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Clofazimine and Linezolid Resistance in Multidrug-Resistant Tuberculosis: Insights from a Single-Centre Study in Iran

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Abstract

Background: Detection of drug resistance in multidrug-resistant tuberculosis (MDR-TB) is essential for effective treatment. This retrospective cross-sectional study compared a World Health Organization (WHO)-recommended proportional method with molecular techniques, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and real-time polymerase chain reaction (RT-PCR), to assess clofazimine and linezolid resistance in MDR-TB isolates collected between 2024 and 2025 at a national referral center in Tehran, Iran.

Objectives: By evaluating phenotypic and genotypic approaches, we aimed to describe resistance patterns and genetic diversity, as well as the value of molecular diagnostics in MDR-TB management.

Methods: The study included consecutive clinical and laboratory-confirmed MDR-TB isolates from patients with confirmed first-line drug resistance based on symptoms, specialist examinations, and radiologic findings. Molecular identification used IS6110-based PCR and hsp65 spacer region PCR-RFLP. Drug susceptibility testing (DST) followed the WHO proportional method on Lowenstein-Jensen and Middlebrook media as the phenotypic reference standard. Primers targeted resistance genes *rv0678* (clofazimine) and *rrl* and *rplC* (linezolid), analyzed by PCR-RFLP and RT-PCR; isolates with indeterminate or missing results were excluded.

Results: Mutations were detected in the *rv0678* gene (17.2%) and in the *rrl* or *rplC* genes (17.2%), associated with resistance to clofazimine and linezolid, respectively. The PCR-RFLP of *rv0678* (720 bp) with NlaIII enzyme showed three distinct patterns: Eighty percent had 80/120/520 bp, 6.7% had 80/120/160/520 bp, and 10% had 80/120/190/520 bp. BsrI enzyme produced uniform 300/400 bp patterns. For *rrl* (860 bp), BsrI showed consistent 105/140/250/260 bp patterns; BbvI revealed 93.3% had a 60/190/290/320/510/530/780 bp pattern, 6.7% lacked the 190 bp fragment. HaeIII enzyme produced 100/770/780 bp in 93.3% and 100/780 bp in 6.7%. The *rplC* gene (400 bp) was highly conserved. BbvI enzyme produced a 75/100/290 bp pattern in 93.3% and a 70/100/290 bp pattern in 6.7%. Despite mutations, only one isolate showed phenotypic resistance to both clofazimine and linezolid, indicating complex resistance mechanisms; no significant genotype-phenotype correlation was observed.

Conclusions: Many MDR-TB isolates carried mutations in genes linked to clofazimine and linezolid resistance, but these did not consistently translate into phenotypic resistance, suggesting complex mechanisms. The retrospective design and small single-centre sample limit generalizability. Future studies using whole genome sequencing (WGS) are recommended to clarify discrepancies and improve MDR-TB treatment strategies.

Keywords: *Mycobacterium Tuberculosis*, Tuberculosis, Multidrug-Resistant, Clofazimine, Linezolid

1. Background

Tuberculosis (TB) remains a critical global health issue, with multidrug-resistant tuberculosis (MDR-TB) posing a significant challenge to disease control and patient management (1). The MDR-TB, defined as resistance to at least isoniazid and rifampicin, is linked to higher morbidity, mortality, and healthcare costs. The World Health Organization (WHO) recognizes MDR-TB as a major obstacle to TB elimination, especially in high-

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burden countries like Iran, where drug-resistant TB rates continue to increase. The emergence of resistance to second-line drugs such as clofazimine and linezolid has further complicated MDR-TB treatment. These drugs are now key components in treatment regimens due to their efficacy and relatively favorable safety profiles. Linezolid, an oxazolidinone antibiotic, is classified by the WHO as a group A drug, recommended as a core agent in MDR-TB therapy (2). Clofazimine, originally developed to treat leprosy, is a group B drug increasingly used in MDR-TB regimens (3). However, increased use has raised concerns about developing drug resistance, which could impair treatment success.

Detecting drug resistance in *Mycobacterium tuberculosis* is essential for guiding effective therapy and improving patient outcomes. Phenotypic drug susceptibility testing (DST), such as the proportional method on Lowenstein-Jensen medium or Middlebrook agar, has been the gold standard for identifying resistance (4). These methods assess *M. tuberculosis* growth in drug-containing media, providing direct evidence of resistance or susceptibility. Despite reliability and clinical relevance, phenotypic DST is slow, often requiring weeks for results, and may not detect low-level or emerging resistance, especially for new or repurposed drugs (5).

