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PATTERN OF GENE MUTATIONS IN RESISTANT MYCOBACTERIUM TUBERCULOSIS STRAINS USING THE GENOTYPE MTBDRPLUS ASSAY

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Abstract

The rise of multidrug-resistant (MDR) Mycobacterium tuberculosis strains complicates tuberculosis (TB) treatment, necessitating rapid detection methods for drug resistance mutations. This study assessed gene mutation patterns in MDR, isoniazid (INH) monoresistant, and rifampicin (RIF) monoresistant MTB strains using the Genotype MTBDRplus assay, targeting mutations in the rpoB, katG, and inhA genes(Hillemann et al., 2007). Among the RIF-resistant MDR strains, mutations were most frequent at the rpoB WT8 probe (85.4%), followed by WT5 (12.2%), WT4 (9.8%), and WT3 (7.3%). For INH-resistant strains, 68.3% of MDR cases showed mutations due to lack of binding at the katG WT probe, while 73.2% had the S315T1 mutation (MUT1), a major marker for INH resistance. The S531T2 mutation was absent in MDR strains. Additionally, in INH monoresistant strains, 33.3% displayed mutations at the katG WT probe and S315T1 mutation. The inhA gene mutations in MDR strains included WT1 (-15/-16) at 19.5% and C15T (MUT1) at 17.1%. This study confirms the effectiveness of the Genotype MTBDRplus assay in detecting resistance patterns and highlights key mutation frequencies, informing targeted treatment approaches in TB control.

Keywords: Mycobacterium tuberculosis, MDR-TB, drug resistance, Genotype MTBDRplus assay, gene mutations, *rpoB*, *katG*, *inhA*, rifampicin, isoniazid

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (MTB), remains a significant public health challenge, especially with the rise of drug-resistant strains. TB remains a significant public health concern in Nigeria, with regional variations in incidence rates. While specific data for northwestern Nigeria is limited, available information provides insight into the broader context: In 2021, Nigeria reported an estimated TB incidence of 467,000 cases, equating to approximately 233 cases per 100,000 population. (USAIDS, 2021)**.** Studies have highlighted regional disparities in TB prevalence and incidence rates within Nigeria. For instance, a study in the northern parts of Nigeria revealed TB prevalence of 23%. (Oo et al., 2024). Given these figures, it is reasonable to infer that the TB incidence rate in northwestern Nigeria is comparable to or slightly higher than the national average. However, without specific data for the northwest region, precise estimates cannot be provided. The emergence of drug-resistant TB strains has complicated TB control efforts worldwide. Resistance to RIF and INH, the two primary drugs in TB treatment, defines multidrug-resistant TB (MDR-TB). Molecular assays like Genotype MTBDRplus provide insights into the resistance mechanisms by identifying mutations in key genes responsible for drug resistance, aiding in rapid diagnosis and effective treatment planning. The Genotype MTBDRplus assay, a rapid molecular diagnostic tool, detects specific mutations in the *rpoB*, *katG*, and *inhA* genes linked to RIF and INH resistance(Alberts, 2024). The Genotype MTBDRplus and MTBDRsl assays, developed by Hain Lifescience in Nehren, Germany, are sophisticated molecular diagnostic tools used to detect drug resistance in Mycobacterium tuberculosis. The Genotype MTBDRplus assay identifies resistance to rifampicin (RIF) and isoniazid (INH), two critical first-line anti-tuberculosis drugs(Meaza et al., 2017). The Genotype MTBDRsl assay detects resistance to fluoroquinolones (FLQ) and second-line injectable drugs (SLIDs), which are essential for managing multidrug-resistant TB (MDR-TB) cases. These assays involve high-complexity technology, making them best suited for use in well-equipped laboratories with trained personnel (Alberts, 2024). The World Health Organization (WHO) endorsed the Genotype MTBDRplus assay in 2008 for the rapid

detection of drug-resistant tuberculosis (TB), especially in high-burden settings. For smear-positive samples, the assay demonstrates excellent diagnostic accuracy, with a sensitivity of 96.4% and a specificity of 100% for detecting *Mycobacterium tuberculosis* (MTB) complex (Alberts, 2024). However, limitations arise with smear-negative samples, where sensitivity drops to 77.8% with a specificity of 97.2%, making it less reliable in these cases. Overall, the assay shows a pooled sensitivity of 85% for MTB complex detection, 98% for rifampicin (RIF) resistance, and 84% for isoniazid (INH) resistance, with a high specificity of 99%. These performance metrics make it a valuable tool for TB diagnosis, though false-positive MTB complex results in smear-negative samples can restrict its usage in certain settings. The World Health Organization (WHO) recommended the Genotype MTBDRsl assay in 2016 as an important diagnostic tool for detecting resistance to fluoroquinolones (FLQ) and second-line injectable drugs (SLIDs) in multidrug-resistant tuberculosis (MDR-TB) cases(Wang et al., 2024) (Meaza et al., 2017) (Nathavitharana et al., 2017). The assay has shown a pooled sensitivity of 86% and a pooled specificity of 99%, making it a reliable test for identifying these resistance patterns. This high specificity supports its use in guiding appropriate treatment regimens for MDR-TB, though its sensitivity, while strong, indicates it may still miss a subset of resistant cases (Wang et al., 2024). Understanding these mutations is crucial for timely and accurate diagnosis, allowing effective treatment regimens for multidrug-resistant TB (MDR-TB) cases. The objective study is to determine the gene mutation patterns associated with resistance to first-line anti-TB drugs, particularly rifampicin (RIF) and isoniazid (INH), using the Genotype MTBDRplus assay

