Heliyon 7 (2021) e07566

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

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

Fatemeh Shahi [a,b], Azar Dokht Khosravi [a,b,c,*], Mohammad Reza Tabandeh [d], Shokrollah Salmanzadeh [a,e]

[a] *Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[b] *Department of Microbiology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[c] *Iranian Study Group on Microbial Drug Resistance, Iran*
[d] *Department of Basic Sciences, Division of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran*
[e] *Tropical Medicine Ward, Razi Teaching Hospital, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*

ARTICLE INFO

Keywords:
Efflux pumps
MDR-TB
Mutation
Tuberculosis

ABSTRACT

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 over-expression 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 resistance 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].

* Corresponding author.
E-mail address: azarkhosravi69@gmail.com (A.D. Khosravi).

https://doi.org/10.1016/j.heliyon.2021.e07566
Received 10 January 2021; Received in revised form 23 February 2021; Accepted 9 July 2021

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 superfamily (MFS) are among the Mtb Eps systems [14]. So far, limited information is available on the expression profile of genes encoding drug EPs in Mtb clinical isolates. The most studied efflux pumps including *Rv1410c*, *Rv2459*, *Rv1258c* (belonging to the MFS family), *mmpL7* (RND family), and the *Rv3065* efflux transporter (only efflux pump from the SMR family) are associated with the reduced susceptibility to dyes and antibiotics such as INH, RIF, erythromycin, tetracycline, aminoglycosides, and fluoroquinolones [12]. We selected these multidrug efflux pump genes based on previous studies that examined the contribution of efflux pumps to the drug resistance of MDR-TB [12, 13, 15, 16].

This study aimed to investigate the overexpression profile of five putative EP genes including *Rv2942* (*mmpL7*), *Rv3065* (*mmr*), *Rv1410c* (*P55*), *Rv2459* (*jefA*), *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 microtitre 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 $\leq$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 (5×), 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 (*rpoB*, *mab-inhA*)/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.jp/tools-bin/clustalw) software to determine the consensus sequences. Consensus sequences were subjected to nBLAST analysis (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^{RT} 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 °C 72 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 $2^{-\Delta\Delta CT}$ 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].

Table 1. Primers used for this study.

Gene	Method	Sequence	Product size(bp)	Tm (°C)
rpoB	Sequencing	F: CGATCACACCGCAGACGTTG R:GGTACGGCGTTTCGATGAAC	318	
katG	Sequencing	F: CATGAACGACGTCGAAACAG R: CGAGGAAACTGTTGTCCCAT	232	
mab-inhA	Sequencing	F: ACATACCTGCTGCGCAAT R: TCACATTCGACGCCAAAC	400	
Rv2942	Real time-PCR	F: GCGATGAAATACGGAAGCCT R: GATAACGGCCAGTAGCACAG	114	81.24
Rv3065	Real time-PCR	F: ATACCTCTTGTGCGCGATCT R: GCCATAACCCACTAGACAGC	109	80.39
Rv1410c	Real time-PCR	F: TGTGGTTCCTTATCGCCCTA R: GGGAAATAAGCCAGTAACCGT	126	79.67
Rv2459	Real time-PCR	F: GATCACCCAGCACTTCCAAA R: GAGGACCCCATTTGTTGACC	131	79.32
Rv1258c	Real time-PCR	F: CTACGAGGCGATCCTCAACC R: CGAGGATGGACAACCCGAAT	125	82.64
polA	Real time-PCR	F: GTCGTGGTTGGACCTTGGAGGG R: GCGTCCGTATCGTCGTCATCG	181	80.09

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 3 represents the comparison in expression levels of five EP genes in the 22 MDR isolates, 5 sensitive isolates, and H37Rv strain. The RT-qPCR results revealed that there were no significant differences ($P > 0.05$) in the expression level of any EPs genes between standard strain and the sensitive strains, the expression level of all EP genes in the MDR isolates were significantly higher compared to sensitive isolates and reference strain with the P value less than 0.05.