Advances in molecular biology have transformed resistance detection by enabling rapid identification of genetic mutations linked to resistance. Techniques such as polymerase chain reaction (PCR), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), and real-time polymerase chain reaction (RT-PCR) offer faster detection than phenotypic methods, often within hours to days, allowing earlier treatment decisions. Molecular approaches also reveal genetic diversity and heterogeneity among resistance genes, improving understanding of resistant strain evolution and transmission.

Linezolid resistance mostly involves mutations in the 23S rRNA gene (*rrl*) and sometimes in the ribosomal protein L3 gene (*rplC*) (6, 7). These mutations alter drug binding, reducing effectiveness. Clofazimine resistance is commonly linked to mutations in the *rv0678* gene, which regulates the MmpL5-MmpS5 efflux pump (8). Mutations here lead to pump overexpression, lowering intracellular drug levels and conferring resistance. Understanding these genetic mechanisms is crucial for developing precise diagnostics and effective treatments.

Despite molecular diagnostics' advantages, genotype-phenotype correlation is complex. Not all mutations cause significant clinical resistance; some resistant phenotypes arise from mechanisms undetectable by targeted assays (9). This discordance highlights the complexity of *M. tuberculosis* resistance and the necessity for comprehensive detection methods. Whole genome sequencing (WGS) provides complete mutation profiles and can clarify genotype-phenotype discrepancies but remains limited in many resource-poor areas where molecular and phenotypic methods predominate.

2. Objectives

In Iran, the rise of MDR-TB, expanded use of linezolid and clofazimine, and lack of genetic data highlight the need for effective drug resistance monitoring. This study examines mutations linked to linezolid and clofazimine resistance in Iranian MDR-TB isolates. By combining phenotypic and molecular methods, including PCR-RFLP and RT-PCR, we assess mutation patterns in *rv0678*, *rrl*, and *rplC* genes. Findings stress continuous surveillance and the value of WGS in cases of genotype-phenotype discordance, enhancing treatment strategies.

3. Methods

3.1. Study Design and Sample Selection

This retrospective cross-sectional study was conducted at the Mycobacteriology Research Center, NRITLD (national referral center), Tehran, Iran (2024 - 2025). Consecutive acid-fast bacilli (AFB) specimens were collected from patients diagnosed with pulmonary TB who had previously been identified as resistant to first-line drugs (isoniazid and rifampicin). Diagnosis was confirmed based on clinical symptoms, specialist examinations, and radiological studies. Samples were stained using the Ziehl-Neelsen technique and cultured on Lowenstein-Jensen medium, with weekly evaluations for 60 days (10). A total of 28 MDR-TB isolates were selected based on AFB positivity, WHO proportional method DST, and molecular tests for *M. tuberculosis*. Indeterminate or contaminated cultures or those with missing data were excluded.

3.2. Molecular Identification of Mycobacterium Tuberculosis

Molecular identification of *M. tuberculosis* was performed using PCR targeting the IS6110 sequence and the *hsp65* gene spacer region. Genomic DNA was extracted from specimens using Qiagen kits (Qiagen, Hilden, Germany), and DNA concentration was measured with a Picodrop device (11). Positive control DNA from *M. tuberculosis* strain *H37Rv* and negative control DNA from *Mycobacterium* fortuitum were

included to ensure the accuracy of the assay. This strategy allowed for precise detection of *M. tuberculosis* in the analyzed samples.

3.3. Drug Susceptibility Testing (Reference Standard)

Phenotypic DST was performed using the WHO-recommended proportional method on Lowenstein-Jensen medium for isoniazid, rifampicin, and ethambutol, at critical concentrations of 0.2, 40, and 2 µg/mL, respectively. Clofazimine and linezolid susceptibility testing was carried out on Middlebrook 7H10 agar at 1 µg/mL. This set of phenotypic methods was chosen based on WHO recommendations and the extensive validation of these methods in the field. A 1 McFarland standard suspension of target colonies was inoculated onto both drug-containing and control media and incubated at 37°C for three weeks. Growth on drug-containing media equal to or exceeding 1% of that on the control media was considered resistant. Results were compared to the *H37Rv* reference strain (12).

