Quick Response Code:

Indian J Med Res 154, July 2021, pp 85-89
DOI: 10.4103/ijmr.IJMR_1168_19

Linezolid resistance in *Mycobacterium tuberculosis* isolates at a tertiary care centre in Mumbai, India

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Received July 8, 2019

***Background & objectives*: Linezolid (LZD) is increasingly being used in tuberculosis (TB) treatment. However, LZD resistance has already been reported, which is highly alarming, given its critical therapeutic role. This study was aimed to phenotypically and genotypically assess LZD resistance in *Mycobacterium tuberculosis* (MTB) isolates at a laboratory in a tertiary care centre in Mumbai, India.**

***Methods*: A sample of 32 consecutive LZD-resistant MTB isolates identified by liquid culture susceptibility testing was subjected to whole-genome sequencing (WGS) on the Illumina NextSeq platform. Sequences were analyzed using BioNumerics software to predict resistance for 12 antibiotics within 15 min.**

***Results*: Sixty eight of the 2179 isolates tested for LZD resistance by MGIT-based susceptibility testing (June 2015 to June 2016) were LZD-resistant. Thirty two consecutive LZD-resistant isolates were analyzed by WGS to screen for known mutations conferring LZD resistance. WGS of 32 phenotypically LZD-resistant isolates showed that C154R in the *rplC* gene and G2814T in the *rrl* gene were the major resistance determinants.**

***Interpretation & conclusions*: LZD resistance poses an important risk to the success of treatment regimens, especially those designed for resistant isolates; such regimens are extensively used in India. As LZD-containing regimens increase in prominence, it is important to support clinical decision-making with an improved understanding of the common mutations conferring LZD resistance and their frequency in different settings.**

Key words Linezolid - Mumbai - mutations - resistance - tuberculosis - whole-genome sequencing

The emergence of extensively drug-resistant (XDR) tuberculosis (TB)[1] [defined as TB resistant to rifampin and isoniazid (multidrug-resistant TB or (MDR-TB) and to fluoroquinolones and any injectable second-line agent] has emphasized on the urgency to develop new active agents to manage drug-resistant TB. Cases of XDR and totally drug-resistant TB have been noted in China, India, Africa and Eastern Europe[2]. This constitutes an increasing public health threat, demanding the development

of novel drugs and dosing regimens for successful treatment.

Of the available anti-TB drugs, linezolid (LZD), approved by the Food and Drug Administration (FDA) in 2000 for the treatment of Gram-positive infections, exhibits robust activity against *Mycobacterium tuberculosis* (MTB)[3]. LZD, therefore, has an important role as an anti-TB agent, and its introduction into the national TB programmes has been scaled up[4]. LZD inhibits bacterial protein synthesis by preventing formation of a 70S initiation complex by binding to the 50S ribosomal subunit near its interface with the 30S unit. Therefore, the development of resistance to LZD is expected to be slow and gradual[3,5,6]. LZD has a minimum inhibitory concentration (MIC) of 0.5~1 mg/l for susceptible isolates of MTB[7].

Despite its clinical efficiency, resistance to LZD has been reported among TB patients. Limited evidence suggests that population-level resistance to LZD may be increasing in the National TB Programmes[4]. This is alarming, given the critical role played by this drug in the treatment of drug-resistant TB and its prominence in upcoming treatment-shortening and injection-sparing regimens for TB[8,9].

Two potential risk factors for LZD resistance have been identified: (*i*) LZD dosing is often reduced because of associated mitochondrial toxicity, leading to suboptimal exposure for efficacy and resistance suppression and to the selection of resistant mutants and (*ii*) LZD may be added to a failing or inadequate regimen, exacerbating the risk of acquired resistance[4]. LZD resistance can also be related to the efflux pump mechanism and low permeability of mycobacterial cell wall[8]. Lack of complete understanding of the underlying molecular mechanism of resistance further compounds the problem of resistance. Mutations in genes encoding the 23S rRNA (*rrl*) peptidyl transferase centre-binding site and the L3 protein (*rplC*), which extends into the binding site, have been identified as the dominant molecular mechanisms underlying LZD resistance[4].

The increase in the extent of resistance, on a global scale, has necessitated molecular analysis of the responsible mutations. Given that commercial molecular techniques such as GeneXpert or line probe assays do not offer any information about LZD resistance, whole-genome sequencing (WGS) is the only currently available alternative. WGS can offer detailed information about the overall resistome of MTB isolates within a relatively short timeframe[9].