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		INH		Mutations			Genes overexpressed
	MIC (μg/mL)	MIC in the presence of CCCP	MIC (μg/mL)	MIC in the presence of CCCP	*rpoB*	*katG*	*inhA*	
MDR-1	64	16	16	8	S531L (TCG→TTG)	WT	WT	Rv1258c
MDR-2	64	16	2	2	S531L (TCG→TTG)	WT	WT	*Rv1410c*
MDR-3	>128	64	32	32	S531L (TCG→TTG)	S315A (AGC→AAC)	WT	-
MDR-4	32	4	8	8	L533P (CTG→CC)	WT	WT	*Rv3065c*
MDR-5	16	8	4	1	H526T (CAC→TA)	S315A (AGC→AAC)	WT	-
MDR-6	4	2	16	4	WT	WT	WT	*Rv3065c, Rv2942*
MDR-7	8	2	16	8	WT	WT	WT	*Rv3065c, Rv2459*
MDR-8	8	4	4	0.125	H526T (CAC→TA)	WT	WT	-
MDR-9	16	0.25	4	4	S531L (TCG→TTG)	WT	C→T(-15)	*Rv1410c*
MDR-10	64	8	16	1	WT	WT	WT	*Rv1410c*
MDR-11	16	8	32	32	S531L (TCG→TTG)	WT	C→T(-15)	-
MDR-12	>128	16	16	2	S531L (TCG→TTG)	WT	WT	*Rv3065c*
MDR-13	8	8	2	0.125	S531L (TCG→TTG)	WT	WT	*Rv1258c*
MDR-14	32	32	16	16	H526T (CAC→TA)	S315A (AGC→AAC)	WT	*Rv2459*
MDR-15	4	2	8	8	S531L (TCG→TTG)	WT	WT	-
MDR-16	64	64	32	16	L533P (CTG→CC)	S315A (AGC→AAC)	WT	-
MDR-17	16	16	4	4	S531L (TCG→TTG)	S315A (AGC→AAC)	WT	-
MDR-18	32	8	4	4	S531L (TCG→TTG)	WT	WT	*Rv1410c*
MDR-19	64	8	16	16	L533P (CTG→CC)	S315A (AGC→AAC)	WT	-
MDR-20	2	0.5	8	1	WT	WT	WT	*Rv1258c*
MDR-21	32	4	4	4	S531L (TCG→TTG)	WT	C→T(-15)	-
MDR-22	64	32	16	16	S531L (TCG→TTG)	WT	WT	*Rv2942*

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 C→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*.

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 EPs 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

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

Isolates	*Rv2942* (*mmpL7*)	*Rv1258c* (*tap*)	*Rv1410c*(*P55*)	*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	3.09	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	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	0.9	1.89	1.02	1.19	1.48
SEN-2	0.067	1.1	1.67	1.03	1.15
SEN-3	1.2	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 $2^{-\Delta\Delta CT}$ 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].

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*, *Rv1218c*, *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 less 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 demonstrated that among MDR isolates 4 isolates lacked any mutation in *rpoB*, *katG*, and *inhA* genes, simultaneously while qRT-PCR revealed that all of these isolates showed efflux pump genes overexpression. Thirteen isolates were without mutation in *katG* and *inhA* genes, these results could indicate that an increase in the expression of EPs can cause INH and RIF resistance in addition to mutations in related genes. Mutations related to INH resistance were identified in 15 unique gene regions that *katG* and *inhA* are the most frequent. Studies have reported highly variable frequencies of these mutations; with *katG*315 mutations considering for 42–95% and *inhA* (-15C→T) mutations accounting for 6–43% of INH resistance [29]. So, the role of other gene mutations in *ahpC-oxyR*, *kasA*, or *ndh* genes even in other regions of the examined genes which were not investigated in this study, cannot be ignored.

Likewise, EPs can play a role in drug resistance even in strains with gene mutations. Decrease in intracellular antibiotics levels caused by EPs overexpression, enabling the survival of certain subpopulations of bacteria until a classical mutation appears and a population with significant antibiotic resistance is established [30].

The MIC results determined that CCCP exposure led to INH (from no fold to a six-fold decrease) and RIF (from no fold to a four-fold decrease) MIC reduction of 45.45% and 77.27%, respectively, which is in agreement with the study findings of Li et al [16] and Jaiswal et al [31]. Jaiswal reported 61% of isolates showed a decrease in MIC of INH with CCCP. Kardan et al [13] found decreased MIC of RIF from no fold changes to an eight-fold was observed in the presence of the efflux pump inhibitor CCCP without drug exposure. These results suggest that efflux pump inhibitors may have potential use in TB treatment.