3.4. Polymerase Chain Reaction

Specific primers, designed and validated in this study (Table 1), were used to amplify genes linked to resistance: *rv0678* for clofazimine and *rrl* and *rplC* for linezolid (13).

Table 1. Primer Patterns Used in This Study

Drugs and Genes	Forward Primer	Reverse Primer
Clofazimine		
rv0678	GGCGATGGCGACCACAAC	CTTGTTGATGCGGTTGCC
Linezolid		
rrl	ATCCGTCGCTCACTAATCCT	GGAGCTTGACTGCGAGACTT
RPLC	TCAACCCACGCCGATACC	ACTGGTCATGGTCCGCAG

3.5. Quantitative Polymerase Chain Reaction

Genomic variations in the *M. tuberculosis* strain *H37Rv* were identified based on its complete genome sequence. An initial BLAST analysis was performed on the samples, followed by an examination of sequence alignments. Primers were designed using Gene-Runner software, and secondary structures were analyzed using Oligo 7. The primers were re-analyzed using Primer-BLAST. The selected genomic regions for detecting drug resistance included the *rrl* and *rplC* genes for linezolid and the *rv0678* gene for clofazimine. Quantitative PCR reactions were prepared according to Table 2. The specifications of the primers and probes can be found in Table 3. All experiments were carried out using the Rotor-Gene device from Qiagen, following specific temperature protocols.

Table 2. Materials Required for Preparing the Mix in the Quantitative Polymerase Chain Reaction Method [a]

Contents	Values
Clofazimine	
DNTP (0.2 mM)	0.5
Mgcl2 (5.5 mM)	4
Buffer (10x)	2.5
FP (30 pM)	1
RP (30 pM)	1
Probe (15 pM)	0.4
Q buffer	0.5
Enzyme (1U)	0.25
DW	9.85
DNA (5 - 20 ngr)	5
Total volume	25
Linezolid	
DNTP (0.2 mM)	0.5
Mgcl2 (5.5 mM)	2
Buffer (10X)	2.5
Primer F (20 pM)	1
Primer R (20 pM)	1
Probe (10 pM)	0.4
Q buffer	0.5
Enzyme (1U)	0.25
DW	11.85
DNA (5 - 20 ngr)	5
Total volume	25

Abbreviations: DNTP, deoxynucleotide triphosphate; Mgcl2, magnesium chloride; FP, forward primer; RP, reverse primer; Q buffer, Qiagen buffer; DNA, deoxyribonucleic acid; DW, distilled water; mM, millimolar; pM, picomolar; ng, nanograms; µL, microliter.

[a] Values are expressed as level (µL).

3.6. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

To investigate bacterial resistance to linezolid, two genes, *rrl* and *rplC*, were examined using PCR-RFLP. Initially, PCR was performed on the *rrl* using the primers listed in Table 1. To prepare a 50 µL mixture, the amounts listed in Table 4 were utilized. The PCR program was performed in a thermocycler (Stack - Japan) similar to the protocol described in Table 5. The PCR products were evaluated by electrophoresis on a 1.5% agarose gel with TAE buffer, using a molecular marker to detect an 820 bp target band. The PCR products were subsequently digested with the restriction enzyme BbvI, with the reaction mixture incubated for 16 hours at 65°C and then inactivated at 80°C for 20 minutes. The digestion patterns were visualized using 8% polyacrylamide gel

Table 3. Primer Patterns and Probes Used in the Quantitative Polymerase Chain Reaction Method

Drugs	Forward Primer	Reverse Primer	Prob Oligo Sequence
Clofazimine	5–ACTGCAGAGG TTGATCAAGAT -3′	5′- GCCCACGCTC ACATGACAG -3′	5′-GCTACGCTGAC CAGACCGCTGT–3′
Linezolid	5–CGTAGGCTA GCTACGCTG -3′	5′- GAATCCGCC CTACCGACAG -3′	5′- FAM-GCTACGCTGACC AGACCGCTGT–3/-TAMRA -3′

Table 4. Materials Required for Preparing the Mix in the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Method [a]

