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Drug resistance of *Mycobacterium tuberculosis* to linezolid and delamanid: a case report from Bukavu, Democratic Republic of Congo

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Abstract
The emergence of resistance is of great concern in the control of TB, especially to the new and repurposed drugs needed for the treatment of rifampicin resistance. We report a patient from South Kivu in the Eastern Democratic Republic of the Congo with primary resistance to delamanid and linezolid without treatment experience with these drugs. The identification of novel resistance mutations raises concerns about the potential global spread and poor outcomes of the WHO-recommended oral treatment regimens, highlighting the need for the urgent rollout of DST.

Keywords Drug resistance, Linezolid and Delamanid, Novel mutations

Background

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis (TB), remains a global health concern due to its high morbidity and mortality rates, especially from its most resistant forms. Together with bedaquiline (BDQ), linezolid (LZD) and delamanid (DLM) are promising new drugs for the treatment of drug-resistant TB, yet resistance to these drugs is already a growing concern.

LZD is an oxazolidinone approved and recommended for the treatment of multi-drug and extremely-drug resistant (MDR/XDR-) TB. Resistance to LZD in MTB is mainly associated with the *rplC* (encoding ribosomal protein L3) gene and *rrl* (encoding 23 S rRNA) gene [1]. Several studies have reported the emergence of LZD-resistant MTB strains, particularly in patients with MDR-TB, often associated with the *rplC*_Cys154Arg mutation, resulting in reduced phenotypic susceptibility to LZD [2].

DLM is a nitroimidazole derivative that inhibits the methoxy-mycolic and keto-mycolic acid biosynthesis pathway in MTB [3]. Mutations in the F_{420}-dependent

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nitroreductase co-enzyme system have been associated with reduced phenotypic susceptibility to DLM. This system includes deazaflavin-dependent nitroreductase (Ddn), encoded by the gene *ddn (Rv3547)*, a glucose-6-phosphate dehydrogenase (G6PD) encoded by *fgd1 (Rv0407)*, and 3 proteins in the F_{420} biosynthetic pathway, FbiA encoded by *fbiA* (Rv3261), FbiB encoded by *fbiB (Rv3262)*, and FbiC encoded by *fbiC (Rv1173)* genes [4]. Genetic variants in these genes are diverse, and for many, their association with phenotypic DLM and pretomanid (PMD) resistance remains to be elucidated.

When introducing new drugs, ongoing surveillance and monitoring of resistance patterns is of key importance. Understanding the mechanisms of resistance and identifying molecular markers for resistance will be crucial for developing rapid molecular tests for new drugs to guide treatment strategies and curb the spread of drug-resistant TB.

Case description

We identified resistance to new drugs in one 33-year-old patient, who was first diagnosed with TB by microscopy in Bukavu in 2019. This patient was confirmed TB positive via sputum smear microscopy, a practice consistent with the prevailing recommendations of the National TB Program in the Democratic Republic of the Congo. According to those guidelines, direct diagnosis using the Xpert MTB/RIF test was reserved for specific high-risk categories, which included retreatment cases (defined as treatment failure, relapse, or treatment after interruption), presumed contacts of drug-resistant TB index cases, new TB cases remaining smear-positive after two or three months of first-line treatment, and individuals belonging to high risk multi-drug resistance tuberculosis (MDR-TB) groups such as people living with HIV, prisoners, artisanal miners, and refugees. Since the patient's initial presentation did not fall within any of these specified criteria, microscopy was the designated primary diagnostic method [5].

He was started on first-line TB treatment with rifampicin (R), isoniazid (H), pyrazinamide (Z), and ethambutol (E), 2RHZE/4RH (the RHZE quadruple combination contains four molecules in a single tablet: Rifampicin, Isoniazid, Pyrazinamide, and Ethambutol. This tablet is dosed as follows: *R* = 150 mg, H = 75 mg, Z = 400 mg, E = 275 mg; the double association RH contains 2 molecules in a single tablet Rifampicin and Isoniazid, and is dosed at *R* = 150 mg and H = 75 mg).

Between 2019 and 2021, he interrupted this treatment three times. In November 2021, he was empirically started on category II treatment - with the addition of streptomycin (S) (2SRHZE/1RHZE/5RHE) - due to repeated non-compliance with the first-line regimen and a lack of available advanced resistance testing assays, especially the pDST and WGS. After 2 months, he stopped treatment again and was restarted on category II treatment in June 2022 for 3 months, returning in January 2023, when he was diagnosed with RR-TB by Xpert MTB/RIF (Xpert). At that time, his sputum sample was sent for culture and extended DST, showing, among others, resistance to LZD and DLM. With support from the local coordination of the National TB Program North Kivu, the patient was recontacted in August 2023, yet the patient declined to resume treatment, stating that he was exhausted and no longer wanted to take conventional medications. He had been using traditional remedies. Prior exposure to LZD, DLM, or other nitroimidazoles was considered unlikely. Their initial diagnosis relied solely on sputum microscopy and Xpert MTB/RIF testing. The more advanced tests, such as pDST and WGS, didn't occur until 2023, primarily due to logistical reasons.