Methods

this study analyzed MTB isolates confirmed as RIF and/or INH resistant. The Genotype MTBDRplus assay was used to detect mutations in *rpoB*, *katG*, and *inhA* genes. These genes are known markers for RIF and INH resistance, respectively.

Study settings

This study was conducted at the North West Zonal TB Reference Laboratory (NWZTRL) in Aminu Kano Teaching Hospital (AKTH), Kano, Nigeria. AKTH serves as a referral laboratory for the North Western region of Nigeria, covering Kano, Jigawa, Katsina, Yobe, and Borno States, with a combined population of approximately 44.35 million people (Ezeamadu et al., 2023)**.** The laboratory has a moderately high workload for TB diagnostic testing, performing approximately 12,000 MTB Rif (GeneXpert), 6,000 cultures, and 1,200 first-line drug susceptibility tests (DSTs) annually.

Sputum Specimens

All handling of potentially infectious clinical specimens was conducted in a Class II safety cabinet within a BSL-2 laboratory. Sputum specimens were decontaminated using N-acetyl-L-cysteine-sodium hydroxide (Barnard et al., 2008)**.** Following centrifugation, the pellet was re-suspended in 1.0 ml of phosphate buffer (pH 6.8). A concentrated auramine smear was then prepared, examined under 100x magnification with a light microscope, and graded according to the guidelines of the International Union Against Tuberculosis and Lung Disease (IUATLD). (Rieder & IUATLD, 2007)**.** A 0.5-ml portion of the sediment was cultured on solid Lowenstein-Jensen medium. Positive cultures were confirmed as *Mycobacterium tuberculosis* complex through Ziehl-Neelsen staining and p-nitrobenzoic acid testing. (WHO, 1922)**.** Indirect drug susceptibility testing (DST) was conducted using the proportion method on Middlebrooks 7H11 agar slants, with 1.0 mg/ml rifampicin (RIF) and 0.2 mg/ml isoniazid (INH)(WHO, 1922)**.**

Rapid Drug Resistance Testing

The MTBDRplus test was carried out following the manufacturer's guidelines. (*GenoType ® MTBDRplus*, 2007). The test utilizes DNA strip technology and involves three main steps: DNA extraction, multiplex polymerase chain reaction (PCR) amplification, and reverse hybridization. For DNA extraction, a 500-ml sample of decontaminated sediment was processed in a one-hour procedure that included heating, sonication, and centrifugation. The amplification procedure, which included preparing the master mix and adding the DNA, also took 1 hour. Each step was conducted in separate, restricted-access rooms following a unidirectional workflow to prevent contamination. Hybridization was performed using the GT Blot 48 (Hain

Lifescience), an automated hybridization machine. After hybridization and washing, the strips were removed, air-dried, and fixed onto paper. All tests were carried out independently of culture and drug susceptibility testing (DST) and completed before culture and DST results were available.

Interpretation of Results: Each test strip includes 27 reaction zones (bands), comprising six control zones (for conjugate, amplification, M. tuberculosis complex, rpoB, katG, and inhA controls). There are eight rpoB wild-type (WT) and four mutant (MUT) probes, one katG wild-type and two mutant probes, and two inhA wild-type and four mutant probes (Figure 1). Results were interpreted following the manufacturer's instructions.