Contents	*rrl*	*rplC*	*rv0678*
dNTP (0.2 mM)	1	1	1
Mgcl2 (5.5 mM)	1	0	1
Buffer (10X)	5	5	5
FP (20 pM)	0.6	0.6	0.2
RP (20 pM)	0.6	0.6	0.2
Q buffer	1	1	3
Enzyme (1U)	0.4	0.4	0.4
DNA (10 ng)	2	2	1
DW	38.4	39.4	38.2
Total volume	50	50	50

Abbreviations: dNTP, deoxynucleotide triphosphate; Mgcl2, magnesium chloride; FP, forward primer; RP, reverse primer; Q buffer, Qiagen buffer; DNA, deoxyribonucleic acid; DW, distilled water; mM, millimolar; pM, picomolar; ng, nanograms; µL, microliter.

[a] Values are expressed as level (µL).

electrophoresis and compared to a standard drug-sensitive strain.

Similarly, the *rplC* gene was analyzed using specific primers and a 50 µL PCR mixture, based on the information provided in Tables 1, 2, and 5. The PCR products were analyzed on a 1.5% agarose gel, showing a 400 bp target band. Digestion with BbvI followed the same procedure as for the *rrl* gene. Resistance to linezolid is often caused by genetic mutations detectable by PCR-RFLP, a method offering high accuracy in identifying drug resistance.

Regarding clofazimine, after reviewing previous studies, the *rv0678* gene was selected as the region with suitable genetic characteristics for evaluating drug resistance. According to Table 1, the primer sequence was chosen, designed, and synthesized. To prepare the necessary mix for this reaction, the quantities listed in Table 4 were utilized. The PCR steps were conducted as per Table 5, and the products obtained from PCR were analyzed using 8% acrylamide gel and a 50 bp marker. The *H37Rv* sample was also included as a control alongside the MDR-TB samples.

3.7. Statistical Analysis

Data were analyzed using SPSS software version 26 (IBM Corp., Armonk, NY, USA). Descriptive statistics, including frequencies, percentages, means, and standard deviations, were calculated to summarize the characteristics of the clinical isolates and resistance patterns. Comparisons between phenotypic drug susceptibility results and genotypic findings (mutations detected via PCR-RFLP and RT-PCR) were assessed using chi-square (χ^2) or Fisher's exact tests, as appropriate, to evaluate the association between gene mutations and resistance status. A P-value of less than 0.05 was considered statistically significant. For analysis of PCR-RFLP patterns, similarity between isolates was evaluated by comparing restriction fragment sizes. The diversity of patterns was quantified, and mutation frequencies were reported for each gene (*rv0678*, *rrl*, and *rplC*). Where applicable, correlation analyses (e.g., Spearman's rank correlation) were performed to explore relationships between the presence of specific mutations and phenotypic resistance levels. All results were presented with 95% confidence intervals where relevant.

3.8. Study Limitations

This study's limited sample size may reduce the generalizability of findings to broader populations. It was conducted in a single center, which may limit geographic representativeness. The use of PCR-RFLP, while a useful molecular tool, has inherent limitations

Table 5. Amplification Program for Polymerase Chain Reaction of Various Genes in Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis

Drugs and Genes	Initial Denaturation (°C, min)	Denaturation (°C, s)	Annealing (°C, s)	Elongation (°C, s)	Final Extension (°C, min)	Cycles (n)
Linezolid						
rrl	95, 5	95 ,30	59, 30	72, 40	72, 5	35
rplC	95, 5	95 ,30	56.5, 30	72, 35	72, 5	35
Clofazimine						
rv0678	95, 5	95 ,30	60, 30	72, 30	72, 5	30

in detecting all resistance-associated mutations. Furthermore, the lack of WGS restricts comprehensive genetic characterization of resistance mechanisms.

4. Results

A total of 30 *M. tuberculosis* isolates were analyzed for susceptibility to clofazimine and linezolid using PCR-RFLP and RT-PCR methods. The sample included 28 MDR-TB clinical isolates, one laboratory reference strain (*H37Rv*), and one wild-type susceptible isolate. Initial RT-PCR susceptibility testing showed that all isolates except isolate 13 were phenotypically susceptible to both clofazimine and linezolid.

