



Molecular identification of multiple drug resistance (MDR) strain of *Mycobacterium tuberculosis*

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Abstract

Background and Objectives Isoniazid and rifampin are the first-line drugs against *Mycobacterium tuberculosis*. Resistance to these important drugs is a serious threat to human public health. Therefore, this study aimed at molecular detection of resistance to these valuable drugs.

Materials and methods In this descriptive cross-sectional study, 111 non-duplicated clinical samples including sputum and Bronchoalveolar lavage (BAL) samples were collected from patients referred to the Ardabil Health Center between 2017 and 2020. The samples were first examined by microscopic method, then their DNA was extracted using the boiling method. Specific primers and MAS-PCR method were employed for the detection resistance to isoniazid and rifampin drugs and identification of MDR strain.

Results of 111 specimens, 15.3% belonged to NTM. In total, the resistance rate to isoniazid and rifampin was 17% and 27% respectively while the resistance rate to isoniazid and rifampin among NTM was 61.54% and 38.46%.

Conclusion In our study, the prevalence of resistance to isoniazid and rifampin among *Mycobacterium tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM) was investigated using the MAS-PCR method. This work highlighted the high anti-tuberculosis resistance rate among NTM compared to MTC strains.

Keywords *Mycobacterium tuberculosis* · PCR · Isoniazid · Rifampin

Abbreviations

DR-TB	Drug-resistant tuberculosis
inhA-RR	InhA-regulatory region
katG315	KatG codon 315
MDR-TB	Multidrug-resistant tuberculosis
XDR-TB	Extensively drug-resistant tuberculosis
WHO	World Health Organization

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Introduction

Mycobacterium tuberculosis (MTB) is an acid-fast bacilli, the causative agent of tuberculosis. Tuberculosis is a chronic and contagious airborne disease that primarily involves the lungs but can affect any part of the body, for this, known as a thousand faces Disease [1]. The increase of MDR, XDR and recently TDR strains, HIV-TB coinfection and the emergence of the new deadly infectious agent, COVID-19, has made it seriously difficult to deal with this bacterium. The first report of resistance against mycobacterial drug

was related to streptomycin, soon after it's introduced in the late 1940s. Initially, it was effective but immediately after wide usage, drug resistance emerged. From that time, multiple drug therapy urgently was recommended to encounter tuberculosis and reduce drug resistance. According to WHO guidelines, Types of drug-resistant TB have been defined including Mono-resistance(resistance to only one first-line drug), poly-resistance(resistance to more than one first-line drug except for isoniazid and rifampicin), Multidrug resistance (MDR)(resistance to both isoniazid and rifampicin), Extensive drug resistance (XDR)(MDR strain that becomes resistant to any fluoroquinolone and at least one injectable anti-TB drug) and Rifampicin resistance (RR)(only resistance to Rifampicin in any form of mono, poly-resistance, MDR or XDR) [2–4]. Several risk factors are reported that directly increase the risk of drug resistance and the development of MDR-TB strains. Inappropriate therapy, patients' compliance, immunosuppressive underlying diseases like HIV and pharmacokinetic differences among Individuals. The development of MDR-TB reflects poor treatment and poor infection control in healthcare settings. Given the socioeconomic burden of MDR-TB strains, timely detection of these strains and adequate treatment of involved individuals are critical in managing the tuberculosis infection in endemic regions [5, 6]. Afghanistan [7], Pakistan [8] and Azerbaijan [9] are a regions with high prevalence of MDR-TB strains therefore continued monitoring of resistance

strains, early detection and appropriate management of the infection would be an effective way to reduce the MDR-TB born infection in IRAN. In this regard, the present study was designed for molecular identification of MDR-TB strains in Ardabil province, northwest of IRAN located in a neighborhood of Azerbaijan County.

Materials and methods

Patients's specimens

In this descriptive, cross-sectional study, a total of 111 non-duplicated sputum and Broncho alveolar lavage (BAL) specimens were obtained from patients admitted to Ardabil Health Center following verbal consent from 2017 to 2020 in Ardabil, Iran. Before sampling, informed consent was obtained from each patient. Inclusion criteria were suspected TB patients with positive smears stained with the Ziehl-Neelsen method while patients with negative smears were excluded.

