


RESEARCH ARTICLE



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Linezolid resistance among multidrug-resistant *Mycobacterium tuberculosis* clinical isolates in Iran

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ABSTRACT

The management of multidrug-resistant (MDR) and extensively drug-resistant tuberculosis (XDR-TB) presents a main challenge and the drug options for treating these infections are very limited. Linezolid (LNZ) has recently been approved for the treatment of MDR and XDR-TB. But, there are narrow data on genotypic and phenotypic LNZ resistance in clinical isolates. So, we aimed to determine the prevalence of LNZ resistance and to identify the mutations associated with LNZ resistance among clinical MDR-TB isolates. The minimum inhibitory concentration (MIC) values of LNZ for 22 MDR-TB isolates were determined by broth microdilution method. All MDR-TB isolates were sequenced in the *rrl* and *rplC* genes conferring LNZ resistance. LNZ resistance was found in 3 (13.6%) of 22 MDR-TB isolates. The MICs of LNZ were 8 µg/mL for two isolates and 16 µg/mL for one isolate. The 421 (A/G) and 449 (T/A) mutations in *rplC* gene were detected in one of the LNZ-resistant isolates. There was no mutation in *rrl* gene. The results reveal that the prevalence of LNZ-resistant isolates is 13.6% among MDR-TB isolates and drug susceptibility testing (DST) against LNZ is useful in the management of complicated and drug-resistant cases. However, further studies could identify other possible genetic mechanism of resistance in TB.

KEYWORDS

Mycobacterium tuberculosis, multidrug-resistance, linezolid, mutation, minimum inhibitory concentration

INTRODUCTION

Drug-resistant tuberculosis (TB) has a major impact on health outcomes in high-burden countries and is predicted to increase during the next two decades [1]. The latest report of WHO showed around 3.4% of new TB cases and 18% of previously treated cases were multidrug-resistant (MDR), and the average proportion of MDR-TB cases with extensively drug-resistant TB (XDR-TB) was 6.2% [2]. Management of MDR and XDR-TB is still a main challenge from both a clinical and public health perspective, needs more expensive drugs with higher toxicity, and takes longer compared with drug-susceptible TB [3, 4].

Over the last decade, MDR treatment success rates have remained static at around 50% so, the international tuberculosis community has recognized that new regimens and drugs with improved efficacy are urgently needed to enhance cure rates [5]. Since 2006, WHO has recommended using linezolid (LNZ) in the treatment of MDR/XDR-TB and this drug now being included in many TB programs around the world [6, 7].

LNZ has been considered as the first oxazolidinone that was developed and approved for clinical use. It has a bacteriostatic activity that acts on the 50S ribosomal subunit, specifically, the peptidyl-transferase center, by blocking the binding of tRNA and thus, inhibiting bacterial cell growth [8–10]. LNZ has exhibited an excellent anti-mycobacterial effect and several studies have shown that it is an effective third-line antibiotic for the treatment of the MDR- and XDR-TB patients, with favorable outcomes [11–13]. It is now classified as group A, the preferred drug group for MDR-TB and rifampin-resistant TB treatment that plays a critical role in the treatment of drug-resistant TB [7, 14].

Although LNZ resistance is rare, it has been reported in multiple countries [9]. In Iran, LNZ is used mainly to treat infections caused by vancomycin-resistant *Enterococcus* species and methicillin-resistant *Staphylococcus aureus*, and the first occurrence of LNZ-resistant among MDR-TB clinical isolates in Iran was described by Kazemian et al. [11]. LNZ resistance is related to a mutation at nucleotide position 2061 in *rrl* (23S rRNA) LNZ peptidyl transferase center (PTC) binding site and the T460C mutation in *rplC* (L3 protein), primarily [3]. But, there are limited data on the association between phenotypic and genotypic LNZ resistance in clinical isolates [1]. Also, drug susceptibility tests, including rapid molecular techniques, are necessary for faultless treatment and diagnosis. So, we aimed to determine the prevalence of LNZ resistance and to identify the mutations associated with LNZ resistance among MDR-TB clinical isolates.

MATERIALS AND METHODS

Bacterial strains

This research was approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.REC.1397.792).

Twenty-two MDR-TB clinical isolates were collected from Ahvaz Regional TB Laboratory, Southwest of Iran in 2020. All of the isolates were isolated from clinical specimens by standard microbiological procedures and determined by the proportion method.