4.1. Genetic Analysis and Statistical Association of rv0678 Gene (Clofazimine Resistance)

The PCR amplification of the *rv0678* gene yielded a uniform 720 bp amplicon across all isolates. Digestion with NlaIII produced three distinct restriction fragment length polymorphism (RFLP) patterns: The predominant pattern with fragments of 80, 120, and 520 bp was observed in 24 isolates (80%; isolates 1 - 7, 13 - 30); isolates 8 and 9 (6.7%) showed an 80/120/160/520 bp pattern; and isolates 10, 11, and 12 (10%) showed an 80/120/190/520 bp pattern. Digestion with BsrI resulted in a uniform 300/400 bp pattern for all isolates, indicating limited sensitivity to genetic variation. Mutations or variant patterns in the *rv0678* gene were found in 6 out of 30 isolates (20%). Among these, only isolate 13 exhibited phenotypic resistance to clofazimine, while the rest remained susceptible. Chi-square and Fisher's exact tests showed no statistically significant association between *rv0678* mutation status and phenotypic clofazimine resistance ($P > 0.05$).

4.2. Genetic Analysis and Statistical Association of rrl Gene (23S rRNA, Linezolid Resistance)

All isolates amplified an 860 bp segment of the *rrl* gene. Restriction digestion with three enzymes revealed: BsrI digestion produced uniform fragment patterns of 105, 140, 250, and 260 bp in all isolates, including the resistant isolate 13; BbvI digestion showed 28 isolates (93.3%) had fragments of 60, 190, 290, 320, 510, 530, and 780 bp, while two isolates (5 and 6; 6.7%) lacked the 190 bp fragment. HaeIII digestion revealed 28 isolates with fragments of 100, 770, and 780 bp, while isolates 5 and 7 (6.7%) lacked the 770 bp fragment. Isolate 5 presented unique fragment patterns with both BbvI and HaeIII, suggesting multiple genetic variations. Despite this, isolate 13, phenotypically resistant to linezolid, showed no unique RFLP patterns. Variant PCR-RFLP patterns in *rrl* were detected in 7 of 30 isolates (23.3%). Only isolate 13 demonstrated phenotypic linezolid resistance. Statistical analysis revealed no significant correlation between *rrl* mutations and phenotypic resistance ($P > 0.05$).

4.3. Genetic Analysis and Statistical Association of rplC Gene (Linezolid Resistance)

The *rplC* gene consistently yielded a 400 bp PCR product in all isolates. Digestion with six enzymes showed: BbvI digestion differentiated 28 isolates (93.3%) with fragments of 75, 100, and 290 bp; isolates 1 and 2 (6.7%) presented fragments of 70, 100, and 290 bp; the other five enzymes (NlaIII, BsrI, BsaJI, TaqI, HaeIII) yielded uniform patterns across all isolates. Mutations in *rplC* were found in 2 of 30 isolates (6.7%), none of which showed phenotypic resistance to linezolid. Statistical testing indicated no significant association between *rplC* mutations and phenotypic resistance ($P > 0.05$).

5. Discussion

The MDR-TB remains a significant public health challenge globally, particularly in countries like Iran where resistance to critical first-line drugs, rifampicin and isoniazid, is rising. Recent meta-analyses estimate Iran's MDR-TB prevalence at approximately 12 - 27%, with a noted increasing trend between 2018 and 2023, underscoring the urgent need for strengthened control efforts (14). This worrying rise is compounded by the country's geographic position, bordering high TB burden nations such as Afghanistan and Pakistan, where

cross-border transmission of resistant strains is a significant concern (15).

Our study contributes valuable insights into the genetic basis of resistance to two critical second-line drugs, clofazimine and linezolid, increasingly incorporated into MDR-TB treatment regimens in Iran. The retrospective nature of this study and the relatively small sample size (n = 30) represent important limitations, which may restrict the generalizability of findings to wider populations. Despite these limitations, the use of standard phenotypic and molecular methods aligned with WHO recommendations and robust performance by the researchers lends credibility to the results. The clinical isolates were obtained from patients with pulmonary TB who were confirmed resistant to first-line drugs based on comprehensive clinical evaluation, specialist examinations, and radiological studies, thereby ensuring accurate diagnosis and relevance of resistance data in this population.

