST, all 22 MDR isolated were resistant to RIF and INH and confirmed as MDR. The overall MIC distributions for these isolates are shown in Table 2, the MIC of RIF and INH ranged from 2 to >128 μg/ml and 2–32 μg/ml, respectively. After efflux pump inhibitor CCCP exposure, 10/22 (45.45%) isolates showed a decrease in MICs of INH, whereas it was observed that the MICs of RIF decreased in 17/22 (77.27%) isolates in the presence of efflux inhibitor. The highest RIF MIC (>128 μg/mL) was detected in MDR-3 and MDR-12 isolates. Furthermore, in the presence of CCCP, 2 isolates changed their MIC phenotypically from resistant to sensitive and the RIF sensitivity was fully restored in one isolate.

Table 2. Isoniazid (INH) and rifampin (RIF) minimum inhibitory concentrations (MICs) in the presence and absence of efflux inhibitor, genes overexpressed and gene mutations of 22 multidrug-resistant (MDR) M. tuberculosis isolates.

| Isolates | RIF MIC (μg/mL) | INH MIC in the presence of CCCP | Mutations | Genes overexpressed |
|----------|-----------------|--------------------------------|-----------|---------------------|
|          | RIF MIC (μg/mL) |                                 |           |                     |
| MDR-1    | 64              | 16                              | SS31L (TCG→TTG) | WT                     |
| MDR-2    | 64              | 2                               | SS31L (TCG→TTG) | WT                     |
| MDR-3    | >128            | 64                              | SS31L (TCG→TTG) | S315A (AGC→AAC)       |
| MDR-4    | 32              | 32                              | SS31L (TCG→TTG) | -                     |
| MDR-5    | 16              | 8                               | LS33P (CTG→CC)  | WT                     |
| MDR-6    | 4               | 2                               | HS26T (CAC→TA)  | S315A (AGC→AAC)       |
| MDR-7    | 8               | 8                               | WT          | WT                   |
| MDR-8    | 8               | 0.125                           | HS26T (CAC→TA)  | WT                     |
| MDR-9    | 16              | 0.25                            | SS31L (TCG→TTG) | WT                     |
| MDR-10   | 64              | 16                              | WT          | WT                   |
| MDR-11   | 16              | 8                               | SS31L (TCG→TTG) | WT                     |
| MDR-12   | >128            | 16                              | SS31L (TCG→TTG) | WT                     |
| MDR-13   | 8               | 2                               | SS31L (TCG→TTG) | WT                     |
| MDR-14   | 32              | 32                              | SS31L (TCG→TTG) | S315A (AGC→AAC)       |
| MDR-15   | 4               | 8                               | LS33P (CTG→CC)  | WT                     |
| MDR-16   | 64              | 32                              | SS31L (TCG→TTG) | S315A (AGC→AAC)       |
| MDR-17   | 16              | 16                              | LS33P (CTG→CC)  | S315A (AGC→AAC)       |
| MDR-18   | 32              | 8                               | SS31L (TCG→TTG) | WT                     |
| MDR-19   | 64              | 16                              | SS31L (TCG→TTG) | S315A (AGC→AAC)       |
| MDR-20   | 0.5             | 8                               | WT          | WT                   |
| MDR-21   | 32              | 4                               | SS31L (TCG→TTG) | WT                     |
| MDR-22   | 64              | 32                              | WT          | WT                   |

| WT: wild type. |
Thirteen isolates (59.1%) showed overexpression (>4-fold) in at least one EP gene. Overexpression of Rv3065, Rv1410c, Rv1258c, Rv2459, and Rv2942 genes was seen in 5 (22.7%), 4 (18.2%), 3 (13.6%), 2 (9%), and 2 (9%) MDR isolates, respectively.

The MIC and gene expression results were analyzed but there was no significant relationship between MICs of the drugs and levels of expression of the pumps (P > 0.05).

Rv3065c shown the highest expression level of 9.14 in MDR-12 followed by Rv1410c which shown an expression level of 6.93 in MDR-10 isolate. Interestingly, Rif and INH MICs were 16 > in both these isolates.