Molecular characterization of resistance mutations

Whole genomic DNA was extracted from each specimen using the boiling method as described previously [10, 11]. The quality and concentration of extracted DNA were confirmed by Nano Drop2000 (Thermo Scientific,USA). Conventional and multiplex allele-specific polymerase chain reaction (MAS-PCR) were used for the detection of IS6110 and specific mutations in *rpoB*, *inhA*, *katG* genes respectively [12]. Master mix was prepared in a total volume of 25 μ L, containing 0.5 μ g of whole genomic DNA, 10 pmol of each primer, 10xbuffer, 0.2mM of each of dNTP, Taq DNA polymerase 1 IU (parsetous,Iran), 1.5mM MgCl₂. The amplification reactions PCR was performed in an Eppendorf thermal cycler (Germany), using the following cycling condition: initial denaturation at 95 °C for 3 min, 94 °C for 40 s, annealing temperature as presented in Table 1 for 60 s, 72 °C for 60 s(35cycles) and 72 °C for 5 min. BCG genome was used as a control. PCR products were run on agarose gel (1%) and stained with the green viewer (parstus, Iran). Stained PCR products visualized with Gel-Doc System (Bio-Rad, USA).

Statistical analysis

Pearson's Chi-square test and significance levels <0.05 were used for statistical analysis and comparing variables (SPSS software v.22).

Table 1 Sequences of primers was used for the amplification of various gene targets and their amplicon size

Primer name	Amplification target gene	Annealing target (°C)	Oligonucleotide sequence	Amplicon size (bp)
IS6110-F	IS6110	62	5'-CTCGTC-CAGCGC-CGCTTCGG-3'	130
IS6110-R			5'-CCTGCGAGCG-TAGGCGTCGG-3'	
RIRm	<i>rpoB</i>		5'-TTGACCCGCGC-GTACAC-3	
<i>rpoB</i> 516			5'-CAGCTGAGC-CAATTCATGGA-3	280
<i>rpoB</i> 531		66	5'-CACAAGCGCC-GACTGTGTC-3	170
<i>rpoB</i> 526			5'-CTGTCTGGGGTT-GACCCA-3	185
<i>KatG</i> -F	<i>katG</i>	58	5'-ATACGACCTC-GATGCCGC-3	292
<i>KatG</i> -R			5'-GCA-GATGGGGCT-GATCTACG-3	
<i>InhA</i> -F	<i>inhA</i>	58	5'-GCGCGGT-CAGTTCCACA-3	270
<i>inhA</i> -R			5'-CACCCCGA-CAACCTATCG-3	

Results

During 2017–2020, 111 specimens were collected from patients suspected of having tuberculosis. In the demographic survey of patients, was found to be most patients were men ($n=73$, 65.76) and in the age range of 50–40 years old ($n=30$, 27.03). Before DNA extraction, a direct smear from each sample was prepared and stained with the Ziehl-Neelsen staining method. In the microscopic examination, bacterial load was calculated as +3 and over ($n=67$, 60.36%), +2 ($n=27$, 24.32%) and 1+ ($n=17$, 15.32%) [13, 14]. The presence and absence of the IS6110 gene is a molecular marker for the identification of mycobacterium tuberculosis complex (MTC) and nontuberculous mycobacteria (NTM) respectively. Our finding indicated that 17 (15.3%) of the examined specimens belonged to the NTM group. Resistance to rifampin is related to a mutation in *rpo* gene. Mutations in codons 516, 531 and 526 are the most common mutation which is associated with rifampin resistance among mycobacterial strains. In the present, study the frequent spot mutation in *rpoB526*, *rpoB516* and *rpoB531* codons were, ($n=16$, 14.41%), ($n=14$, 12.61%) and ($n=9$, 8.1%) respectively, of which 5, 5, 1 specimens were found to be infected with NTM strains which harbor respective mutated allele respectively. Simultaneous dual and triple mutations in 516, 531 and 526 codons were observed only in NTM strains. As reported previously, spot mutations in *inhA* and *KatG* genes are strongly associated with resistance to isoniazid antibiotic. Our finding revealed that 19 (17%) of the infected specimens had spot mutation in *inhA* or *KatG* which leading to isoniazid resistance [15]. Overallly, it is estimated that 27% of patients are infected with the MDR strain.