Our molecular analyses focused on mutations in the *rv0678*, *rrl*, and *rplC* genes, revealing genetic heterogeneity among the isolates. However, the correlation between genotypic mutations and phenotypic resistance was not straightforward, with only one isolate (isolate 13) exhibiting phenotypic resistance to both clofazimine and linezolid, despite several isolates harboring mutations or variant PCR-RFLP patterns in the examined genes.

Linezolid acts by binding to domain V of the 23S rRNA encoded by the *rrl* gene, inhibiting protein synthesis. Mutations in *rrl* and *rplC* (ribosomal protein L3) genes can confer resistance (6). Clofazimine's mechanism involves disruption of the bacterial respiratory chain and generation of reactive oxygen species; resistance is linked primarily to mutations in *rv0678*, which impact regulation of the MmpS5-MmpL5 efflux pump and cross-resistance with bedaquiline (16, 17). Our finding of multiple *rv0678* PCR-RFLP variants parallels reports from other regions, though many such mutations did not translate into phenotypic resistance, suggesting that not all genetic changes confer functional resistance or that compensatory mechanisms may be at play.

The absence of a significant statistical association between mutations in these genes and phenotypic resistance in our study highlights the complexity of MDR-TB resistance mechanisms. The RFLP-based detection methods, while useful for genetic diversity analysis, may miss or fail to discriminate key mutations responsible for resistance, especially outside the restriction sites analyzed. This complexity aligns with earlier studies emphasizing the limitations of targeted gene assays and underpins the recommendation for more comprehensive approaches such as WGS or next-generation sequencing (NGS) for precise identification of resistance mutations (18).

Iran's treatment protocols for MDR-TB have increasingly incorporated clofazimine and linezolid, reflecting WHO guidelines and international best practices (15). However, access to these drugs remains a challenge, and resistance emergence threatens their efficacy. Our finding that most isolates remain susceptible is encouraging, yet the identification of genetic variants even in phenotypically susceptible strains calls for vigilant surveillance to detect emerging resistance early. Regional studies from high TB burden countries like China and South Africa have similarly documented notable clofazimine resistance linked to *rv0678* mutations, reinforcing the need for local resistance data to guide empiric treatment and stewardship (19).

Given the relatively small sample size in our study (n = 30), these findings are preliminary and indicate the necessity for larger-scale epidemiological studies in Iran to fully characterize molecular resistance patterns and inform treatment algorithms. Ultimately, controlling MDR-TB in Iran requires a multifaceted strategy combining accurate and rapid diagnostic methods, optimized drug regimens, patient adherence support, and robust surveillance systems. The potential of advanced molecular tools like NGS to identify known and novel resistance mutations promises timely and tailored interventions, which are crucial given the high social and economic burden of MDR-TB (20). Continuous monitoring of drug resistance evolution with integration of molecular and phenotypic data will be essential. Strengthening laboratory capacities and expanding access to second-line drugs including clofazimine and linezolid, alongside public health interventions targeted at vulnerable populations such as refugees and migrants from neighboring high-burden countries, are critical steps toward curbing the MDR-TB epidemic in Iran.

5.1. Conclusions

This retrospective study highlights the complex genetic landscape underlying clofazimine and linezolid resistance in *M. tuberculosis* isolates from Iran. While notable genetic variations were identified in the *rv0678* and *rrl* genes, the *rplC* gene exhibited high conservation among the isolates. Given these findings, continued and expanded molecular surveillance is vital to detect emerging resistance early and guide effective treatment strategies. Employing advanced genomic technologies such as NGS will provide comprehensive insight into

known and novel resistance-conferring mutations beyond the limitations of targeted PCR-RFLP assays. Importantly, the presence of these mutations did not consistently correlate with phenotypic resistance, underscoring the multifactorial nature of drug resistance mechanisms in MDR-TB.

The limited sample size is an important limitation that may affect the generalizability of results to broader populations. The selection of clinical isolates from patients with pulmonary TB resistant to first-line drugs, with confirmation based on clinical symptoms, specialist evaluations, and radiological studies, further supports the relevance of findings while acknowledging this as a constraint affecting external applicability. Given these considerations, continued and expanded molecular surveillance involving larger cohorts is crucial to detect emerging resistance early and to inform effective treatment strategies. Employing advanced genomic technologies such as WGS or NGS is recommended to overcome the limitations of targeted PCR-RFLP assays by providing comprehensive insight into known and novel resistance-conferring mutations. Strengthening diagnostic capabilities and integrating genotypic and phenotypic data are critical steps toward optimizing MDR-TB management. Ultimately, sustained efforts in vigilant monitoring, timely diagnosis, and appropriate use of second-line drugs like clofazimine and linezolid are essential to limit the spread of resistant strains and improve treatment outcomes in Iran and similar high-burden settings.

