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Original Article

Microbiology Section

Molecular Identification of Mutations Associated with Pyrazinamide-Resistance in Multidrug-Resistant Tuberculosis in Eight Provinces of Iran

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ABSTRACT

Introduction: Multidrug-Resistant Tuberculosis (MDR-TB) is a growing concern which has always played a role in controlling infectious diseases. Pyrazinamide (PZA) is one of the four front line cures of tuberculosis during the first two months of the treatment course. Diagnosis of PZA resistance is imperative in order to optimize the efficacy of new treatment regimens and minimize the risk of developing resistance to new drugs. In addition, high prevalence of PZA resistance, especially among those afflicted with MDR-TB, points to the need for developing those medicinal regimens that can be used in patients with PZA resistant MDR-TB.

Aim: The aim of this study was to determine anti-tuberculosis drug resistance rate and identify the correlation between MDR-TB and of mutations in *pncA* gene among *Mycobacterium tuberculosis* isolates and frequency of mutations associated with PZA resistance in all the isolates.

Materials and Methods: This is a cross-sectional descriptive study conducted from April 2014 to June 2015. A total of 118 *Mycobacterium tuberculosis (M. tuberculosis)* clinical strains were isolates from patients referred to TB reference laboratory of Kermanshah from 8 provinces including: Kermanshah, Lorestan, Hamadan, Ilam, Kurdistan, Ardabil, Uromia and Tabriz. Antimicrobial susceptibility testing was performed using the proportional method and Minimum Inhibitory Concentration (MIC) and mutations in *pncA* for the PZA resistant isolates were studied using monoplex- Polymerase Chain Reaction (PCR) and then PCR products were sent for sequencing.

Results: Among the 118 clinical samples of *M. tuberculosis* investigated in various parts of Iran, 10 isolates (8.5%) were resistant to Isoniazid, 10 isolates (8.5%) were resistant to Rifampin, 7 isolates (6%) were MDR, and 23 isolates (19.5%) were resistant to PZA. Only did one MDR-TB isolate resistant (14.3%) to PZA show inactive mutation at Glu-122 codon that was found in *pncA* gene. According to our results, a significant correlation was found between MDR strains and of mutations in *pncA* gene (pv=0.049). *pncA* gene was not isolated from any of the PZA resistant isolates.

Conclusion: Our findings indicated that only one MDR-TB isolate resistant to PZA showed a mutation in the *pncA* gene (14.3%) and mutations were not observed in the other PZA-resistance isolates. The reason for resistance to PZA in the other PZA-resistance strains might be related to mutations in other genes or to some other factors. Thus, these reasons need to be further investigated in our study population.

Keywords: *Mycobacterium tuberculosis*, *pncA* gene, Polymerase chain reaction

INTRODUCTION

Despite massive efforts, Tuberculosis (TB) is one of the most important diseases that affect humans. In 2015, the World Health Organization (WHO) estimated that one-third of the world's population was infected and reported an outbreak afflicting 9 million people with the total mortality of 1.7 million of them [1].The rise and spread of drug-resistant strains of *M. tuberculosis*, in particular MDR strains, are crucial threats to the control of tuberculosis and constitute an important public health issue. Patients with MDR strains, as a species resistant to both Rifampin (RIF) and Isoniazid (INH), are hard to treat and more presumably stay as sources of infection for a longer duration than do patients with drug-sensitive strains [1-3]. Pyrazinamide (PZA) is the main first-line anti-tuberculosis (anti-TB) drug that is applied in short-period chemotherapy and is one of the main drugs in curing MDR [4]. PZA seems to destroy at least 95% of the half-persistent bacterial number persisting in a low pH setting because its activity is present only in the acidic environment found in active inflammation [5,6]. *PncA* gene encodes the pyrazinamidase enzyme which is an essential step to the activation of PZA. A mutation in the *pncA* gene leads to a decrease in the function of PZase enzyme which is the main resisting mechanism to Pyrazinamide. PZA needs enzyme transformation into its active type, Pyrazinoic acid, by the bacterial Pyrazinamidase (PZase), which is encoded by the 561- nucleotide (nt) *pncA* gene [7,8]. Mutations in the *pnc*A end can stop or reduce PZase activity, which needs to be taken into account as the initial mechanism of PZA resistance in *M. tuberculosis* [9]. However, the diversity level of *pnc*A mutations as it is described at present can be served as a marker when tracing the outbreak or transmission of PZA-resistant *M. tuberculosis* isolates. Interpretation of *pnc*A mutation results for epidemiologic purposes needs to be cautiously done [10]. Specifying the frequency of mutations in resistant isolates of *M. tuberculosis* to PZA is significant in presenting proper treatment of tuberculosis in order to avoid the spread of infection in the society. Thus, the present study was to determine anti-tuberculosis drug resistant rate and identify the correlation between MDR-TB and of mutations in *pncA* gene among 118 *M. tuberculosis* strains and frequency of mutations associated with PZA-resistance in the *M. tuberculosis* Isolates who were referred to the reference TB laboratory of Kermanshah, west of Iran.

