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Comparison of toxin-antitoxin expression among drug-susceptible and drug-resistant clinical isolates of *Mycobacterium tuberculosis*

Abstract

Introduction: *Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis (TB), is a significant global public health threat. Besides extensive multidrug resistance, MTB possesses several properties for long-term viability in the host as well as stress adaptation and resistance in harsh conditions. The role of toxin-antitoxin (TA) systems in disseminating and maintaining antimicrobial resistance in bacterial populations has also been demonstrated. This study aimed to evaluate differences in expression of MazEF (a well-known TA system) related genes (*mazE3*, *mazF3*, *mazE6,* and *mazF6*) amongst drug-susceptible and resistant MTB isolates in Iran.

Material and methods: A total of 20 confirmed clinical isolates of MTB including 10 drug-susceptible and 10 drug-resistant (nine MDR, and one XDR) species were included in this study. *M. tuberculosis* H37Rv was used as the standard strain. RNA extraction, cDNA synthesis, and relative quantitative real-time PCR were performed according to the standard procedures.

Results: Our analysis indicated significant enhanced expression of the *mazE6* antitoxin gene in drug-susceptible isolates compared to drug-resistant isolates and the standard strain. The expression of the *mazF6* toxin gene was also increased in drug-susceptible isolates compared with the standard strain. In drug-resistant isolates, the expression levels of *mazF3* and *mazF6* genes were significantly higher than that in the susceptible isolates and the standard strain.

Conclusions: In this study, there was significant overexpression of *mazE6* in drug-susceptible isolates. As well, *mazF3* and *F6* were overexpressed in drug-resistant isolates when compared with the standard strain. The changes in expression levels of MazEF6 associated genes were greater than that of MazEF3 in both groups of isolates.

Key words: *Mycobacterium tuberculosis,* toxin-antitoxin system, MazEF, gene expression

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Introduction

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), is a significant global public health threat [1]. According to World Health Organization (WHO) reports, there are more than 1.5 million TB-related deaths annually despite the extensive efforts to eradicate TB [2]. Multi-drug resistant (MDR) and extensively drug-resistant (XDR) TB are also major challenges in the elimination of TB [3].

MTB possesses several properties for its long-term viability in hosts such as its special cell wall structure and metabolism [4]. Another important strategy for stress adaptation and resistance in harsh conditions (i.e. poor nutrition, oxidative stress, low pH, and hypoxia) is its Toxin-Antitoxin (TA) system [5-8]. The toxin molecule is

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a stable protein and the antitoxin could either be a labile protein or non-coding RNA. During stressful conditions, the antitoxin is degraded and free active toxins interfere with essential cellular functions [5, 9].

MTB strains harbor several TA systems with MazEF being a well-known type [10]. MazEF consists of MazE (antitoxin) and MazF (toxin), and both components create a complex in normal conditions. This system has 10 loci in the MTB genome which are supposed to be effective in adapting to the environment, programmed cell death, cell cycle inhibition, persistence, and latency. MazF3, MazF6, and MazF9 inhibit mycobacterial growth. Also, they are important in expression of virulence factors. Further, MazF6 plays an essential role in cell survival [7, 9].

The role of TA systems in disseminating and maintaining antimicrobial resistance in bacterial populations has been demonstrated [11]. Moreover, previous studies indicate that *mazE* and *mazF* genes are differentially expressed in drug-susceptible and drug-resistant bacteria [12, 13]. This study aimed to evaluate differences in expression of MazEF related genes (*mazE3*, *mazF3*, *mazE6,* and *mazF6*) between drug-susceptible and drug-resistant MTB isolates in Iran.

Material and methods

Bacterial strains

A total of 20 confirmed clinical isolates of MTB including 10 drug-susceptible and 10 drug-resistant (nine MDR, and one XDR) strains were included in this study [14]. Drug susceptibility testing (DST) and detection of resistance-determinant mutations amongst the isolates were also determined in a previous study [14]. *M. tuberculosis* H37Rv was used as the standard strain. This study was approved by the Ethics Committee of Ilam University of Medical Sciences (Register code: IR.MEDILAM.REC.1395.34).

Total RNA extraction, cDNA synthesis and relative quantitative Real-time PCR

Total RNA of mycobacterial colonies was extracted by an RNA extraction kit (Thermo, Dreieich, Germany) for each strain. The RevertAid First Strand cDNA Synthesis Kit (Thermo) was used for cDNA synthesis according to the manufacture's protocol. Relative quantitative real-time PCR (RT-qPCR) was performed using SYBR Green Low ROX Master Mix (Amplicon, Brighton, UK) and specific primers (Table 1) [12, 14]. Reaction mixtures were made to a final volume of 25μL including 12.5μL of 2X SYBR Green master mix, 1μL of template cDNA (50 ng/μL), 0.4 pM of forward/reverse primers, and 9.5μL of ddH_2O in a 0.2 mL PCR microtube. The PCR reactions were done according to the following protocol: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 20 s, 58°C for 20 s, and 72°C for 30 s. To ensure the single amplicon production, melting curve analyses were applied. The heat shock protein 65 gene (*hsp65*) was used as an internal control. The genome of H37Rv standard strain was used as the external control. All tests were performed twice.