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Footnotes

Authors' Contribution: All authors have equally and actively participated in all stages of the research, including study design, data collection, analysis and interpretation of results, drafting the initial manuscript, and important content revisions. Each author has taken full responsibility for a portion of the work and has contributed to the approval of the final version of the manuscript.

Conflict of Interests Statement: The authors declare conflict of interest.

Data Availability: The datasets generated and/or analyzed during the current study are not publicly available due to confidentiality and ethical considerations but are available from the corresponding author upon reasonable request.

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References

1. Chen Z, Wang T, Du J, Sun L, Wang G, Ni R, et al. Decoding the WHO Global Tuberculosis Report 2024: A Critical Analysis of Global and Chinese Key Data. *Zoonoses*. 2025;**5**(1). https://doi.org/10.15212/zoonoses-2024-0061.
2. Oehadian A, Santoso P, Menzies D, Ruslami R. Concise Clinical Review of Hematologic Toxicity of Linezolid in Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis: Role of Mitochondria. *Tuberc Respir Dis (Seoul)*. 2022;**85**(2):111-21. [PubMed ID: 35045688]. [PubMed Central ID: PMC8987663]. https://doi.org/10.4046/trd.2021.0122.
3. Nugraha RV, Yunivita V, Santoso P, Aarnoutse RE, Ruslami R. Clofazimine as a Treatment for Multidrug-Resistant Tuberculosis: A Review. *Sci Pharm*. 2021;**89**(2). https://doi.org/10.3390/scipharm89020019.
4. Rupasinghe APR. *Identifying and addressing the gaps impeding fast and accurate phenotypic drugsusceptibility testing of Mycobacterium tuberculosis[Dissertation]*. Antwerp, Belgium: University of Antwerp; 2024.
5. Sanchini A, Lanni A, Giannoni F, Mustazzolu A. Exploring diagnostic methods for drug-resistant tuberculosis: A comprehensive overview. *Tuberculosis (Edinb)*. 2024;**148**:102522. [PubMed ID: 38850839]. https://doi.org/10.1016/j.tube.2024.102522.
6. Gan WC, Ng HF, Ngeow YF. Mechanisms of Linezolid Resistance in Mycobacteria. *Pharmaceuticals (Basel)*. 2023;**16**(6). [PubMed ID: 37375732]. [PubMed Central ID: PMC10303974]. https://doi.org/10.3390/ph16060784.
7. Sengupta S, Jain P, Ratnam R, Pandey BK, Singh U, Kumar V, et al. Agreement between Phenotypically Detected Linezolid Resistance and Mutations in rrl and rplC Genes of Mycobacterium tuberculosis Isolates Using Nanopore Sequencing. *Int J Mycobacteriol*. 2024;**13**(3):331-6. [PubMed ID: 39277897]. https://doi.org/10.4103/ijmy.ijmy_139_24.
8. Velayati AA, Mitaria S, Farnia P, Farnia P, Ghanavi J, Maleknia M, et al. Exploring the Genetic Landscape of Mycobacterium tuberculosis: Unlocking the Differences in Between Latent and Active Tuberculosis. *Int J Mycobacteriol*. 2024;**13**(4):410-9. [PubMed ID: 39700163]. https://doi.org/10.4103/ijmy.ijmy_212_24.
9. Banerjee R, Patel R. Molecular diagnostics for genotypic detection of antibiotic resistance: current landscape and future directions. *JAC Antimicrob Resist*. 2023;**5**(1):dlad018. [PubMed ID: 36816746]. [PubMed Central ID: PMC9937039]. https://doi.org/10.1093/jacamr/dlad018.