In this study, a retrospective investigation was performed using WGS to identify mutations associated with LZD resistance among consecutive phenotypically LZD-resistant clinical MTB isolates in a tertiary care centre in Mumbai, India.

Material & Methods

This non-randomized, retrospective, non-interventional, experimental study was undertaken at the Section of Microbiology, department of Laboratory Medicine, P. D. Hinduja Hospital and Medical Research Centre, Mumbai, India. The study was approved by the institutional ethics committee of the hospital. Consent was waived for this observational laboratory research study of de-identified cultured clinical isolates of MTB.

Laboratory records of all consecutive, culture-positive, pulmonary and extrapulmonary TB samples submitted to this tertiary care centre for TB diagnostics from June 2015 to June 2016 were reviewed to identify the number of samples that were subjected to Mycobacterial Growth Indicator Tube (MGIT) culture and LZD susceptibility testing for clinical purposes by MGIT drug susceptibility testing (DST); critical concentration, 1 mg/l. The MGIT-positive vials of LZD-resistant MTB isolates were used for this study (which involved a second round of MGIT DST for LZD and WGS), provided adequate volume of the culture suspension was available for performing these experiments. Thirty two consecutive LZD-resistant MTB isolates fulfilling this requirement, representative of the same pool and chosen by convenient sampling, were included in this study. To reduce selection bias, all consecutive isolates with LZD resistance were included. All isolates were reconfirmed for LZD resistance by a second MGIT DST for LZD[10].

Culture suspension from the same MGIT vial was used to subculture 0.2 ml on Lowenstein–Jensen (LJ) slopes (bioMérieux SA, Marcy l'Etoile, France), which were incubated for four weeks or until confluent growth of MTB was observed. The growth was scraped from LJ slants and transferred into PrimeStore Molecular Transport Medium (Longhorn Diagnostics, TX, USA) for storage until transportation to the external sequencing infrastructure provider. About 400 µl of the homogenized culture suspension was used for DNA extraction using the commercial QIAamp DNA Kit (QIAGEN Diagnostics GmbH, Hilden, Germany) with modifications, including bead beating to enhance

mycobacterial cell lysis. DNA quality was determined spectrophotometrically (A260/280 and A260/230) and fluorometrically (Qubit 2.0; Thermo Fisher Scientific, MA, USA) using the dsDNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, MA, USA). The quantified DNA was included into the Nextera DNA Sample Prep Kit (Illumina, CA, USA) for tagmentation. The library was sequenced on an Illumina NextSeq (Illumina, CA, USA) in a shared run using the Illumina Mid Output 2*150 paired-end sequencing programme (Illumina, CA, USA). The resulting .fastq files were quality and adaptor trimmed by using CutAdapt (version 1.14; National Bioinformatics Infrastructure, Essen, Germany) before alignment with the reference MTB H_{37}Rv genome (NCBI Reference Sequence: NC_000962.3). The sequences were used for wgSNP analysis using the BioNumerics software (Applied Maths NV, bioMérieux, Sint-Martens-Latem, Belgium), which involved resistance prediction for 12 antibiotics based on known mutations [minimum depth (DP) of 10, mapping quality score of 40 and a minimum quality per depth of 2]. The entire pipeline is integrated in this software and was implemented on a scalable high-throughput calculation environment, providing results within 15 min of submission. Sequencing was attempted a maximum of twice in case of non-interpretable findings.

Results & Discussion

During the study period, a total of 6518 isolates were tested, of which 2869 (44.0%) tested MDR. Of these, 2179 isolates were evaluated for LZD resistance (based on patient's request), and of these, 68 were found to be resistant to LZD (3.1%).

Thirty two isolates, representative of this pool of 68 LZD-resistant isolates and with adequate volume of culture suspension available for performing this study, tested positive in a second, confirmatory round of MGIT DST, performed at a critical concentration of 1 mg/l. Based on the findings of WGS, performed using the macroscopic growth on LJ slants for each isolate (n=24 isolates), the following known LZD mutations were detected:

rplC gene (50S ribosomal protein L3): Cys154ARG (C154R) at the genomic position 801268 in H37Rv (n=16) and *rrl* gene (23S rRNA): G2814T at the genomic position 147647 in H37Rv (n=3). Eight isolates did not yield readable sequences and were excluded because of low quality (n=4) or failed sequencing (n=4). This could be attributed to the inadequate limit of detection of sequencing, errors in variant calling, incorrect interpretation of variants, errors in phenotypic testing and/or contamination with non-target DNA[11]. Sequencing failures can also be associated with the smear status of the sample (smear-negative samples had more sequencing failures), and the sequencing length of the given gene target[12].