Methods

Culture, isolation, and drug susceptibility testing

Two sputum specimens were provided in 2 sterile screw-cap universal disposal containers. Ziehl-Nielsen slides and Xpert MTB/RIF assay were performed on the first sputum specimen, while culture, pDST, and TLA were performed on the second specimen. Primary isolation was performed in both liquid (BBL MGIT, Becton Dickinson, USA) and solid (in-house Lowenstein-Jensen) media, following standard protocols. Growth from both methods was confirmed using Bioline TB Ag MPT64 to identify the MTB complex before initiating the indirect DST with the MGIT AST/SIRE System (Becton Dickinson, Sparks, MD, USA). The critical concentration for RIF used in BBL™ MGIT™ Becton–Dickinson was 0.5 mg/L, as per the WHO guidelines [6]. The drug-containing tubes were prepared with critical concentrations of Linezolid (1.0 µg/mL) and Delamanid (0.016 µg/mL), as recommended by WHO. Control tubes without drug were also included. Monthly testing of H37Rv as a quality control strain had consistently shown correct DST results. Thin Layer Agar (TLA), a direct culture method, was also performed and showed growth [7].

Whole genome sequencing

Colonies from solid culture were harvested in 150 µL 0.5 M Tris-EDTA buffer and heat-killed, followed by genomic DNA extraction using an in-house optimized protocol [8]. WGS sequencing was outsourced to CDgenomics, applying the Illumina Novaseq PE150 strategy, which generated ~ 0.5 GB of data (~ 50x depth coverage).

For sequence analysis, non-MTBC reads were filtered out from Fastq datasets using Centrifuge v1.0.3 [9]. Reads mapping to any variant in the MTBC were retained. Reads were next mapped to the H37Rv reference genome

Table 1 Mutations found by the WGS and their interpretation by the WHO catalog V2

Drug	Mutation	% of reads by WGS	Interpretation by the WHO mutation catalog V2
INH	(katG_p.Tyr337Ser	99.37%	Variant is not present in the WHO catalog V2
DLM	fbiC_p.Pro843Ala	99.37%	Variant present in the WHO catalog V2 with "uncertain significance"
LZD	rplC_p.Ile57Val	99.37%	Variant is not present in the WHO catalog V2
Aminoglycosides	rrs_n.–187 C > T	99.37%	Variant is not present in the WHO catalog V2

MTBseq (9) using with default values, where the filter for variants was set at 75%. TB-Profiler v5.0.1 was used to define WGS-based resistance profiles with the tb db mutation database and re-analysed against the WHO catalog V2 [10]. Phylogenetic analysis was performed using a representative sample of isolates from various lineages and sub-lineages of M. tuberculosis [11]. The SNP alignment was input into RAxML-NG v1.2.0 [11], and the tree was built using a GTR + G model that accounted for constant sites in the genome, along with a Stamatakis ascertainment bias correction.

Results

Xpert showed resistance to RIF with a very low bacterial load. However, pDST done on samples using BACTEC MGIT 960, with H37Rv as sensitive control, showed resistance to INH, a core first-line drug, and to second-line drugs DLM and LZD, and susceptibility to RIF, BDQ, LFX, and CFZ. Direct culture, using the TLA test, showed resistance to RIF and INH, but susceptibility to LFX and BDQ. Subsequent WGS analysis showed resistance to INH (katG_p.Tyr337Ser mutation), to DLM (fbiC_p.Pro843Ala mutation) to LZD (rplC_p.Ile57Val mutation) and a possible resistance to aminoglycosides (rrs_n.–187 C > T mutation) (Table 1). The specific katG_p.Tyr337Ser variant is not mentioned in the WHO catalogue V2. However, katG_p.Tyr337Asp and katG_p.Tyr337Cys are mentioned at that codon position, but are considered "uncertain significance". The fbiC_p.Pro843Ala variant in the catalog with clofazimine, but again with "uncertain significance" (Table 1). The rrs_n.–187 C > T variant is not mentioned in the WHO catalogue V2 (Table 1). The isolate was identified as phylogenetically belonging to L4, between L4.6 and L.4.7, yet does not belong to any currently described sub-lineage.

Discussion and conclusion

The emergence of new mutations causing resistance to drugs used for rifampicin-resistant TB is of great concern in TB control. The development of resistance to Both LZD and DLM poses a significant threat to disease management, precluding the use of the WHO-recommended BPaLM regimen. The identification of previously unreported mutations highlights the dynamic nature of MTB resistance. It highlights the importance of understanding the selection pressures, raising concerns about their potential global spread and associated health implications. Comprehensive knowledge of the spectrum of mutations conferring resistance to these drugs in MTB and the resultant mutant phenotypes is critical for timely and accurate diagnosis and the design of optimal treatment, promoting the safe and effective use of these drugs in clinical settings.

