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Original Research Article

Investigation of efflux pump genes in isoniazid resistant *Mycobacterium tuberculosis* isolates

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ABSTRACT

Background: Tuberculosis (TB) is one of the most important infectious diseases worldwide. Resistance to antituberculosis drugs develops because of genetic mutations that render drug-activating enzymes inactive, changes in cell wall permeability, and increased expression of efflux pump genes and also combination therapy with efflux pump inhibitors may be more effective in drug-resistant TB patients.
Aims: To investigate the effect of verapamil (VR) on isonicotinic acid hydrazide (INH) resistance and the expression of 21 efflux pump genes in INH monoresistant MTBC clinical isolates.
Study design: In vitro study.
Methods: In our mycobacteriology laboratory, 10 INH monoresistant and 10 primary anti-TB drug-susceptible MTBC clinical isolates were selected. Drug susceptibilities for INH and VR were studied by resazurin microtiter plate method and minimum inhibitory concentration (MIC) was determined. Additionally, mRNA gene expressions were investigated by quantitative Real Time Polymerase Chain Reaction for 21 efflux gene regions.
Results: While no change was observed in INH MICs of susceptible isolates under VR effect, 6 (60%) of the 10 INH-resistant isolates showed a decrease of less than one dilution in INH MIC under VR effect. VR significantly reduced resistance in resistant isolates ($p < 0.05$). INH monoresistant MTBC isolates showed a 2.85-fold expression increase in the *Rv1634* region of the Major Facilitator Superfamily efflux family under INH stress ($p = 0.029$). No statistically significant change was observed in other efflux gene regions. Herein, increased expression was observed in the *Rv1634* region, consistent with other studies in the literature, and this was associated with drug resistance. No significant change in expression was detected in other gene regions.
Conclusion: The effect of efflux pump inhibitor VR on INH MIC levels is promising for the treatment of resistant TB. However, studies with more resistant strains are needed to evaluate the efficacy of efflux pump genes.

1. Introduction

Tuberculosis (TB) remains one of the leading causes of death from infectious agents. According to World Health Organization reports, 6.4 million people were diagnosed with TB in 2021 [1]. TB continues to be a public health problem in Türkiye as it is worldwide. In 2020, the number of TB cases reported in Türkiye was 8925 and the incidence was 10.6 per 100,000 cases [2]. The increasing prevalence of multidrug-resistant TB (MDR-TB) and extensive drug-resistant TB (XDR-TB) complicates disease management. One of the main mechanisms leading to the development of resistance to anti-TB drugs is the increased activity of the efflux pump system [3]. Studies with *Mycobacterium tuberculosis* complex (MTBC) isolates have demonstrated the presence of efflux pumps. Gene regions belonging to the "major facilitator superfamily" (MFS), "ATP-binding cassette" (ABC), "resistance-nodulation-division" (RND), and "small multi drug resistance" efflux pump gene families have been identified in MTBC [4,5]. The aim of the present study was to investigate efflux pump genes in isonicotinic acid hydrazide (INH) monoresistant MTBC isolates and the effect of efflux pump inhibitor verapamil (VR) on INH resistance.

Abbreviations: INH, Isonicotinic acid hydrazide; MDR-TB, Multidrug-resistant TB; MIC, Minimum inhibitory concentration; MTBC, *Mycobacterium tuberculosis* complex; TB, Tuberculosis; VR, Verapamil.

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2. Materials and methods

2.1. Study design

Our study was carried out in the laboratory of the Mersin University Medical Microbiology Department between 2019 and 2022. A study was designed with isolates (10 susceptible and 10 INH-monoresistant clinical MTBC isolates and H37Rv standard strain for control) selected from the culture collection, which were isolated from clinical specimens that came to our laboratory in the last 10 years.

2.2. Activating the clinic isolates

Among the MTBC isolates identified and stored in the Mycobacteriology Laboratory of Mersin University Hospital, 10 INH-monoresistant isolates and 10 isolates sensitive to anti-TB drugs were selected. Löwenstein Jensen (LJ) storage cultures at +4 °C were passaged to Middlebrook 7H9 liquid medium containing 10% oleic acid, albumin, dextrose, catalase and 0.5% glycerol. After 14 days of incubation, growth and contamination were checked. 100 µl of each culture was passaged onto LJ solid medium. Cultures were incubated for 21 days at 37 °C in 5% CO_2. MTBC strain H37Rv was used as the standard strain.

