



MOLECULAR CHARACTERIZATION OF GENE MUTATIONS ASSOCIATED WITH ISONIAZID AND RIFAMPICIN RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS ISOLATES USING THE GENOTYPE MTBDRPLUS ASSAY IN KANO, NIGERIA

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ABSTRACT

The rise of multidrug-resistant tuberculosis (MDR-TB), particularly strains resistant to rifampicin (RIF) and isoniazid (INH), poses a major challenge to TB control efforts in Nigeria. Rapid identification of resistance-conferring mutations is critical for timely and effective treatment. To determine the pattern of gene mutations associated with resistance to first-line anti-TB drugs using the Genotype MTBDRplus assay in *Mycobacterium tuberculosis* isolates from Kano State Nigeria, alongside socio-demographic and culture-based insights. This study was carried out at the North-West Zonal Tuberculosis Reference Laboratory, Aminu Kano Teaching Hospital (AKTH), Kano. Socio-demographic information of the study participants was obtained using structured data collection tools. A total of 401 sputum specimens were cultured, and the resulting *Mycobacterium tuberculosis* isolates were analyzed using the Genotype MTBDRplus assay to identify resistance-associated mutations in the *rpoB*, *katG*, and *inhA* genes. The distribution of mutation frequencies was subsequently compared across multidrug-resistant (MDR-TB), rifampicin monoresistant, and isoniazid monoresistant strains. Culture and drug susceptibility testing revealed MDR-TB in 41 (10.2%) patients, RIF monoresistance in 18 (4.5%), and INH monoresistance in 6 (1.5%). Males constituted 71.6% of participants, and the predominant age group was 25–34 years (35.4%). The most common mutations in MDR strains were *rpoB* S531L (41.5%), *katG* S315T1 (73.2%), and *inhA* C15T (17.1%). The *rpoB* WT8 probe was affected in 85.4% of MDR and 61.5% of RIF monoresistant strains. Among INH monoresistant isolates, mutations were equally distributed between *katG* S315T1 and *inhA* C15T (33.3% each). Less frequent *inhA* mutations such as A16G, T8C, and T8A were also observed. The Genotype MTBDRplus assay effectively detects resistance-associated mutations in MTB. The integration of molecular and demographic data offers a comprehensive understanding of MDR-TB epidemiology. The high prevalence of *rpoB* and *katG* mutations supports the assay's role in guiding rapid MDR-TB diagnosis and region-specific treatment strategies in Nigeria.

Keywords: Mycobacterium tuberculosis, MDR-TB, Genotype MTBDRplus, drug resistance, *rpoB*, *katG*, *inhA*, rifampicin, isoniazid, socio-demographics, culture

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INTRODUCTION

Tuberculosis (TB) remains one of the world's leading infectious diseases, with an estimated 10.6 million new cases and 1.3 million deaths reported in 2022 [1]. Nigeria ranks among the 30 high TB burden countries globally, contributing significantly to the incidence of drug-resistant TB (DR-TB) [2]. The emergence of multidrug-resistant tuberculosis (MDR-TB), defined as resistance to at least isoniazid (INH) and rifampicin (RIF), poses a major threat to global TB control and complicates treatment outcomes [3].

Kano State, located in northwestern Nigeria, represents a unique epidemiological setting due to its high population density, urban–rural mix, and extensive migration and cross-border mobility [4]. According to the National Tuberculosis and Leprosy Control Programme (NTBLCP), the state contributes substantially to Nigeria's TB burden [5]. While national reports provide estimates of TB and MDR-TB prevalence, there is a paucity of subnational data describing the magnitude of resistance and its molecular determinants in Kano State.

Advances in molecular diagnostics, such as the Genotype® MTBDRplus assay, now enable rapid detection of drug resistance-associated mutations in *Mycobacterium tuberculosis*, targeting critical regions of the *rpoB*, *katG*, and *inhA* genes [6–8]. These tools complement conventional culture and drug susceptibility testing (DST) methods and provide timely information essential for patient management and surveillance. However, their use remains under-reported in many parts of Nigeria, including Kano.

Socio-demographic and clinical risk factors, including male sex, previous TB treatment, HIV co-infection, and poor adherence to therapy, have been consistently linked with rifampicin resistance and MDR-TB [9–12]. Given this context, the present study was conducted to determine the prevalence of MDR-TB in Kano State using Lowenstein–Jensen (LJ) culture and DST, and to characterize the molecular mutations conferring resistance to INH and RIF. By integrating socio-demographic, clinical, and molecular data, this study provides important insights to guide TB control strategies and improve treatment outcomes in high-burden Nigerian settings.