Drug susceptibility testing

To confirm the collected TB isolates as MDR-TB, drug susceptibility testing (DST) was done by the proportional method according to the WHO guidelines [15], the test was performed on Lowenstein–Jensen medium with the following antibiotic concentrations: RIF (40 µg, mL^{−1}) and

INH (0.2 µg, mL^{−1}). Then MDR-TB isolates were subjected to this study.

Minimum inhibitory concentration determination

To determine the minimum inhibitory concentration (MIC) of LNZ against MDR-TB isolates, a range of concentrations, from 0.125 to 16 mg/L was prepared for LNZ using serial two-fold dilutions. Each 2 × antibiotic working solution (50 µL) was added to a microtiter plate well, except for the growth control well. MDR-TB clinical isolates were grown on Lowenstein–Jensen medium. A suspension with a turbidity of 1 McFarland standard ($\sim 5 \times 10^7$ colony-forming units [CFU]/mL) was prepared in phosphate-buffered saline (PBS) and was subsequently diluted 1:100 in 7H9 broth medium then, 50 µL was added to each well containing the 2 × antibiotic working solutions. The inoculum was added to all wells except the negative control well (including the growth control wells), which was inoculated with 50 µL of 2 × 7H9 broth medium then, all plates were incubated at 37 °C for 2 weeks. Quality control was done under similar conditions to those of the experiment using H37Rv ATCC 27294. the MIC was considered as the lowest concentration of the LNZ that prevented visible growth of mycobacteria. Phenotypic resistance was defined by an MIC > 0.5 mg/L, the recognized critical concentration for LNZ [3].

PCR and sequencing

DNA extraction was conducted on all MDR isolates using DNA isolation kit (Bio Basic, Canada). The primers used for polymerase chain reaction (PCR) and sequencing of genes related to LNZ resistance, *rrl* and *rplC* were designed in this study and are listed in Table 1. Primers were designed for each target gene using NCBI Primer-BLAST [16].

PCR was done under the following conditions: 5 min at 94 °C followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s, 72 °C for 1 min, with a final extension at 72 °C for 7 min [3]. 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 *Mycobacterium tuberculosis* strain H37Rv.

RESULTS

The LNZ MICs distribution is presented in Table 2. Also, the MIC values corresponding with phenotypic resistance and mutations are shown in Table 3. Based on the critical concentration (>0.5 mg/L) used for LNZ 3 isolates (13.6%) of the tested isolates were resistant that MIC concentration were 8 mg/L and >16 mg/L for two and one isolates, respectively. Eighteen isolates (87.4%) had the MICs range from 0.125 to 0.5 mg/L and were susceptible to LNZ. The MIC for the H37Rv strain was 0.25 mg/L.



Table 1. Primers used for PCR and sequencing

Gene	Gene function	Sequence	Size (base pair)
<i>rrl</i>	23S rRNA gene	F1: GTAAGTGTCTAAGGGCGCAT	1000
		R1: ATCCAGTAGCTCTACCTCCG	
		F2: TAAGCTCCGTACGTCGAAAAG	1041
		R2: AAATTCTCCGCTTCACCCTT	
		F3: AACTGTCTCAACCATAGACTCG	893
<i>rplC</i>	50S ribosomal protein L3	R3: TTAGCACCAGTTCCCTACAC	
		F: CTGGGTATGACGCAGGTATT	621
		R: CTTCTCACCTCGTTTGATCG	

Table 2. MIC distribution of linezolid among MDR isolates

MIC	0.125	0.25	0.5	1	2	4	8	>16
MDR-TB Isolates (%)	5(23)	13(59)	1(4.5)	0	0	0	2(9)	1(4.5)

MIC: minimum inhibitory concentration, MDR: multi drug resistant.

Table 3. MIC and mutations identified in linezolid resistant isolates

Isolate	Linezolid MIC (mg/L)	<i>rrl</i> mutation	<i>rplC</i> mutation
MDR-1	8	–	–
MDR-2	8	–	–
MDR-3	>16	–	421 (A/G), 449 (T/A)

MIC: minimum inhibitory concentration.

The sequencing data of the *rplC* and *rrl* genes were compared with the standard sequences of H37Rv. The genetic analysis of the MDR isolates illustrated just 1 of the 3 LNZ resistant MDR-isolates sequenced carried substitution mutations at nucleotide 421 (A/G) and 449 (T/A) compared to the reference strain and susceptible isolates, resulting in amino acid exchange from valine to isoleucine at codon 141 and isoleucine to asparagine at codon 150, respectively. However, no resistance-related mutations were indicated in isolates with MICs below or at the critical concentration. None of the isolates harbored mutation in *rrl* gene. Furthermore, there was no mutation in *rplC* and *rrl* genes among LNZ susceptible isolates.