To conclude, our data showed that drug resistance of MDR-TB is a combination of drug efflux and the presence of target gene mutations. Hence, the expressional differences of some EPs genes (*Rv2942*, *Rv3065*, *Rv1410c*, *Rv2459*, and *Rv1258c*) between MDR and sensitive isolates could be helpful in MDR-TB diagnosis and treatment. Efflux pump activity inhibitor CCCP can reduce the phenotypic level of INH and RIF resistance. In addition, to clarifying the actual roles of EPs in drug resistance, further researches on more isolates and more EP genes regarding efflux pump inhibitors are required.

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 Salmanzadeh: Analyzed and interpreted the data.

Funding statement

This study is a part of Ph.D. thesis by Fatemeh Shahi, which was approved in Infectious and Tropical Diseases Research Center, and was financially supported by a grant (OG-9736) from Research Affairs, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

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.

Acknowledgements

We are greatly thankful to the staff of Regional TB Reference Laboratory especially Nazanin Ahmad Khosravi for their collaboration.

References

[1] M. Raviglione, G. Sulis, Tuberculosis 2015: burden, challenges and strategy for control and elimination, Infect. Dis. Rep. 8 (2) (2016) 33–37.
[2] M.K. Calgin, F. Sahin, B. Turegun, D. Gerceker, M. Atasever, D. Koksal, et al., Expression analysis of efflux pump genes among drug-susceptible and multidrug-resistant *Mycobacterium tuberculosis* clinical isolates and reference strains, Diagn. Microbiol. Infect. Dis. 76 (3) (2013) 291–297.
[3] T.B. Stop, Partnership U. Global Plan to End TB; Revised Funding Estimates 2018–2022, 2018.
[4] A.F.L. Iacobino, F. Giannoni, Drug-resistant tuberculosis 2020: where we stand, Appl. Sci. 10 (6) (2020) 2153.
[5] WHO. Consolidated Guidelines on Drug-Resistant Tuberculosis Treatment, World Health Organization, Geneva, Switzerland, 2019. Available from: https://apps.who.int/iris/bitstream/handle/10665/311390/WHO-CDS-TB-2019.3-eng.pdf.
[6] A.R.A. Matteelli, A.C. Carvalho, Extensively drug-resistant tuberculosis: epidemiology and management, Clin. Epidemiol. 6 (2014) 111.
[7] WHO, Global Tuberculosis Report 2019. License: CC BY-NCSA 3.0 IGO, World Health Organization, Geneva, 2019.
[8] A.G.K. Narang, S. Porwal, A. Bhandekar, K. Shrivastava, A. Giri, et al., Potential impact of efflux pump genes in mediating rifampicin resistance in clinical isolates of *Mycobacterium tuberculosis* from India, PloS One 14 (9) (2019), e0223163.
[9] E. Bottger, The ins and outs of *Mycobacterium tuberculosis* drug susceptibility testing, Clin. Microbiol. Infect. 8 (17) (2011) 1128–1134.
[10] J. Palomino, A. Martin, Drug resistance mechanisms in Mycobacterium tuberculosis, Antibiotics 3 (3) (2014) 317–340.
[11] H. Ghajavand, M.K. Kamakoli, S. Khanipour, S.P. Dizaji, M. Masoumi, F.R. Jamnani, et al., Scrutinizing the drug resistance mechanism of multi-and extensively-drug resistant *Mycobacterium tuberculosis*: mutations versus efflux pumps, Antimicrob. Resist. Infect. 8 (1) (2019) 70.
[12] D. Machado, T.S. Coelho, J. Perdigão, C. Pereira, I. Couto, I. Portugal, et al., Interplay between mutations and efflux in drug resistant clinical isolates of *Mycobacterium tuberculosis*, Front. Microbiol. 8 (2017) 711.
[13] J. Kardan-Yamchi, H. Kazemian, M. Haeili, A.A. Harati, S. Amini, M.M. Feizabadi, Expression analysis of 10 efflux pump genes in multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* clinical isolates, J. Glob. Antimicrob. Resist. 17 (2019) 201–208.
[14] J.P. Sarathy, V. Dartois, E.J.D. Lee, The role of transport mechanisms in *Mycobacterium tuberculosis* drug resistance and tolerance, Pharmaceuticals 5 (11) (2012) 1210–1235.
[15] J.K. Yamchi, M. Haeili, S.G. Feyisa, H. Kazemian, A.H. Shahraki, F. Zahednamazi, et al., Evaluation of efflux pump gene expression among drug susceptible and drug resistant strains of *Mycobacterium tuberculosis* from Iran, Infect. Genet. Evol. 36 (2015) 23–26.
[16] G. Li, J. Zhang, Q. Guo, Y. Jiang, J. Wei, L-l Zhao, et al., Efflux pump gene expression in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates, PloS One 10 (2) (2015), e0119013.
[17] World Health Organization, Policy Quidance on Drug-Susceptibility (DST) of Second Line Antituberculosis drugs.WHO/HTM/TB/2018.392, World Health Organization G, switzerland, 2018.
[18] J.-C. Palomino, A. Martin, M. Camacho, H. Guerra, J. Swings, F. Portaels, Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*, Antimicrob. Agents Chemother. 46 (8) (2002) 2720–2722.
[19] F. Nateche, A. Martin, S. Baraka, J.C. Palomino, S. Khaled, F. Portaels, Application of the resazurin microtitre assay for detection of multidrug resistance in *Mycobacterium tuberculosis* in Algiers, J. Med. Microbiol. 55 (7) (2006) 857–860.
[20] N.K. Taneja, J.S. Tyagi, Resazurin reduction assays for screening of anti-tubercular compounds against dormant and actively growing *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*, J. Antimicrob. Chemother. 60 (2) (2007) 288–293.