MATERIALS AND METHODS

Study setting and population

This study was conducted in Kano State, located in northwestern Nigeria (latitude 11°30'N, longitude 8°30'E). Kano, one of Nigeria's most populous states (>15 million; 2023 projection), comprises 44 Local Government Areas (LGAs) and experiences a high burden of tuberculosis (TB) [13]. Its population density, urban-rural mix, and cross-border mobility create a distinct epidemiological setting relevant to TB transmission [14]. The study population comprised patients diagnosed with smear-positive pulmonary tuberculosis (PTB) attending Directly Observed Treatment Short-course (DOTS) clinics across the state's 44 LGAs between January 2018 and December 2019. Nineteen strategically located DOTS clinics served as diagnostic hubs covering all LGAs.

Study design

A cross-sectional study design was employed to determine the prevalence of multidrug-resistant tuberculosis (MDR-TB) and molecular characterization of gene mutations among smear PTB patients in Kano State, Nigeria.

Sample size determination

The minimum sample size was calculated using Cochran's formula [15] for a cross-sectional study design.

$$n = Z^2 \cdot p \cdot q / d^2$$

Where:

- $Z = 1.96$ (for 95% confidence level)
- $p = 0.27$ (estimated TB prevalence in Nigeria [16])
- $q = (1 - p) = 0.73$
- $d = 0.05$ (5% margin of error)

This yielded a baseline sample size of $n = 303$. To account for methodological complexities including anticipated attrition, non-response bias, and cluster design effects inherent in the multi-site sampling strategy, a design effect multiplier of 1.51 [17, 18] was applied. This adjustment, consistent with recommendations for accommodating non-response [19], elevated the target sample size to 458 participants. However, only 401 participants responded for the survey.

Ethical considerations

Ethical approval was obtained from the Kano State Ministry of Health and Aminu Kano Teaching Hospital Ethics Committees. Written informed consent/assent was obtained. Data were anonymized, and laboratory procedures followed strict biosafety protocols.

Sampling technique and participant recruitment

Proportionate stratified sampling was used. The 19 DOTS clinics served as strata. Sample size allocation per clinic was proportionate to the clinic's patient load, based on the 2018 Kano State TB and Leprosy Control Programme (KSTBLCP) report, ensuring representation across the state. Smear-positive PTB patients aged 5–88 years presenting at the participating DOTS clinics during the study period were eligible. Despite implementing tailored contact protocols and incentives to maximize response rates [20], the final analysable sample comprised 401 participants.

Data collection

Questionnaire administration: Trained personnel administered a structured questionnaire to participants (or guardians for minors) between January 2019 and December 2021, collecting data on demographics, socioeconomic status, HIV status, history of TB infection (previous 2 years), history of TB treatment, comorbidities (e.g., diabetes), and behavioral factors (e.g., alcohol consumption, injection drug use). Written informed consent was obtained prior to any data or specimen collection.

Sputum specimen collection: Two early morning sputum samples (approx. 5 mL each, expectorated sputum, not saliva) were collected within one week from each participant into clean, sterile, leak-proof containers [21].

Specimen transport and storage: Samples were placed in primary sealable biohazard bags, then into secondary sealable biohazard bags, and transported upright in double-walled containers maintaining a cool

temperature (as low as possible) [21]. Batches of 15–20 specimens were transported promptly. Upon arrival at the laboratory (Aminu Kano Teaching Hospital), specimens were refrigerated (2–8°C) and processed as soon as possible accordingly.

Laboratory processing and analysis

All laboratory procedures were conducted at the Aminu Kano Teaching Hospital.

- **Digestion and decontamination:** The NALC-NaOH method was used [22, 23]. Sputum was transferred to a 50 mL centrifuge tube if necessary. An equal volume of NALC-NaOH solution was added, vortexed, and incubated. After treatment, PBS was added to 50 mL, vortexed, and centrifuged at 3000 g for 20 minutes (4°C). The supernatant was decanted, and the sediment was re-suspended in 2 ml PBS.

Lowenstein-Jensen culture procedure

The Lowenstein-Jensen (LJ) medium is a traditional egg-based culture medium used for the isolation and drug susceptibility testing (DST) of *Mycobacterium tuberculosis* (MTB). Although it is highly sensitive and remains a reference standard, it is technically demanding, requires biosafety precautions, and has a long turnaround time [24, 25].