MATERIALS AND METHODS

Mycobacterial Isolates

In the present study 135 smear-positive sputum samples were collected from patients suspected of TB from 8 provinces including Kermanshah, Lorestan, Hamadan, Ilam, Kurdistan, Ardabil, Uromia and Tabriz who were referred to the reference center in Kermanshah, from April 2014 to June 2015. Among these 135 samples, only 118 cases had positive culture (Positive culture refers to cases in which at least one colony was grown on Lowenstein-Jensen medium) in LJ medium and thus only these cases were included in the present study. Cases irrespective of age and sex were included. Samples that were culture negative were excluded from the present study. Colony form, growth rate and presence or absence of pigment was recorded and these 118 samples were identified as *M. tuberculosis* based on standard biochemical tests. Among these 118 patients 39 patients were under the age of 50, and 79 of them were aged above 50.

Bacterial culture was accomplished in the Lowenstein-Jensen (LJ) medium at 37^{0}C for 3 to 4 weeks and the isolates were identified by Ziehl-Neelsen method after decontamination by 4% NaOH [11] and biochemical tests such as: niacin production and nitrate and catalase tests [3]. Antimicrobial Susceptibility Testing (AST) was performed using the proportional method following the current recommendations from the World Health Organization (WHO) [12]. The critical concentrations were 0.2 mg/l for INH, 40mg/l for RIF, 2mg/l for Ethambutol (EMB), and 100 mg/l for PZA [13]. Determination of Minimum Inhibitory Concentration (MIC) for PZA in sputum preparation by the Broth Microdilution Method with 7H9 Broth [14]. Freshly grown colonies from LJ medium were transferred to a tube containing phosphate buffer saline to equal the density of 0.5 McFarland standard for use. All isolates were cultured in Middle brook 7H9 supplemented with Albumin-Dextrose-Catalase (ADC) at pH 5.5 containing PZA concentrations ranging from 100 to 800 mg/l. The PZA-susceptible *M. tuberculosis* strain H37Rv (ATCC 27294) was used as control. DNA extraction and PCR amplification chromosomal DNA were extracted from *M. tuberculosis* strains using the Qiagen kit according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany). A 670-bp segment of the *pncA* gene was amplified by using the conditions and the set of primers PF5 -bof 35 cycles of 95°C for 30 seconds for denaturation and 57°C for 30 seconds for annealing and 72°C for 45 seconds [15] elongation was performed with a BioRad thermal cycler PCR C1000 [11]. The expected size of the *pncA* PCR products was about 670 bp [16]. After electrophoresis of PCR products on 1% Agarose gel (Merck Co, Germany) and staining with Ethidium bromide, the DNA bands were visualized by Gel documentation apparatus (BioRad, USA). PCR, sequencing and bioinformatic analyses were performed to identify mutations in *pncA* genes. To determine the *pncA* sequence, PCR products was sent for sequencing. The sequencing was performed using Dye terminator sequencing method (ABI 3730XL DNA analyser apparatus Macrogen Inc., Korea). Sequenced data were edited by using BioEdit software version 7.05.3, and the results were compared with the H37Rv genome. The GenBank accession number for *pncA* gene in this work was KY659393 [17].