Discussion

Despite many attempts over the past decades, to provide effective drugs, vaccines and surveillance systems, tuberculosis (TB) remains a serious public health problem with high mortality and morbidity worldwide. Stop TB strategy is a WHO 's program, under this strategy, the incidence rate of the disease will drop by 80% by 2035 worldwide [16, 17]. A world free of tuberculosis is a global goal to end the new cases of disease, suffering and death due to tuberculosis. To achieve this goal monitoring, prevention, control and improve the outcomes of treatment of drug resistance tuberculosis (DR-TB) patients play critical roles [17].

The precise prevalence of drug strain of MTB remains ambiguous due to limited access to mycobacterial standard culture and drug susceptibility testing, however, it is estimated that 4.1% of all new tuberculosis cases and

19% percent of previously treated cases are multidrug-resistant [18, 19]. MDR strain is defined as resistance to potent first-line anti-tuberculosis treatment including rifampin and isoniazid. Compared to second and third-line drugs, first-line drugs have the least side effects and the greatest effect on tuberculosis. The treatment of tuberculosis caused by MDR strains is very difficult and expensive, and the mortality rate caused by these strains is higher than sensitive strains. Long-term treatment with anti-tuberculosis agents and use of inappropriate dosage, will lead to the development of drug resistance in tuberculosis bacteria [4].

A study in Iran has shown that the specific treatment of MDR strains can be accompanied by neurological complications, hearing toxicity, hepatitis, skin rashes and kidney toxicity [20]. According to WHO statistics, the prevalence of detected drug resistance between 2019 and 2020 was around 22%. Statistics have also shown that 2 out of every 3 people with MDR strains did not have access to proper treatment. Control and elimination of tuberculosis occur when new cases of infection are quickly diagnosed and treated effectively. Over the past decades, several molecular methods have been developed to detect drug resistance, such as Xpert® MTB/RIF, Ultra, MTB-XDR, BDMax, Line probe assay (LPA), Loop-mediated isothermal amplification (LAMP) and Whole genome sequencing (WGS). All these methods are different from each other in terms of cost, sensitivity, turnaround time, expertise of the operator, and the type of mutation they detect. However, the high cost, lack of access in many endemic areas and the need for high expertise to perform and interpret the results have limited their use. In comparison, MAS-PCR is a simple, cost-effective and time-consuming method for verifying drug resistance [4, 21]. According to a study in Iran, the prevalence of MDR stain 2013–2020 is estimated at 6.3% which is not compatible with our findings [22].

In a study in northwest Iran, 32 Azerbaijani patients and 48 Iranian patients were examined in terms of anti-tuberculosis susceptibility patterns. This study revealed that among Azerbaijani patients the resistance rate to Isoniazid, Rifampin and ethambutol are 72%, 69% and 56% while among Iranian patients were 4%, 8% and 4%. Since there is a lot of movement and community among Azerbaijani to Iran, it is very necessary to identify infected individuals to prevent the spread of infection [23]. Finally, in our study the resistance rate to Isoniazid, Rifampin and the prevalence of MDR strain among patients from northwest Iran were investigated, also, the rate of infectivity with NTM strains and molecular drug susceptibility among them was highlighted.

Conclusion

Understanding the epidemiology of MDR strains would be critical in the effective treatment and control of infection. Considering the neighboring of Ardabil province with Azerbaijan, the region with a high rate of tuberculosis infection, identifying infected passengers and quarantining them is very important. Given that a considerable antibiotic resistance was detected among NTM strains, identifying these strains would be critical in providing effective and functional treatment.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11033-023-08867-7>.

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Authors' contributions Roghayeh teimourpour was involved in the study design. Mahin Sadeghnezhad and Shabnam Sohrabi were implicated in sample preparation. Zahra Hosseiali, Majid Esmaelizad and Hafez Mirzanejad-Asl performed all experiments. Jafar Mohammadshahi, Mohsen Arzanlou and Amir Teimourpour were assigned to manuscript writing. All authors read and approved the final manuscript.

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Data availability All data obtained during this study are included in the manuscript.

Declarations

Ethics approval and consent to participate This study was approved by the Institutional Ethics Committee of the Ardabil University of Medical Sciences (IR.ARUMS.REC.1400.201). Written consent was obtained from each patient before sampling. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication All authors expressed their consent for publication of the current manuscript.

Competing interests All authors declare to have no competing of interests.

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