[21] M. Sirous, A.D. Khosravi, M.R. Tabandeh, S. Salmanzadeh, N. Ahmadkhosravi, S. Amini, Molecular detection of rifampin, isoniazid, and ofloxacin resistance in Iranian isolates of *Mycobacterium tuberculosis* by high-resolution melting analysis, Infect. Drug Resist. 11 (2018) 1819–1829.

[22] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method, Methods 25 (4) (2001) 402–408.

[23] A.A. Velayati, M.R. Masjedi, P. Farnia, P. Tabarsi, J. Ghanavi, A.H. ZiaZarifi, et al., Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran, Chest 136 (2) (2009) 420–425.

[24] T.S. Oh, Y.J. Kim, H.Y. Kang, C.-K. Kim, S.Y. Cho, H.J. Lee, RNA expression analysis of efflux pump genes in clinical isolates of multidrug-resistant and extensively drug-resistant *Mycobacterium tuberculosis* in South Korea, Infect. Genet. Evol. 49 (2017) 111–115.

[25] M. Balganesh, N. Dinesh, S. Sharma, S. Kuruppath, A.V. Nair, U. Sharma, Efflux pumps of *Mycobacterium tuberculosis* play a significant role in antituberculosis activity of potential drug candidates, Antimicrob. Agents Chemother. 56 (5) (2012) 2643–2651.

[26] X. Jiang, W. Zhang, Y. Zhang, F. Gao, C. Lu, X. Zhang, et al., Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR, Microb. Drug Resist. 14 (1) (2008) 7–11.

[27] D. Machado, I. Couto, J. Perdigao, L. Rodrigues, I. Portugal, P. Baptista, et al., Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*, PloS One 7 (4) (2012), e34538.

[28] D. Machado, D. Pires, J. Perdigão, I. Couto, I. Portugal, M. Martins, et al., Ion channel blockers as antimicrobial agents, efflux inhibitors, and enhancers of macrophage killing activity against drug resistant *Mycobacterium tuberculosis*, PloS One 11 (2) (2016), e0149326.

[29] M. Seifert, D. Catanzaro, A. Catanzaro, T.C. Rodwell, Genetic mutations associated with isoniazid resistance in *Mycobacterium tuberculosis*: a systematic review, PloS One 10 (3) (2015), e0119628.

[30] J. Fonseca, G. Knight, T. McHugh, The complex evolution of antibiotic resistance in *Mycobacterium tuberculosis*, Int. J. Infect. Dis. 32 (2015) 94–100.

[31] I. Jaiswal, A. Jain, S.K. Verma, P. Singh, S. Kant, M. Singh, Effect of efflux pump inhibitors on the susceptibility of *Mycobacterium tuberculosis* to isoniazid, Lung India 34 (6) (2017) 499.