STATISTICAL ANALYSIS

All data were analysed by using SPSS version 23.0 for the correlation between gene mutation and the resistance by Chi-square test. Statistical significance was defined as p-value ≤ 0.05.

Ethics

The project was approved by the Ethics Committee and we have received code of Ethics with number IR.KUMS.REC.1394.504.

RESULTS

A total of 118 *M. tuberculosis* clinical strains were exclusively isolated from sputum samples collected from patients who were diagnosed with susceptible or resistant TB reference laboratory of Kermanshah for MDR-TB cases [Table/Fig-1]. 69 (58.4%) of them were male and 49 (41.5%) were female. The average age of patients was 47 years. While 117 of them were newly infected, 1 of them was reinfected. Among 118 studied patients, 10 samples (8.5%) were resistant against isoniazid, 10 samples (8.5%) were resistant against Rifampin, 7 samples (6%) were MDR, and 23 (19.5%) were resistant to PZA [Table/Fig-2]. MIC for four different concentrations is shown in [Table/Fig-3]. Analysis of mutations associated with PZA resistance isolates [Table/Fig-4] showed that only in one isolate was an one-point mutation for codon of Glu-122 in (GAG to GAA) the *pncA* gene. The GenBank accession number for *pncA* gene in this work was KY659393. According to our results, there was no significant relation between resistance to PZA and mutations in *pncA* gene (p-value ≥0.05) and a significant correlation between was found MDR strains and of mutations in *pncA* gene (p=0.049). *pnc*A gene was not isolated from any of the PZA resistant isolates.

DISCUSSION

Tuberculosis, which is caused by *M. tuberculosis*, has long been recognized as a major global calamity. It is recognized as the eighth cause of death worldwide and the second most prevalent fatal infectious disease [18]. Although different kinds of medications have been used to cure tuberculosis for many years, it has continued to remain as a major universal concern. The advent of drug-resistant strains and genotypes has posed serious hurdles in the path to control and treat this disease. Some strains of the bacterium have drawn more attention for the same reason [19]. Multi-drug resistance of various strains of *M. tuberculosis* has turned into one of the most serious challenges in the treatment of tuberculosis [20,21]. Zhang H et al., reported a multiple-drug resistance of 77% in China [22]. In a systematic review study in Iran, the findings of Nasiri MJ et al., showed that 23 percent of new cases of tuberculosis and 65.6 percent of previous cases of the disease treated in the past were resistant to at least one the anti-TB medications [23]. Our findings

Province	Positive sample of MTB	Rifampin resistance	Isoniazid resistance	Pyrazinamide resistance
Ardabil	9 (7.62%)	0	1 (10%)	1 (4.34%)
Tabriz	21 (17.7%)	2 (20%)	2 (20%)	6 (26%)
Urmia	10 (8.47%)	0	0	3 (13.04%)
Ilam	4 (3.38%)	0	0	1 (4.34%)
Kurdistan	27 (22.8%)	0	0	3 (13.04%)
Kermanshah	17 (14.4%)	2 (20%)	1(10%)	2 (8.69%)
Lorestan	19 (16.1%)	0	0	4 (17.39%)
Hamadan	5 (4.2%)	0	0	2 (8.69%)
Others	6 (5%)	6 (60%)	6(60%)	1 (4.34%)
Total	118	10	10	23