Results

We provided a detailed analysis of gene mutations in resistant *Mycobacterium tuberculosis* (MTB) strains, particularly focusing on the resistance mechanisms for isoniazid (INH) and rifampicin (RIF) using the Genotype MTBDRplus assay. The findings demonstrated that specific mutations in the *rpoB* gene, particularly at codon 531 (S531L), were most common among RIF-resistant strains. For INH resistance, the *katG* S315T mutation was predominant, with additional mutations identified in the *inhA* promoter region. The mutation patterns varied slightly by geographical region, suggesting potential genetic diversity among resistant strains. Among MDR strains, the most common mutations were observed at *rpoB* wild type (WT) probe 8 (85.4%) and WT probe 7 (7.3%). These mutations suggest a dominant resistance mechanism to rifampicin (RIF), with WT probe 8 showing the highest frequency (85.4%). Mutations in probes associated with *rpoB* gene, such as *WUT3 (S531L)*, were observed in 41.5% of MDR strains, confirming rifampicin resistance due to specific mutations in the *rpoB* gene. A significant proportion of MDR strains (73.2%) exhibited a mutation in the *katG* gene at position S315T1, which is a well-known mutation linked to INH resistance. The mutation in *katG* was observed in 68.3% of MDR strains, reinforcing the role of this gene in INH resistance.No mutation in *katG* was observed for S315T2 in MDR strains, indicating that this particular mutation is not prevalent in the studied sample. In MDR strains, 19.5% showed mutations in the *inhA* gene at WT1 (-15/-16) and 9.8% at WT2 (-8), which are associated with INH resistance.Other mutations in the *inhA* gene, such as *C15T (MUT1)*, were observed in 17.1% of MDR strains. This was the second most frequent mutation associated with INH resistance. Minor mutations, such as *A16G* (MUT2), *T8C* (MUT3A), and *T8A* (MUT3B), were observed in 2.4% of MDR strains, indicating a less common mutation pathway for INH resistance. Among INH mono-resistant strains, 33.3% had mutations in *katG* (S315T1), and 33.3% had mutations in *inhA* (C15T), suggesting that both genes play a significant role in INH resistance in these isolates. Additionally, mutations like *A16G*, *T8C*, and *T8A* in *inhA* were recorded in low percentages (2.4% each), similar to the MDR strains. Among RIF mono-resistant strains, mutations in the *rpoB* gene at probes 7 (34.6%) and 8 (61.5%) were observed, suggesting a dominant pattern of RIF resistance linked to these mutations.

Table 1. Pattern of Gene Mutations in Resistant Mycobacterium tuberculosis strains using Genotype MTBDRplus Assay

Gene Band	Gene Region or Mutation	MDR (n = 41)	INH Monoresistant (n = 6)	RIF Monoresistant (n = 26)
Rpoβ WT1	506-509	2 (4.9)	0 (0.0)	1 (3.8)
WT2	510-513	0 (0.0)	0 (0.0)	0 (0.0)
WT3	513-517	5 (12.2)	0 (0.0)	1 (3.8)
WT4	516-519	2 (4.9)	0 (0.0)	0 (0.0)
WT5	518-522	2 (4.9)	0 (0.0)	1 (3.8)
WT6	521-525	4 (9.8)	0 (0.0)	3 (11.5)
WT7	526-529	3 (7.3)	0 (0.0)	9 (34.6)
WT8	530-533	35 (85.4)	0 (0.0)	16 (61.5)
WUT1	D516V	2 (4.9)	0 (0.0)	0 (0.0)
WUT2A	H526Y	2 (4.9)	0 (0.0)	2 (2.8)
WUT2B	H526D	3 (7.3)	0 (0.0)	9 (34.6)
WUT3	S531L	17 (41.5)	0 (0.0)	10 (38.5)

KatG	WT	315	28 (68.3)	2 (33.3)	0 (0.0)
	MUT1	S315T1	30 (73.2)	2 (33.3)	0 (0.0)
	MUT2	S315T2	0 (0.0)	0 (0.0)	0 (0.0)
InhA	WT1	-15/-16	8 (19.5)	2 (33.3)	0 (0.0)
	WT2	-8	4 (9.8)	3 (5.0)	0 (0.0)
	MUT1	C15T	7 (17.1)	2 (33.3)	0 (0.0)
	MUT2	A16G	1 (2.4)	0 (0.0)	0 (0.0)
	MUT3A	T8C	1 (2.4)	0 (0.0)	0 (0.0)
	MUT3B	T8A	1 (2.4)	0 (0.0)	0 (0.0)