2.3. Determination of INH and VR MICs

After incubation, INH and VR susceptibility testing was performed with the resazurin microtiter plate method (REMA). After the minimum inhibitory concentrations (MIC) were determined, INH MIC values were re-determined for each isolate under ½ VR MIC value. The critical concentration for INH was taken as 0.2 µg/mL [6].

2.4. RNA extraction and cDNA transcription

The isolates were resuscitated for molecular testing. 5 groups (Fig. 1) were created. Four groups were formed from 10 INH resistant isolates. Group 1 was incubated without drug effect, group 2 under drug stress with only ½ INH MIC, group 3 under drug stress with only ½ VR MIC, group 4 under drug stress with both ½ INH and ½ VR for 48 h. No stimulation was applied to the susceptible isolates (Group 5). RNA extraction was then performed. The cells were then collected by centrifugation at 7000×*g* for 10 min. Using an RNA extraction kit (High Pure RNA Isolation Kit, Roche, Ref No: 1182866001), RNAs were extracted and stored at −20 °C until complementary DNA (cDNA) synthesis.

RNAs were converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoCat. No: 4368814) protocol.

2.5. Real Time Polymerase Chain Reaction (qRT-PCR)

Efflux pump gene expression levels of each INH-resistant isolate under INH stress (group 2), under INH and VR co-drug stress (group 4), and without drug inhibition (group 1) were determined by qRT-PCR. In addition, qRT-PCR was applied to 10 clinical MTBC isolates sensitive to all first-line anti-TB drugs without drug stress (group 5) with the same protocol.

For qRT-PCR, a mixture containing 10 µl master mix, 6 µl PCR grade water, and 1 µl primer was prepared. 3 µl cDNA was added to the mixture, and the device was loaded. qRT-PCR conditions were 95 °C for 5 min followed by 45 cycles of amplification at 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 15 s, warming up at 95 °C for 10 s, 65 °C for 60 s, 97 °C for 1 s, and then cooling down at 40 °C for 30 s. Expression of the genes listed in Table 1 was measured.

2.6. Statistical analysis

According to REMA results, statistical significance of the change in INH MIC value in the presence of VR was evaluated with Fisher's Exact Test.

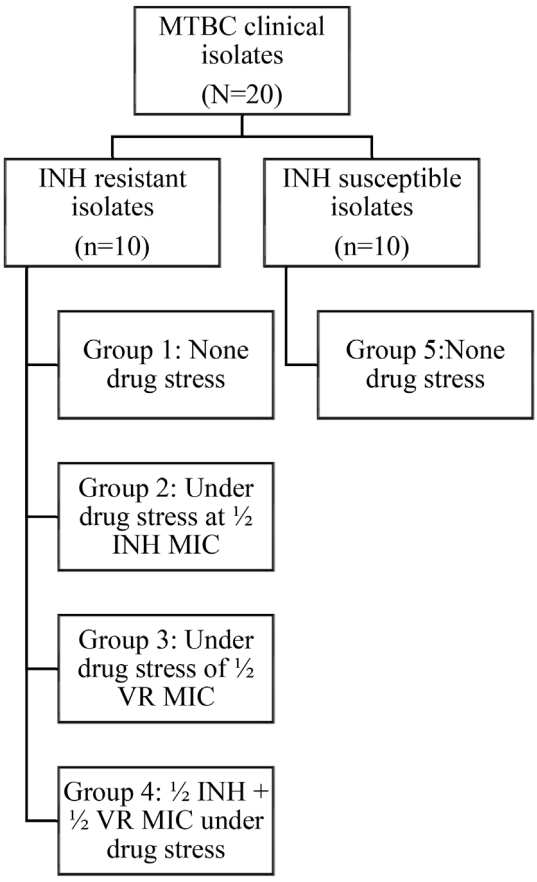

Fig. 1. Isolate groups for RNA isolations.