The observed resistance may be due to novel mutations that have not yet been reported in global databases (like the WHO catalog of mutations). This is particularly relevant in geographically isolated settings or in cases of *de novo* acquisition of resistance under specific treatment pressures. Given the prior context of an inadequate initial regimen (due to false RR and true INH resistance), there could have been a strong selective pressure for the bacterium to adapt. This could have driven the selection of rare or novel resistance mechanisms.

Previous studies have described the spontaneous high-level resistance of *M. tuberculosis* (MTB) to the nitroimidazole prodrugs DLM and PMD through mutations that occur in one of the five genes previously associated with nitroimidazole activation and resistance, including the *fbiC* gene [8]. Their classification in the WHO catalog v2 [12] may vary. Still, they are categorized as group 2 mutations for DLM and PMD. They are associated with reduced susceptibility to both drugs and mutations affecting the biosynthesis of coenzyme F_{420}, the target of PMD and DLM. While resistance to LZD is linked with mutations in the *rplC* gene, these mutations may be classified as Category I or II mutations in the WHO catalogue, indicating varying levels of resistance to LZD. They can be associated with increased MIC values for LZD and mutations in the ribosomal protein L3, the target of LZD [12].

It is unknown whether higher doses of nitroimidazoles and LZD may overcome resistance to achieve the desired therapeutic effect without excess toxicity associated with especially LZD use [3]. Higher doses could lead to alterations in the metabolism of DLM and LZD within the bacterial cell, potentially impacting the ability of these drugs to target and inhibit specific enzymes or pathways involved in bacterial growth and survival [3].

Xpert provided a RR result at the start, while pDST and WGS yielded a discordant result. The discrepancies

observed between Xpert MTB/RIF and the pDST and WGS in rifampicin resistance detection were predominantly driven by delayed probe binding by Xpert MTB/RIF, in this case in sample with low bacillary load. Probe dropout or significantly delayed probe hybridization (indicated by elevated ΔCt values exceeding the recommended threshold of 4 cycles) can falsely indicate rifampicin resistance even in the absence of true mutations [13–15]. Other studies have found that the accuracy of the Xpert in detecting RR can be influenced by the amount of bacteria present in the sample. When there are very few bacteria (paucibacillary samples), the test may occasionally yield incorrect results indicating resistance [16].

This case report highlights the need for an active, ongoing surveillance and monitoring of emerging drug resistance in MTB, also in low resource settings, where limited access to healthcare, poverty and social determinants of health, and high burden of HIV/AIDS have been identified as key factors contributing to the rise of DR-TB [16]. Additionally, the lack of access to universal DST in resource-limited areas with a high prevalence of DR-TB poses a significant barrier to timely diagnosis, further exacerbating the spread of DR-TB within these communities. Moreover, the high burden of DR-TB in these regions underscores the importance of prioritizing resources and conducting targeted studies to understand the local drivers of DR-TB emergence and transmission [17]. By addressing these challenges and implementing comprehensive strategies, it is possible to mitigate the impact of DR-TB and improve treatment outcomes in these vulnerable populations.

In conclusion, the lengthy diagnostic journey outlined in the case study highlights the challenges in diagnosing drug resistance, and in supporting patients to take the full treatment course. As shown here, multiple diagnostic modalities are necessary to accurately characterize drug resistance profiles in MTB strains. The identification of baseline resistance to second-line drugs DLM and LZD emphasizes the evolving landscape of drug resistance in tuberculosis and the challenges in managing DR-TB.

Supplementary Information

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Supplementary Material 1.

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Clinical trial number
Not applicable.

Authors' contributions
BCB, EB, SC and BdJ contributed to the Bukavu approved version of the InnoR3TB protocol, study implementation, data collection, interpretation of results, drafting and revisions of the manuscript. MK, VB, J-CS N, CJM, WM, ICM, LR, FB, and MK contributed to data interpretation, revisions, and writing of the manuscript. All authors approved the final version of the manuscript.

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Data availability
Sequence data that support the findings of this study have been deposited in the European Nucleotide Archive with the primary accession code PRJEB85890 (https://www.ebi.ac.uk/ena/browser/text-search?query=PRJEB85890).

Declarations

Ethics approval and consent to participate
Ethics approval was received from the Institutional Ethics Committee of the Université Catholique de Bukavu (reference number UCB/CIES/NC/015/2022). The patient voluntarily agreed to participate in the study.

Consent for publication
The patient gave written informed consent for the publication of the data.

Competing interests
The authors declare no competing interests.

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