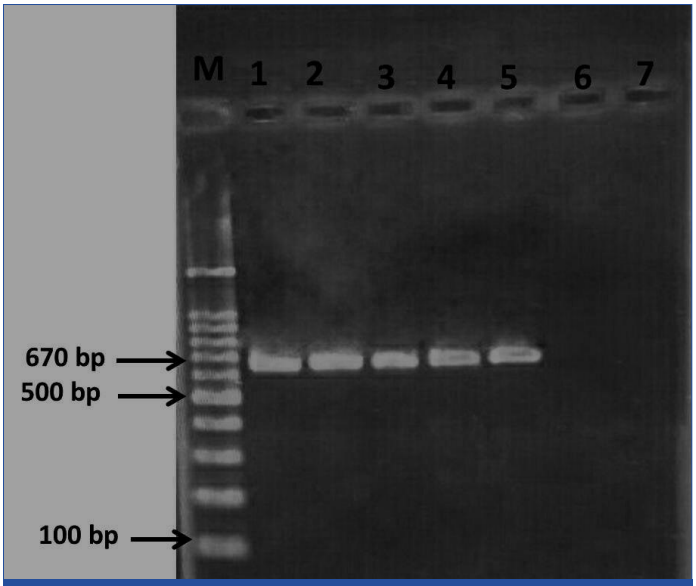
[Table/Fig-1]: Prevalence of MTB drug resistance in this study.

Anti-microbial drug	Resistance; No. (%)	Sensitive; No. (%)	Total
Isoniazid	10 (8.5%)	108 (91.5%)	118 (100%)
Rifampin	10 (8.5%)	108 (91.5%)	118 (100%)
Pyrazinamide	23 (19.5%)	95 (80.5%)	118 (100%)
MDR	7 (6%)	111 (94%)	118 (100%)

[Table/Fig-2]: Results in proportional method.

MIC and Anti-microbial Test	100 µg/ml	200 µg/ml	400 µg/ml	> 800 µg/ml
Pyrazinamide	9 (39.1%)	3 (13%)	4 (17.39%)	7 (30%)

[Table/Fig-3]: MIC of Pyrazinamide resistant.



[Table/Fig-4]: Polymerase chain reaction image of the PCR products (*pncA* gene) on a 1.2% agaros gel. Of right to left: Lane M = 100-bp ladder DNA size marker; lane 1-5 = *M. tuberculosis* clinical isolates (positive sample for *pncA* gene); lane 6 and 7, negative control.