The nucleotide sequences obtained in the present study have been deposited in the GeneBank database under the MT429771, MT429770, MT429772 (*rplC*), MT431515, MT431516, and MT431517 (*rrl*) accession numbers.

DISCUSSION AND CONCLUSION

The management of MDR and XDR-TB presents a main challenge and the drug options for treating these infections are very limited. Although, several studies demonstrated the usefulness of LNZ in the treatment of MDR-TB, its mechanisms of resistance are not well determined. In the present study we evaluated the susceptibilities of MDR-TB clinical isolates to LNZ, to our knowledge, this is the first study in Iran that analyzed the LNZ susceptibility of MDR-TB isolates with possible mutations conferring resistance. In the

current study, the majority of MDR isolates (86.4%) were susceptible to LNZ and exhibited an MIC of ≤ 0.5 mg/L but 13.6% LNZ resistance with MICs of ≥ 16 mg/L ($\text{MIC} \geq 1$) is reported here. This finding is consistent with Zhang et al. [17] study that indicated 10.8% LNZ resistance among MDR-TB isolates circulating in China and a study by Kazemian et al. [11] in Iran that activity of LNZ against 39 MDR isolates and a single XDR was evaluated and 10% LNZ resistant MDR-TB was reported which all isolates had MICs > 16 mg/L. It seems that there is a trend towards increasing percent of LNZ resistance among MDR isolates.

Previous studies have been reported that the G2061T, G2576T, C2848A, A2810T, G2270C, and G2270T mutations in the *rrl* gene are associated with LNZ resistance or reduced susceptibility to LNZ [18, 19]. As well as, Beckert et al. [10] and Zhang et al. [19] identified T460C as a dominant mutation in *rplC* in LNZ-resistant TB isolates which this mutation was associated with higher LNZ MIC values (> 2 mg/L). The genetic analysis of this research revealed, although no nucleotide mutation in *rplC* and *rrl* was detected among susceptible isolates, two mutations were found in *rplC* genes (421 (A/G), 449 (T/A)) in one LNZ-resistant isolate. To our knowledge, these positions of nucleotide mutation are in proximity to the T460C mutation position in the L3 protein that extends into the LNZ binding site [20] and were different from those reported before and are the first report in clinical isolates. These mutations in *rplC* were associated with higher LNZ MIC values (≥ 16 mg/L). However, since two (2/3) LNZ-resistant isolates showed no mutations in these genes, the mechanism of resistance in these isolates has remained unknown. In previous studies, Yang et al. [3] reported 0.2% (one isolates) and Richter et al. [21] found 1.9% (4 isolates) LNZ resistant MDR, however, they did not detect specific mutations associated with resistance to LNZ in *rrl*, *rplC* and 23S rRNA, the *rplV*, *rplD* genes, respectively.

The emergence of resistant isolates without mutations allows us to assume other potential mechanisms of LNZ resistance, such as other non-ribosomal alterations or the possible role of efflux pumps as has been shown in *Mycobacterium smegmatis* mutants [22–24]. Sander et al. [23]

proposed a non-ribosomal resistance mechanism for strains that showed wild-type growth characteristics, unaltered peptidyl transferase activity, and had MICs of 4–8 µg/mL. Escribano et al. [22] indicated that the efflux pump systems are also involved in the decrease in the LNZ susceptibility of *M. tuberculosis*. The low permeability of the mycobacterial cell wall, with its unusual structure, is another major factor conferring drug resistance. Also, the cell wall thickness is considered a useful indicator for cell wall permeability [17]. Efflux pumps and other natural mechanisms always result in the emergence of low-level resistance, in contrast, high-level resistance is associated with mutations in the target genes. The development of high-level resistant isolates without mutations indicates that non-ribosomal mutation mechanisms may be conferred LNZ resistance in TB isolates.

In conclusion, this research reveals that the prevalence of LNZ-resistant isolates is 13.6% among MDR-TB isolates and DST against LNZ may be useful in the management of complicated and drug-resistant cases. However, further studies on ribosomal and non-ribosomal mutations in LNZ-resistant isolates as well as analysis of efflux pumps, could reveal possible mechanism of resistance in TB. The limitation of this study is that since Iran has a low prevalence of MDR-TB, we enrolled 22 isolates, more LNZ-resistant isolates should be analyzed to evaluate the prevalence of mutations associated with LNZ resistance.

Conflicts of interest: The authors report no conflicts of interest in this study.

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