The results of qRT-PCR were obtained as raw cycle threshold (Ct) count and the delta–delta Ct method (approximation method) was used for evaluation [7]. The fold change of target gene expression in the target sample compared to the control group was determined. For statistical analysis, p-values and repeated $2^{(-\Delta\Delta CT)}$ values for each gene in the control and experimental groups were obtained using Student's t-test to determine the fold change in gene expression increase or decrease. "Mann Whitney *U* test" was used to compare the obtained data between the groups. P-values <0.05 were considered statistically significant.

3. Results

In 60% (n = 6) of the INH-resistant samples, at least one dilution decrease in INH MIC value in the presence of VR was detected by the REMA method (Table 2). For all 10 isolates susceptible to first-line anti-TB drugs and the control isolate (H37Rv), no decrease in INH MIC values was detected below the MIC value of ½ VR. Compared to the susceptible group, the presence of six isolates with at least one dilution decrease in INH MIC value in INH-resistant isolates under VR effect revealed that VR significantly reduced INH resistance ($p = 0.011$).

Among 21 efflux gene regions, the quadrilateral region (*Rv0849, Rv1456c, Rv1457c, Rv1634*) showing a two-fold or more expression increase under INH stress was identified in resistant isolates compared to the drug-free control group. Only the increase in expression in the *Rv1634* region of the MFS efflux gene family was statistically significant ($p = 0.029$) (Table 3). However, expression levels were compared for 21 gene regions of group 1, taking group 5 as the control group. No significant change was observed in the expression levels of the INH resistant group compared to the susceptible isolates (p > 0,05).

When the change in MICs of INH-resistant isolates with VR, an efflux pump inhibitor, was analyzed, 60% (n = 6) of the isolates showed at least

Table 1
Gene regions investigated by qRT-PCR.

Gene	Gene base size (bp)	Sequence (5′–3′)	Efflux Pump Family
Rv0194 *	202	F:CGACCTACTTGCTGATGTACG R: TTGGGCACATCGAACAGC	ABC
Rv1272c **	220	F:CAAGACCACGCTGGTGAACCTG R: CCTGGCGGCTTCTACTATCTCG	ABC
Rv1273c *	203	F:TAGTCACCGAGCAGGAGGAGATG R: CACGTCAAGTGCGGAGAAC	ABC
*Rv1456c***	195	F:ATCGCCGCAATCGTCATCC R: CACCGCAAACGTGACCATC	ABC
*Rv1457c***	229	F:TGCTGGTCGGGCTGACTTTG R: AACACCACGGCAACCACTG	ABC
Rv1458c *	190	F:GCAGCATTGAGGTACTTGGAC R:TCGGTGAGACCCAAGGTGTC	ABC
*Rv1686c***	211	F:GCTTGCCTGGTGCTACTGG R: GGAACCAGAACGCCACAATG	ABC
*Rv1687c***	216	F:GGACCCCCGACCATTTACAACG R: TCGAGCACTAGCAGATCAGG	ABC
Rv1819c *	200	F:GATCAGTGCCATCTGTGTGTGC R: TGTCGTTGCCTTGGTAGCTG	ABC
jefA (Rv2459) **	235	F:TCGCCCTGATCGCATACA R: ATCACCATCAGAGTCCCGA	MFS
*Rv0842***	220	F:TCTGGCAACGCTCACGAC R: ACAAAGAGTCACCCACACTCC	MFS
*Rv0849***	170	F:TGCGGTCATGGCATTGGTG R: CCAGATTTCCGACGAGGACTC	MFS
*Rv0876c***	211	F:AGTGCCAACCGCTATCTCC R: AGGTCAATGCCGTCAGTCC	MFS
Rv1250 *	200	F:GAACTTCAGCTTGTCCAACCTC R:ACAACGGATGGGACCTGTC	MFS
Rv1634 *	206	F:CGTGAGCTACTGAGCAGGTATC R:CGCAGCAACATCGTATTGAC	MFS
Rv1877 *	201	F:GTGCCCTATCAGGTCTTTG R:GGCTTCGAATTGGTGGTCTG	MFS
*Rv2265***	195	F:TGCTGTCGTTTCACATGGTCAG R:CGTTGTCCGTGAAGGCGAATAG	MFS
*Rv2456c***	200	F:GCTGATGTCGTCGTGCATC R: CTCCGATACCGTCGAGCAAC	MFS
Rv0507 *	210	F:GTGGGAACGGCAATTGTG R:CTCGATCAACAGCACCTCAG	RND
Rv0676c *	212	F:ATCCGCAGCTACTTCTACTGG R:TGCGCCTTCATGCTCTTC	RND
Rv3823c *	199	F:GGGCTGAATGTAGACCAAC R:ATCCGACGACAGACACCTTG	RND
*Rv2703 (sigA)**	200	F:GTGGCAGCGACCAAAGCAAG R:GTGTCCTGGGGTGCCGAG	–