10. Farnia P, Farnia P, Ghanavi J, Besharati S, Velayati AA. Comparison of Proline-glutamate-proline-glutamate-polymorphic GC-rich Sequences Family Protein Wag22 (Rv1759c), PE_PGRS31 (Rv1768), PE_PGRS32 (Rv1803), and PE_PGRS33 gene (Rv1818c) in Exponential State and Under In vitro Model of Latency in Same Clinical Isolates of Mycobacterium tuberculosis: Frameshift Mutation in Extensively Drug-resistant and Totally Drug-resistant tuberculosis Bacilli. *Biomed Biotechnol Res J*. 2023;**7**(4):621-32. https://doi.org/10.4103/bbrj.bbrj_271_23.
11. Ayoubi S, Farnia P, Farnia P, Aghajani J, Ghanavi J, Velayati AA. Prevalence of non-tuberculosis mycobacteria among samples deposited from the National Tuberculous Reference Laboratory of Iran (2011-2018). *Asian Pac J Trop Med*. 2021;**14**(10):451-5. https://doi.org/10.4103/1995-7645.329007.
12. Web Annex C. *WHO operational handbook on tuberculosis. Module 3: diagnosis*. 3rd ed. Geneva, Switzerland: World Health Organization,; 2024.
13. Yang J, Pang Y, Zhang T, Xian X, Li Y, Wang R, et al. Molecular characteristics and in vitro susceptibility to bedaquiline of Mycobacterium tuberculosis isolates circulating in Shaanxi, China. *Int J Infect Dis*. 2020;**99**:163-70. [PubMed ID: 32738481]. https://doi.org/10.1016/j.ijid.2020.07.044.
14. Ayoubi S, Farnia P, Farnia P, Ghanavi J, Velayati AA. Prevalence and Temporal Trends of Multidrug-resistant Tuberculosis in Iran from 1981 to 2023: A Systematic Review and Meta-analysis. *Int J Mycobacteriol*. 2024;**13**(3):320-30. [PubMed ID: 39277896]. https://doi.org/10.4103/ijmy.ijmy_162_24.
15. Khelghati F, Nasirpour Seilakhori F, Goudarzi M, Malekloo S, Shahidi Bonjar AH, Goudarzi H, et al. Multidrug-resistant tuberculosis in Iran: a multicenter study. *Monaldi Arch Chest Dis*. 2025;**95**(1). [PubMed ID: 38214397]. https://doi.org/10.4081/monaldi.2024.2844.
16. Farnia P, Besharati S, Farina P, Ayoubi S, Marjani M, Ghanavi J, et al. The Role of Efflux Pumps transporter in Multi-drug Resistant Tuberculosis: Mycobacterial memberane protein(MmpL5). *Int J Mycobacteriol*. 2024;**13**(1):7-14. [PubMed ID: 38771273]. https://doi.org/10.4103/ijmy.ijmy_37_24.
17. Ghodousi A, Rizvi AH, Baloch AQ, Ghafoor A, Khanzada FM, Qadir M, et al. Acquisition of Cross-Resistance to Bedaquiline and Clofazimine following Treatment for Tuberculosis in Pakistan. *Int J Mycobacteriol*. 2021;**10**(Suppl 1). https://doi.org/10.4103/2212-5531.307111.
18. Meehan CJ, Goig GA, Kohl TA, Verboven L, Dippenaar A, Ezewudo M, et al. Whole genome sequencing of Mycobacterium tuberculosis: current standards and open issues. *Nat Rev Microbiol*. 2019;**17**(9):533-45. [PubMed ID: 31209399]. https://doi.org/10.1038/s41579-019-0214-5.
19. Padmapriyadarsini C, Vohra V, Bhatnagar A, Solanki R, Sridhar R, Anande L, et al. Bedaquiline, Delamanid, Linezolid and Clofazimine for Treatment of Pre-extensively Drug-Resistant Tuberculosis. *Clin Infect Dis*. 2022;**76**(3):e938-46. [PubMed ID: 35767251]. [PubMed Central ID: PMC9907500]. https://doi.org/10.1093/cid/ciac528.
20. Jin W, Wang M, Wang Y, Zhu B, Wang Q, Zhou C, et al. Targeted next-generation sequencing: a promising approach for Mycobacterium tuberculosis detection and drug resistance when applied in paucibacillary clinical samples. *Microbiol Spectr*. 2025;**13**(7). e0312724. [PubMed ID: 40492765]. [PubMed Central ID: PMC12211088]. https://doi.org/10.1128/spectrum.03127-24.