Five phenotypically resistant isolates did not yield any mutations known to be associated with LZD resistance. This could be because of the following reasons: resistance due to extra-chromosomal induction of efflux pump activity, the presence of mutations not known to impact LZD activity such as mutations outside the loci of interest, low/inadequate limit of detection or coverage of sequencing method, mutations that changed MICs without crossing the critical concentration in MGIT, heteroresistance, mixed populations, technical errors, assay-related reasons (inoculum, contamination, inactive drug, improper drug concentration, inaccurate drug dilution and medium *p*H) and/or non-association of mutations with phenotypic resistance (silent/synonymous mutations or neutral polymorphisms)[13].

Some earlier studies have reported mutations imparting LZD resistance; these have been listed in the Table[14,15]. *In vitro* studies have also reported some mutations[16-18]. However, these mutations have not been validated in the present study.

The first study to report LZD resistance, conducted in Germany, found that four of the 210 MDR isolates (1.9%) were resistant to LZD[19]. A research study

Table. Linezolid mutations listed in literature and the present study

Region/country, where the study was performed	*rplC* gene	*rrl* gene
Asia[8,14-16]	T460C	G2270C, G2270T, G2746A, A2810T and C2848A
South Africa[4,15]	T460C	G2270C, G2270T, A2810C, G2814 and A2572C
Moscow[5]	T462G	G2294A and G2814
Present study	C154R	G2814T

conducted at our hospital found that of the 2750 samples that underwent 14-drug MGIT DST, 250 were XDR-TB, of which 44 (17.6%) showed resistance to LZD[20]. A per-patient analysis of 286 MDR-TB patients visiting the same hospital found that 105 patients underwent testing for LZD resistance, of whom three (1%) showed resistance to LZD[21]. A study from China reported LZD resistance in about 11 per cent of MDR-TB isolates, with about 30 per cent carrying mutations in the *rrl* or *rplC* gene[8].

According to the available literature, a limited number of mutations are known to impart LZD resistance; this can be considered a positive factor for the development of rapid molecular diagnostics, which are needed to support the rollout and widespread use of LZD in the National TB Programmes[4].

Our study had some limitations. MIC determination using broth dilution method was not performed, and therefore, these molecular findings were not correlated with MICs. Susceptible isolates were not included in the study. Further, only data collected as part of routine laboratory service were used in this study; patients' history and follow up data were not recorded.

The increase in LZD resistance, associated infrequency of mutations and the fact that most (but not all) resistant isolates, harbour known mutations emphasize that introduction of novel drugs (with maximized efficacy and minimized toxicity) must be accompanied by continuous phenotypic susceptibility testing and determination of genetic mutations conferring resistance[5]. It is, therefore, necessary to undertake comparative analysis of genotypic and phenotypic findings before compiling a list of high-confidence mutations.

In conclusion, our study involving WGS of 32 phenotypically LZD-resistant isolates identified the presence of C154R in the *rplC* gene and G2814T in the *rrl* gene as the major resistance determinants. Similar studies will help improve the understanding of the common mutations conferring LZD resistance and their frequency in different settings, which will, in turn, aid clinical decision-making.

***Acknowledgment*:** The authors thank the staff of the Mycobacteriology Laboratory, Section of Microbiology, Department of Laboratory Medicine, PD Hinduja Hospital for their support.

***Financial support & sponsorship*:** This work was supported by bioMérieux India, New Delhi, India. The second author (JAT) was supported by the United States National Institutes of Health (NIH), National Institute of Allergy and Infectious Diseases (grants K23AI135102 and R21AI122922), the Johns Hopkins University School of Medicine Clinician Scientist Career Development Award, and the NIH Office of the Director, Fogarty International Center, Office of AIDS Research, through the Fogarty Global Health Fellows Program Consortium (R25TW009340).

***Conflicts of Interest*:** None.

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