ABC: ATP-binding cassette; MFS: major facilitator superfamily; RND: resistance-nodulation-division; Rv2703 (sigA), reference gene region. *(13); **(12).

Table 2
Isonicotinic acid hydrazide, verapamil, and combined minimum inhibitory concentration values of the isolates.

Isolate no	INH MIC[a] (μg/mL)	VR MIC (μg/mL)	Combined MIC (INH MIC values in the presence of ½ VR MIC)
1	32	62,5	**16**
2	0.5	15.62	**0.25**
3	8	125	**2**
4	4	62.5	**2**
5	0.25	15.62	0.25
6	1	31.25	1
7	2	625	2
8	4	3125	**2**
9	0.25	62.5	0.25
10	8	62.5	**4**
11	0.031	15.62	0.031
12	0.015	62.5	0.015
13	0.015	15.62	0.015
14	0.007	7.81	0.007
15	0.007	15.62	0.007
16	0.031	15.62	0.031
17	0.007	7.81	0.007
18	0.007	62.5	0.007
19	0.007	15.62	0.007
20	0.015	62.5	0.015
H37Rv	0.003	62.5	0.003

INH: isoniazid.
VR: verapamil.
MIC: minimum inhibitory concentration.
Decreased MIC values in the presence of INH and VR compared with only INH or VR are shown in bold.
[a] The critical concentration for INH was 0,2 μg/mL.

Table 3
Expression analysis according to gene regions under INH stress compared to drug-free control group.

Gene	Fold Change	p Value
Rv0194	1.19	0.415796
Rv0507	1.04	0.379825
Rv0676c	1.48	0.306574
Rv0842	1.43	0.276028
Rv0849	2.65	0.200341
Rv0876c	1.14	0.473836
Rv1250	1.46	0.257161
Rv1272	1.13	0.522289
Rv1273c	0.84	0.473877
Rv1456c	2.13	0.132772
Rv1457c	5.33	0.199126
Rv1458c	1.39	0.109027
Rv1634	**2.85**	**0.029493**
Rv1686c	1.04	0.372907
Rv1687c	1.34	0.331671
Rv1819c	1.08	0.316084
Rv1877	1.13	0.27783
Rv2265	1.57	0.324648
Rv2456c	1.42	0.105801
Rv2459c	1.51	0.324423
Rv3823c	1.03	0.891513

Statistically significant results are shown in bold.

one dilution decrease in MIC value. However, no significant change was found in gene expression levels under both drug stresses compared to the drug-free control group and INH stress only group ($p > 0.05$).

4. Discussion

Numerous studies have revealed that the efflux pump is a universal bacterial mechanism that contributes to antibiotic resistance and also that the activity of antibiotics exposed to efflux can be enhanced by the combined use of efflux pump inhibitors. However, the contribution of efflux to the overall drug resistance levels of *M. tuberculosis* clinical isolates is not fully understood. For this reason, this mechanism is still overlooked. Previous studies have identified efflux pump genes belonging to different families in MTBC [8]. VR is a synthetic papaverine derivative that exerts its effect through calcium channel blockade. Studies have found that VR inhibits the efflux pump of *Mycobacteria*. It also shows bactericidal activity by disrupting cell energy metabolism in *Mycobacteria* [9].