suggest that out of the 118 cases of the study, 7 cases (6%) of the isolates possessed multi-drug resistance. Similar studies reported the following percentages of 7 multi-drug resistance among the isolates: 3.4% in Golestan, 2.9% in Tabriz and 15% in Tehran Province [24-26]. PZA is considered as one of the four first line drugs for curing TB during the first two months of the treatment course [27]. Today, a high prevalence of resistance to PZA has been reported worldwide. Various studies have deduced that different mechanisms play a part in the development of PZA resistance [28]. In our study, 23 isolates (19.5%) out of 118 samples were PZA resistant, whereas the findings of Xia Q et al., in China, Maslov DA et al., in Russia and Jonmalung J et al., in Thiland indicated that 43.7%, 74.3% and 49% of the isolates were PZA resistant, respectively [15,29,30]. However, Sreevatsan S et al., pointed out that 72% of the PZA resistant isolates in their study possessed a mutation in the *pncA* gene [31]. In addition, the findings of Cuevas-Córdoba B et al., in Mexico, Jnawali HN et al., in Korea, Zhao L-I et al., in China, and Perdigao J et al., in Portugal showed 81%, 87.8%, 11.5%, and 25% of the PZA resistant isolates revealed mutations in the *pncA* gene, respectively [32-35]. According to our findings, out of the 23 PZA resistant cases and 7 MDR isolates, a mutation in *pncA* gene was observed only in one case (14.3%). The results of Doustdar F et al., study on *M. tuberculosis* isolates resistant to PZA in Tehran showed that in 8 PZA resistant isolates with negative PZase activity, there was no mutation in the *pncA* gene [28]. In a study by Akhmetova A et al., in Kazakhstan, out of 36 pyrazinamide-sensitive cases (46.7%), two mutations (5.5%) in the *pnc*A were detected [36]. The results of Li H et al., in China also showed that there were mutations in the *pnc*A gene in three pyrazinamide-sensitive mycobacterium cases [37]. Sreevatsan S et al., indicated that in 28% of pyrazinamide-resistant isolates there was no mutation in the *pnc*A gene [31]. However, the results of other studies have also shown that the *rps*A gene, which encodes the S1 ribosomal protein and is a vital protein involved in the transfer of protein and ribosome secretion, can cause pyrazinamide resistance as well. Alanine deletion in the C-terminal of *rps*A gene leads to dramatic increase in PZA resistance [38]. In a similar study by Xia Q et al., on 118 pyrazinamide-resistant mycobacteria and 161 sensitive cases, 92 resistant and 5 sensitive cases in the *pnc*A gene and also 5 pyrazinamide-resistant and 6 sensitive cases in the *rps*A gene exhibited mutations [15]. Cui Z et al., studied 423 cases of *M. tuberculosis* in China and found that in four pyrazinamide-resistant cases with no change in the *pnc*A gene, there was deleting mutation in the *rps*A gene [39]. The findings of Zhang S et al., showed that out of 174 pyrazinamide-resistant mycobacterium cases, in 5(3%) of them no mutations in the *pnc*A and *rps*A genes were observed. It also revealed that mutation in the *pan*D gene was closely related to pyrazinamide resistance. This gene is encoded by the aspartate decarboxylase enzyme [40]. The results of sequencing 30 cases of pyrazinamide-resistant mycobacterium by Shi W et al., disclosed no mutations in the *pnc*A and *rps*A genes while 24 cases (80%) revealed mutations in the *pan*D gene [38]. Two more mechanisms recently proposed for pyrazinamide resistance include efflux pumps and a flaw in the absorption of pyrazinamide by the organism [41]. Zimic M et al., showed that changes in the amount of bacterial Pyrazinoic acid (POA) exit (POA output) affects PZA resistance. These changes might depend on the levels of PZAse activity, PZAse intracellular concentration, and the efficiency of POA exit pump [41]. Although different results have indicated that PZA resistant mutation in the *pnc*A gene are not only highly variable and disperse throughout the gene but various levels of PZA resistance might be observed. The results of our study and similar ones in Iran, however, showed that the frequency of mutations related to PZA resistance in the *pnc*A gene among *M. tuberculosis* cases is very rare in the west of Iran. As mentioned, numerous mechanisms play a role in pyrazinamide resistance. Given the widespread development of *M. tuberculosis* and highly dynamic mutations in the *pnc*A gene related to PZA resistance, it is expected that newer mutations in the *pnc*A gene and the effects of other genes on pyrazinamide resistance among clinical cases of *M. tuberculosis* in the west of Iran be observed in the future.

LIMITATION

Our study has several limitations; one limitation of our study is that a small number of MDR isolates were observed in our samples. Therefore, the possibility of resistance to PZA and the presence of mutation in the *pncA* gene has also been low, leading to a decrease in the prevalence of resistance to PZA due to mutations in the *pncA* gene in our study. Another limitation of our study concerns investigating mutations only in the *pncA* gene, ignoring resistance caused by mutations in the other genes if mutations in the other genes had also been studied. Despite these limitations, our study is important because it demonstrates the problem of resistance to PZA in eight provinces of Iran and treated for PZA-resistance *M. tuberculosis* strains.

CONCLUSION

Our findings indicated that only one MDR-TB isolate resistant to PZA showed a mutation in the *pncA* gene (14.3%) and mutations were not observed in the other PZA-resistance isolates. The reason for resistant to PZA in the other PZA-resistance strains might be related to mutations in other genes or to some other factors in our study population. Therefore, there is a higher possibility for mutations to occur in the MDR strains of *M. tuberculosis*. Our results illustrate the need for further research to investigate a more resistant gene association with PZA resistance in PZA-resistance *M. tuberculosis* strains.

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