Table 1. Sequences of primers used in relative quantitative real-time PCR

Gene	Sequence (5'–3')
mazE3	F: CCAGCGTATCCAGATCACC R: GCGGGTGCATACCAAACT
mazF3	F: TATGACACCACCCAATCG R: ACCTATCCACTACGCACAGC
mazE6	F: TCACCACTCATCGTCCTG R: ATGAAGACAGCTATTTCTCTGCC
mazF6	F: GGTCGGTGAGGTCAGTCTTG R: GGTGATTAGTCGTGCCGAGAT
Hsp65	F: AAGTCGGTGGCGGTCAAG R: GCGTTCTCCAGCGTCAGG

Data and statistical analysis

For gene expression analysis, data were analyzed using the Line-Gene K software on the BIOER detection system using the Livak method (Livak. KJ, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{[-\text{Delta Delta C(T)}]}$ method). SPSS 21 was used for statistical analysis. Data are represented as means ± SEM and differences between groups were analyzed using t-test. $P < 0.05$ was considered as statistically significant.

Results

Our analysis indicated increased expression levels of *mazE3* and *mazE6* antitoxin genes in drug-susceptible isolates. It was negligible for *mazE3* but enhanced expression of *mazE6* was significant compared to drug-resistant isolates and the standard strain H37Rv ($p < 0.01$). In drug-resistant isolates, reduced expression of *mazE3* gene was observed, but *mazE6* exhibited

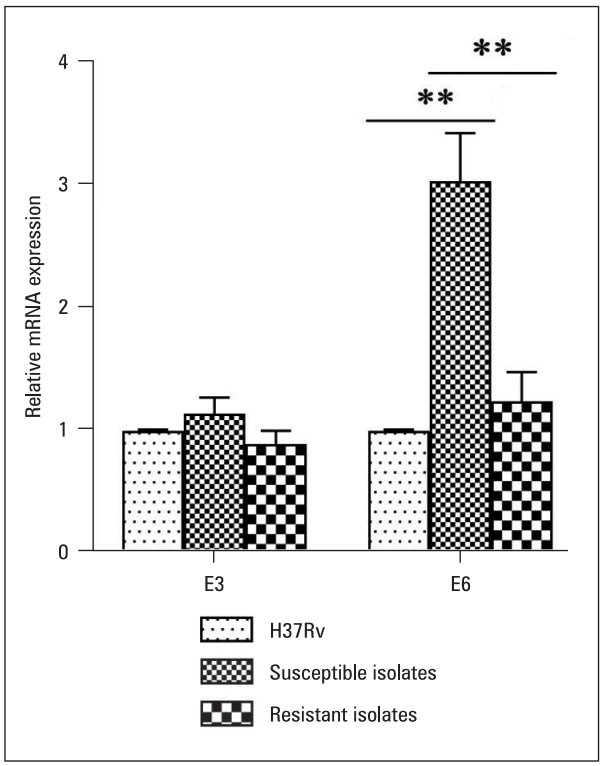

Figure 1. mRNA expression profile of *mazE3* and *mazE6* in drug -susceptible and drug-resistant MTB clinical isolates compared to standard strain H37Rv. **p < 0.01

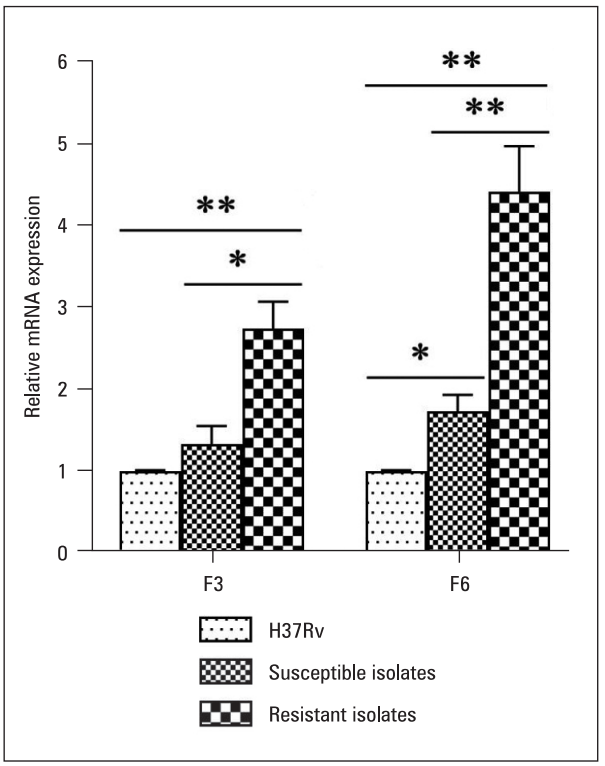

Figure 2. mRNA expression profile of *mazF3* and *mazF6* in drug -susceptible and drug-resistant MTB clinical isolates compared to standard strain H37Rv. *p < 0.05; **p < 0.01

non-significantly increased expression when compared with the standard strain (Figure 1).