When the effect of VR on INH MIC was examined in the present study, 60% (n = 6) of INH monoresistant isolates had at least one dilution decrease in INH MIC. This finding supports our hypothesis that VR, an efflux pump inhibitor, may be effective in reducing INH resistance. In our study, according to qRT-PCR results, no significant change was observed in the gene expressions investigated after VR + INH exposure compared to the control group (Group 1). However, there are studies in the literature showing that there is a significant decrease in efflux pump gene expressions. In a study conducted by Caleffi-Ferracioli et al., on the *M. tuberculosis* H37Rv strain, a decrease was observed in the expression of efflux pump genes after 72 h of Rifampicin (RIF) and Verapamil (VP) exposure [10]. A study with macrophages evaluated the effect of rifampicin and verapamil on MTBC. VR has been shown to cause down-regulation in some efflux pump genes [11].

In the present study conducted with INH monoresistant strains, no significant change was found in the expression of the *Rv0194* gene region, a member of the ABC efflux pump gene family, with drug exposure. However, there are studies reporting that the *Rv0194* region is associated with amikacin and capreomycin resistance [12]. In a study conducted with an INH-resistant isolate (MIC = 1.5 mg/L), it was emphasized that *Rv1273c and Rv1250* regions showed increased expression, but mutations in *inhA* and *katG* associated with INH resistance were not found [13]. In a study analyzing *M. tuberculosis* H37Rv, it was reported that *Rv1250* and *Rv1273c* regions of efflux pump genes had significantly higher expression after exposure to ½ MIC INH compared to drug exposure at $^1/_3$ and ¼ MIC INH values [14]. In the present study, no significant increase in the expression of *Rv1273c* and *Rv1250* regions was detected in INH-resistant isolates.

Hao et al. investigated resistance mechanisms in MDR-TB and XDR-TB isolates and reported increased expression of *Rv1456c-Rv1457c-Rv1458c* efflux pump genes [15]. In another study, INH monoresistant isolates were studied and it was reported that the expression of the *Rv1457c* region increased more than two-fold [16]. In our study, more than two-fold increase in expression was observed in the *Rv1456c* and *Rv1457c* regions and a 1.39-fold increase was observed in the *Rv1458c* region, which are efflux pump genes from the ABC family; however, the increase was not statistically significant ($p > 0.05$).

In a study on drug resistance mechanisms in TB, it was emphasized that the *Rv1686c* region, which showed a significant increase in expression, may cause kanamycin resistance [17]. According to the results obtained in the present study, no significant change was observed in the expression of *Rv1686c-Rv1687c* regions in INH monoresistant isolates under INH stress.

In a study examining the relationship between drug exposure at different sub-inhibitor concentrations (½, $^1/_3$, and ¼) and expression levels of efflux pump genes that cause drug resistance in *M. tuberculosis*, it was reported that the *Rv1819c* gene region was significantly overexpressed at $^1/_3$ MIC INH exposure [14]. In the present study, under INH stress (group 2) there was no statistically significant increase in the expression of the Rv1819c gene region compared to the drug-free control group (group 1) ($p = 0.316$).

When an isolate with high-level INH resistance (INH MIC 1.5 mg/L) was investigated for 24 efflux pump genes, despite no *katG* or *inhA* mutation, overexpression in 8 efflux pump genes *(Rv1273c, Rv0194, Rv1634, Rv1250, Rv3823c, Rv0507, jefA,* and *p55*) was observed. In the same study, it was reported that no increased expression was observed in the *Rv0876c* gene region [13]. In the present study, *Rv2459 (jefA)* region showed a 2.04-fold increase in expression when compared to susceptible isolates as the control group, but the difference was not statistically significant ($p > 0.05$). In INH monoresistant strains, no significant alteration of the *Rv0876c* region was found under drug effect.

Although there are studies indicating that overexpression of the *Rv0842* efflux pump gene is associated with kanamycin resistance, increased expression of this gene region has not been reported in INH-resistant strains [13,17]. Consistent with the literature, no significant change was observed in the expression of *Rv0842* in INH monoresistant strains in our study.