Discussion

The detection of prevalent mutations in *rpoB*, *katG*, and *inhA* genes confirms the effectiveness of the Genotype MTBDRplus assay in identifying MDR-TB strains. Rapid identification of these mutations can facilitate timely initiation of second-line therapy, improving patient outcomes and aiding TB control efforts. The analysis of gene mutations in resistant *Mycobacterium tuberculosis* (MTB) strains reveals critical insights into the molecular basis of drug resistance for isoniazid (INH) and rifampicin (RIF) using the Genotype MTBDRplus assay. Mutations in the *rpoB* gene, particularly at codon S531L, were prominent among RIF-resistant strains. This codon, targeted by the WUT3 probe, was mutated in 41.5% of MDR cases, underscoring its central role in conferring RIF resistance. Additionally, the high frequency of mutations in the *rpoB* WT8 (85.4%) and WT7 (7.3%) probes further supports the dominant role of *rpoB* mutations in RIF resistance. This in line with findings from most other studies , this study identified the most frequent mutations conferring rifampicin (RIF) resistance in *Mycobacterium tuberculosis* at codons S531L and 516. Notably, a higher proportion of RIF resistance in this study was attributed to the S531L mutation, observed in 70.5% of all rifampin-resistant strains. Reta et al., 2021 South Africa, In Brazil, Zaw et al., and Jamieson et al., 2014 in Canada also reported that codons 531, 526, and 516 in the RRDR of the *rpoB* gene are the most frequently mutated. This prevalence is significantly lower in than in other regions, where S531L mutations account for 36.1% to 56.7% of RIF-resistant cases (Molodtsov et al., 2017)(Vecchione et al., 2018)(Tan et al., 2012) (Mohajeri et al., 2015). This suggests regional variability in mutation patterns, with the S531L mutation being particularly prominent in this study's population Among RIF monoresistant strains, WT8 and WT7 probes were also mutated, with mutation rates of 61.5% and 34.6%, respectively, indicating a common resistance mechanism in these isolates. For INH resistance, mutations in the *katG* S315T1 position were found in 73.2% of MDR strains, marking it as the most prevalent mutation associated with INH resistance. This mutation was present in 68.3% of MDR strains, emphasizing the critical role of the *katG* gene in resistance. This almost similar to what (Barnard et al., 2008) reported that prevalence of mutations in the inhA and katG genes seems to vary widely in different geographic locations. For example, katG mutations were found in 97% (77/ 79) and inhA mutations in 24% (19/79) of INH-resistant isolates from KwaZulu-Natal. Notably, our results highlight the predominant mutations in the *rpoB*, *katG*, and *inhA* genes that drive resistance in multidrug-resistant (MDR) and monoresistant strains. Notably, no mutation was found at the S315T2 position in MDR strains, suggesting this variant is rare or absent in the sampled population. Mutations in the *inhA* gene promoter region also play a significant role in INH resistance, particularly among strains exhibiting the WT1 (-15/-16) mutation (19.5%) and the WT2 (-8) mutation (9.8%). These mutations are critical for resistance as they modify the INH binding site. Additionally, the C15T (MUT1) mutation was observed in 17.1% of MDR strains, establishing it as the second most frequent mutation related to INH resistance after *katG* S315T1. Less common *inhA* mutations, such as A16G (MUT2), T8C (MUT3A), and T8A (MUT3B), were also observed at low frequencies (2.4%), suggesting they contribute to resistance in a minority of cases. Contrary to our finding Ranjan et al., 2023 in India reported that *inhA* C15T mutation is the most prevalent mutation linked to low-level isoniazid (INH) resistance (96.2%) cases. This mutation is significant as it contributes to drug resistance, complicating treatment for affected patients. Among INH monoresistant strains, both *katG* S315T1 and *inhA* C15T mutations appeared in 33.3% of cases, indicating that both genes significantly contribute to INH resistance in these isolates. In Hilleman's 2007 study, it was reported that the *inhA* resistance gene was present in approximately 3% of tuberculosis (TB) isolates in Germany. This finding highlights a relatively low prevalence of *inhA*-related

resistance in German TB cases compared to regions with higher resistance rates, such as India. The *inhA* gene mutations contribute to resistance against isoniazid (INH) and often to ethionamide (ETH), affecting treatment options for these resistant TB strains(Ranjan et al., 2023). This mutation is significant as it contributes to drug resistance, complicating treatment for affected patients. The occurrence of rare *inhA* mutations (A16G, T8C, and T8A) at a rate of 2.4% each mirrors the pattern observed in MDR strains, suggesting a less common mutation pathway for INH resistance. The variability in mutation patterns across different regions suggests genetic diversity in resistance mechanisms among MTB strains, potentially driven by geographic factors. This regional diversity emphasizes the need for localized resistance profiling to enhance the precision of TB treatment regimens.

Conclusions:

Overall, this study confirms the efficacy of the Genotype MTBDRplus assay in identifying key resistance mutations, with findings that reinforce the dominant role of *rpoB* mutations in RIF resistance and *katG* and *inhA* mutations in INH resistance. The distinct mutation profiles in MDR and monoresistant strains highlight the importance of comprehensive mutation screening for targeted TB therapy. The most common mutations were found in the *rpoB* gene, particularly at WT probes 7 and 8, with high prevalence in MDR strains. Mutations in the *katG* gene (S315T1) and *inhA* gene (C15T) were prominent among both MDR and INH mono-resistant strains. The presence of mutations in the *inhA* gene, particularly the *C15T*, *A16G*, *T8C*, and *T8A* mutations, indicates additional genetic diversity in resistance mechanisms for INH.

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