According to the sequencing results, of 22 MDR isolates, 18 (81.8%) showed a mutation in rpoB gene. Three mutational profiles were detected for these isolates, S531L (TCG→TTG) was the most prevalent mutation (12 isolates) followed by H526T (CAC→TAC) mutation (3 isolates), and L533P (CTG→CCG) mutation (3 isolates).

Nine (40.9%) isolates carried a mutation in INH resistance-related genes, which 6 isolates of them showed S315A (AGC→AAC) mutation in katG gene and 3 isolates had mutation A→T (−15) mutation in inhA promoter region while there was no mutation in INH resistance-related genes in 13 (59%) of isolates. Four MDR isolates (18.2%) did not display any mutation in any investigated genes. The overexpression was indicated in 4 and 10 isolates lacked genetic mutation in the surveyed regions of the rpoB + katG + inhA and katG + inhA genes, respectively. Three MDR isolates showed neither INH resistance-related mutations nor overexpression in EP.

The following accession numbers for the nucleotide sequences obtained in this study have been deposited in the GeneBank database: MW916092, MW916093 for rpoB, MW913094 for katG, and MH734237 for mab-inhA.

### Table 3. Expression profile of EP genes among different isolates of M. tuberculosis.

| Isolates | Rv2942 (mmpl7) | Rv1258c (tap) | Rv1410c(F55) | Rv2459 (jefA) | Rv3065 (mmr) |
|----------|----------------|---------------|---------------|---------------|---------------|
| MDR-1    | 4.32           | 0.02          | 1.4           | 0.12          | 1.05          |
| MDR-2    | 1.4            | 3.91          | 4.59          | 1.67          | 1.04          |
| MDR-3    | 3.64           | 0.96          | 1.51          | 2.9           | 0.03          |
| MDR-4    | 1.1            | 0             | 3.64          | 0             | 4.53          |
| MDR-5    | 1.19           | 1.23          | 3.2           | 0             | 0.026         |
| MDR-6    | 3.48           | 4.1           | 3.19          | 3.03          | 6.06          |
| MDR-7    | 3.55           | 1.93          | 1.67          | 6.25          | 4.61          |
| MDR-8    | 3.6            | 3.96          | 1.12          | 3.04          | 2.23          |
| MDR-9    | 5.3            | 3.4           | 6.49          | 3.13          | 3.13          |
| MDR-10   | 1.3            | 2.25          | 6.93          | 3.02          | 1.3           |
| MDR-11   | 3.46           | 1.14          | 1.23          | 3.18          | 1.09          |
| MDR-12   | 3.9            | 2.36          | 3.93          | 1.03          | 9.14          |
| MDR-13   | 3.3            | 5.4           | 3.99          | 2.06          | 1             |
| MDR-14   | 3.1            | 3.06          | 1.51          | 4.63          | 3.32          |
| MDR-15   | 2              | 1.21          | 3.62          | 1.15          | 3.96          |
| MDR-16   | 1.02           | 3.12          | 3.14          | 2.26          | 1.2           |
| MDR-17   | 3.06           | 3.1           | 2.14          | 1.96          | 2.15          |
| MDR-18   | 3.55           | 1.63          | 5.2           | 1.48          | 3.01          |
| MDR-19   | 0              | 3.06          | 3.16          | 3.1           | 2.03          |
| MDR-20   | 0.93           | 6.16          | 1.9           | 1.14          | 4.53          |
| MDR-21   | 3.9            | 3.14          | 2.08          | 3.02          | 2.67          |
| MDR-22   | 2.69           | 4.5           | 3.96          | 2.26          | 3.53          |
| SEN-1    | 0.97           | 1.89          | 1.02          | 1.19          | 1.48          |
| SEN-2    | 0.067          | 1.1           | 1.67          | 1.03          | 1.15          |
| SEN-3    | 1.12           | 0.62          | 1.02          | 1.13          | 1.25          |
| SEN-4    | 1.52           | 1.1           | 0.9           | 1.62          | 0.91          |
| SEN-5    | 1.11           | 1.19          | 1.36          | 1             | 0.92          |
| H37Rv    | 1              | 1             | 1             | 1             | 1             |

The mean value was considered as an expression level of each gene against the reference strain (MTB strain H37Rv) after normalization to the polA housekeeping gene. The 2^−ΔΔCT method was used for determination of the relative expression fold changes of mRNAs in comparison with the H37Rv reference strain. Result equal to one indicates that the expression level of the gene is the same as the reference strain, expression levels above 1 were considered to be increased and an overexpression level of >4-fold is considered as the cut-off for distinguishing overexpressed samples [16].