In a study conducted by Li et al. with 9 MDR-TB and 10 all anti-TB drugs susceptible isolates, INH and RIF drug resistance and expression of efflux pump genes were examined, and it was emphasized that *drrA, drrB, jefA (Rv2459), mmr, Rv1634, Rv1250,* and *Rv0849* showed increased expression under INH and RIF stress [18]. Consistent with these data, in the present study, a significant increase in expression was observed in the Rv1634 gene region, while the Rv0849 gene region from the MFS family was also evaluated and no significant overexpression was found.

The *Rv1634* region is an efflux pump gene belonging to the MFS family that has been tested in many studies. In a study by Narang et al. overexpression was found in six isolates that were determined to be INH resistant [13]. Studies conducted in Türkiye reported that the expression of *Rv1634* region in MDR-TB isolates increased under drug stress [19,20].

Consistent with the studies conducted in the world and in Türkiye, an increase in expression was detected in the *Rv1634* region in our study ($p < 0.005$). For this gene region, no significant difference was observed when the expression levels of INH-resistant and susceptible isolates were compared, while a 2.85-fold increase in expression was observed in resistant isolates compared to controls that were not under drug stress ($p = 0.029$).

In a study conducted with *M. tuberculosis* H37Rv strain, the expression of efflux pump genes was examined after exposure to first-line anti-TB drugs INH, RIF, streptomycin (SM), and ethambutol (EMB) and overexpression of *Rv1877* region, a member of the MFS family, was observed when exposed to ¼ MIC RIF and SM. However, it was reported that INH exposure at subinhibitory concentrations did not induce expression in this gene region [14]. In the present study conducted with 10 INH monoresistant isolates, no significant change was observed in the *Rv1877* region compared to the control group.

Studies investigating the Rv2265 gene region belonging to the MFS efflux pump gene family have reported that it is overexpressed in MDR-TB strains [13,18]. However, in the present study, no significant increase in expression in the *Rv2265* region was detected in INH monoresistant isolates compared to the control group.

Rv2456c region is one of the drug efflux carriers that belongs to MFS family. In one study, its expression was investigated in INH-resistant isolates and no overexpression was detected [13]. Consistent with this study in the literature, no significant change in expression was detected in this gene region in the present study conducted with INH monoresistant strains.

In a study examining the *Rv0507* (*Mmpl2*) region from the RND efflux gene family in MTBC, expression of some efflux pump genes was measured after INH (½, $^1/_3$, and ¼ MIC) exposure at subinhibitory concentrations to investigate its relationship with INH resistance and increased expression was reported in this region at all subinhibitory concentrations [14]. There are studies indicating that the *Rv3823c* region is overexpressed in INH-resistant isolates that do not carry *katG* and *inhA* mutations [13]. In the present study, no significant change was found in the expression levels of *Rv0507, Rv0676c, Rv3823c* regions belonging to the RND efflux family under drug effect.

4.1. Limitations of the study

A limited number of isolates were included in the study due to budget constraints, and mutations causing INH resistance could not be examined. In addition, expression levels could be measured by exposing resistant isolates to only ½ INH MIC value, and expression levels after drug exposure at different subinhibitory concentrations could not be evaluated.

5. Conclusion

The data obtained in the present study suggests that VR may reduce INH resistance. The reduction in INH MICs with VR reflects that resistance may be caused by efflux pumps. The data also showed that VR had no effect on the expression of efflux pump genes we investigated. In the expression of 21 efflux pump regions we investigated, only one region showed a significant increase in expression under INH drug effect. Further studies with more resistant isolates are necessary. There are limited studies on efflux pump genes in MTBC isolates conducted in Türkiye. Therefore, the data obtained in the present study will contribute to the literature.

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Conflicts of interest/Competing interests

None. The article was presented at 6th International Health Science and Life Congress as a online oral presentation on 2nd of March 2023.

Ethics approval

Approval for the study was obtained from Mersin University Rectorate Clinical Research Ethics Committee with the board decision dated September 04, 2019 and numbered 2019/378 (78017789/050.01.04/1152419).

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