Figure 2 shows *mazF3* and *mazF6* expression levels in the clinical isolates compared to the standard strain. The expression of *mazF3* and *mazF6* toxin genes were increased in drug-susceptible and drug-resistant isolates compared with the standard strain. In drug-resistant isolates, the expression levels of both genes were significantly higher than that in the susceptible isolates and the reference strain ($p < 0.05$). The mean ± SEM of the relative expression changes are mentioned in Supplementary Table 1.

Discussion

Drug-resistant TB is still a serious global health problem. Besides extensive multidrug resistance, drug tolerance or persistence are other possible responses to antibiotic treatment of MTB infection [15, 16]. Stabilization in adverse conditions encountered in the host is one of the functions of TA systems and they are important in both the mycobacterial evolution and in the process of infection. MazEF induces a reduction in metabolic activity, persistence, and cell arrest via inhibition of protein synthesis leading to the bacteria being protected from antimicrobial agents [9, 17].

The present study was conducted to investigate *mazE3*, *mazF3*, *mazE6,* and *mazF6* gene expression in drug-susceptible and drug-resistant MTB isolates. We observed no significant increase in the expression level of *mazE3* and *mazF3* genes in susceptible isolates compared with the H37Rv standard strain (Figure 1 and 2). In a study which was conducted by Zhao *et al.*, similar *mazF3* results in susceptible MTB strains were reported [12]. Also, no considerable changes in expression levels of MazEF encoded genes were previously described in susceptible *Staphylococcus aureus* isolates [13].

We found significant enhanced expression levels of *mazE6* and *mazF6* in susceptible isolates when compared with the standard strain. However, overexpression of *mazE6* was higher than *mazF6* (Figure 1 and 2). The *mazF6* upregulation and significant *mazE6* downregulation in drug-susceptible MTB strains was reported in a previous study [12]. Regardless, a higher concentration of antitoxin may justify antibiotic susceptibility amongst our studied isolates. In other words, MazE6 can neutralize MazF6 toxin and prevent its endoribonuclease activity lead-

ing to a normal microorganism metabolism and susceptibility to related antibiotics.

According to our results, no remarkable changes were observed in the expression level of *mazE3* and *mazE6* antitoxin genes in drug-resistant isolates in comparison with the standard strain (Figure 1). However, *mazF3* and *mazF6* were overexpressed significantly when compared to the drug-susceptible isolates and the standard strain. Overexpression of *mazF6* was also higher than *mazF3* (Figure 2). Reduced expression in antitoxin associated genes (*mazE3, E6*) and notable increased expression in both toxin genes (*mazF3, F6*) among drug-resistant strains was shown in previous research [12]. Accumulation of *mazF3* and *mazF6* may be due to various antibiotics and other exposures to stress of drug-resistant isolates. A high existence of MazF3 and MazF6 ribonucleases contributes synergistically to MTB growth inhibition and persistence and mediates resistance to antimicrobial agents [5]. Moreover, these proteins facilitate MTB survival in macrophages, increase resistance to oxidative stress, cause nutrient deprivation, and may cause chronic infection [5, 7, 18].

Conclusion

In this study, upregulation of *mazE6* in drug-susceptible isolates and *mazF3* and *F6* in drug-resistant isolates were observed when compared to the standard strain H37Rv. Expression differences in MazEF6 associated genes were greater than in MazEF3-related genes in both groups of isolates. It seems that the role of MazEF6 in MTB persistence is greater than that of MazEF3. Knowing the role and expression level of the genes encoding TA systems among drug-resistant bacteria may be helpful for the development of novel therapeutic approaches. MazEF associated genes, especially toxin-encoding genes, are a potential target for the treatment of drug resistant and latent TB infections alongside antibiotic therapy.

Limitations

In the present study, investigation of the studied genes (*mazE3, mazF3, mazE6* and *mazF6*) and other MazEF associated genes (*mazE5, mazF5, mazE9, and mazF9)* in various conditions (i.e. presence of several stresses or antibiotics) was required.

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Conflict of interest

The authors declare that there is no conflict of interest.

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