### 4. Discussion

In the current study, we investigated the overexpression EP genes plus gene mutations in MDR-TB clinical isolates in Iran, the country where for the first time the first totally drug-resistant (TDR) strains of Mtb were identified [23].

The qRT-PCR assay results demonstrated the possible contribution of EPs overexpression to the resistance phenotype of the studied MDR isolates. It was indicated that expression level differences between sensitive and reference strain were not statistically significant (P > 0.05). On the other hand, MDR isolates showed significantly different rates of EPs expression compared with sensitive and reference strains. In agreement with our findings, Li et al., in China [16] and Kardan et al., in Iran [13], previously found that significantly different rates of expression were found between the sensitive and MDR isolates and overexpression of analyzed ESs is associated with MDR property in Mtb strains. In this study, 59.1% (n = 13) of MDR isolates showed overexpression in EP genes (>4-fold) that was more than Kardan et al. study [13] and Oh et al. study [24] in South Korea that showed overexpression in 48% and 42% of MDR isolates, respectively, without drug exposure. A possible explanation for this high level of overexpression is due to different EPs that were investigated in cited studies.

The Rv3065, and Rv1410c showed the highest expression level additionally were the most commonly overexpressed EPs in 5 and 4 MDR isolates, respectively. The EPs encoded by the Rv3065 appear to play significant roles in the efflux of multiple drugs and chemical classes [25]. Another study by Kardan et al. [15] revealed increased expression of certain
EP genes even in the absence of antibiotic pressure. They compared the expression level of 4 EPs in 31 clinical isolates of Mtb and reported that \(Rv1410c\) shown overexpression in 7 of 21 M/XDR-TB isolates. In the Balganesh et al. [25], study \(Rv0849\), \(Rv2128c\), \(Rv3065\), and \(Rv1258c\) were tested, and they concluded that \(Rv3065\) and \(Rv1218c\) play an important role in mediating the efflux of different antibiotics and \(Rv0849\) and \(Rv1258c\) mediate the efflux of antibiotics, but to a lesser extent. Also, Jiang et al. [26], showed upon RIF or INH exposure, \(Rv1258c\) and \(Rv1410c\) were overexpressed and \(Rv1819c\) was overexpressed only in the presence of INH. Other surveys suggested that the overexpression of these and other EPs in MDR and XDR strains of Mtb, is believed to decrease the intracellular levels of the antibiotics and prevent the drug to reach its cellular target, allowing the development of drug resistance [24, 27, 28]. The DNA sequencing results showed upon RIF or INH exposure, \(Rv1258c\) and \(Rv1410c\) were overexpressed and \(Rv1819c\) was overexpressed only in the presence of INH. Other surveys suggested that the overexpression of these and other EPs in MDR and XDR strains of Mtb, is believed to decrease the intracellular levels of the antibiotics and prevent the drug to reach its cellular target, allowing the development of drug resistance [24, 27, 28]. The DNA sequencing results showed upon RIF or INH exposure, \(Rv1258c\) and \(Rv1410c\) were overexpressed and \(Rv1819c\) was overexpressed only in the presence of INH. Other surveys suggested that the overexpression of these and other EPs in MDR and XDR strains of Mtb, is believed to decrease the intracellular levels of the antibiotics and prevent the drug to reach its cellular target, allowing the development of drug resistance [24, 27, 28].

**Declarations**

**Author contribution statement**

Fatemeh Shahi, Azar Dokht Khosravi: Conceived and designed the experiments; Wrote the paper.

Mohammad Reza Tabandeh: Contributed reagents, materials, analysis tools or data.

Shokrollah Salanzadeh: Analyzed and interpreted the data.

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**Data availability statement**

The authors do not have permission to share data.

**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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