In this study, sputum specimens were processed using the standard NALC-NaOH method. After centrifugation, sediments were inoculated onto LJ slopes—drug-free and drug-containing media. Inoculations were done in a Class II biosafety cabinet. Cultures were incubated at 35–37°C and examined weekly for up to eight weeks. MTB colonies were identified by rough, buff-colored, cauliflower-like morphology. Contaminated cultures were discarded. Drug susceptibility testing (DST) was performed using the proportion method on LJ medium containing critical concentrations of isoniazid, rifampicin, and ethambutol. Strains were considered resistant if >1% of the bacterial population grew in drug-containing media.

Despite its long turnaround time, the LJ method remains valuable in resource-limited settings due to low cost and sensitivity, but requires BSL-2 infrastructure and skilled personnel [24, 25].

Molecular detection of drug resistance using Genotype® MTBDRplus Assay

The Genotype® MTBDRplus version 2.0 assay (Hain Lifescience GmbH, Nehren, Germany) was utilized for detecting mutations associated with rifampicin (RIF) and isoniazid (INH) resistance in *M. tuberculosis* isolates. It is endorsed by the WHO for rapid detection of MDR-TB, targeting *rpoB*, *katG*, and *inhA* genes [26, 27].

DNA extraction: MTB DNA was extracted from LJ-positive isolates by heating colonies at 95°C for 30 minutes. Supernatant containing DNA was used as template. To minimize the risk of contamination, all sputum samples were processed using the standard N-acetyl-L-cysteine/sodium hydroxide (NALC/NaOH) decontamination method in accordance with Centers for Disease Control and Prevention (CDC) guidelines. Sample processing and molecular procedures were conducted in designated work areas free from amplified DNA to prevent cross-contamination. Specimens were heat-treated at 95–105 °C for a minimum of 15 minutes to ensure complete cell lysis and inactivation of vegetative bacteria. Molecular biology-grade water was used throughout all procedures, and centrifugation was performed using aerosol-tight rotors. All manipulations were carried out within a Class II biosafety cabinet under strict aseptic conditions. [26, 27].

Multiplex PCR amplification: PCR used primers from the kit targeting RRDR of *rpoB*, codon The GenoType MTBDRplus assay uses proprietary multiplex PCR primers to amplify resistance-associated regions of the *rpoB* gene (codons ~507–533), *katG*, and the *inhA* promoter, followed by hybridization to membrane-bound probes to detect wild-type and resistance-conferring mutations; the primer sequences are proprietary and not publicly disclosed. 315 of *katG*, and *inhA* promoter region. [26, 27]. Standard cycling conditions were applied [26, 27].

Hybridization and detection: The GenoType MTBDRplus assay is based on reverse hybridization of biotin-labeled PCR amplicons to specific oligonucleotide probes immobilized on nitrocellulose membrane strips. Detection is performed using a streptavidin-alkaline phosphatase conjugate and a chromogenic substrate, producing visible bands for interpretation. Successful amplification and assay validity were confirmed by the presence of internal amplification and conjugate control bands on each strip, as recommended by the manufacturer; external verification of amplicon size by gel electrophoresis was not required. Amplified DNA was hybridized on nitrocellulose strips with immobilized WT and MUT probes. Detection used streptavidin-alkaline phosphatase. Banding patterns were interpreted visually. Mutations in *rpoB* (notably S531L), *katG* (S315T1), and *inhA* (C15T) were predominant.

Statistical analysis plan and sample size discrepancy

The study analyzed cross-sectional laboratory and epidemiological data comprising mainly categorical variables, which were summarized using frequencies and percentages. Continuous variables were presented as means (\pm SD) or medians (IQR), as appropriate. The discrepancy between the design-effect-adjusted target sample size ($n = 458$) and the achieved sample size ($n =$

401) was assessed for its potential impact on statistical power and precision, and sensitivity analyses were considered. Statistical analyses were conducted using SPSSv25 with statistical significance set at $p < 0.05$ [28, 29].

RESULTS

A total of 401 participants were enrolled in the study. The mean age was 35.0 ± 14.8 years, with the majority falling within the 25–34-year age group (35.4%), followed by 35–44 years (20.2%). Participants aged 5–14 years constituted only 3.0%, while those aged 65 years and above accounted for 6.2%. Males were predominant (71.6%), giving a male-to-female ratio of approximately 2.5:1.

Most respondents were married (58.9%), while 41.1% were single. Nearly all participants were of Hausa ethnicity (98.8%), with a few belonging to the Yoruba, Idoma, and Igbo ethnic groups. Regarding educational attainment, half (50.9%) had tertiary education, 35.2% had secondary education, 7.7% had primary education, while 6.2% had no formal education.

In terms of occupation, self-employment was the most common (45.6%), followed by students (31.2%) and wage-employed individuals (14.0%). A smaller proportion were unemployed (5.7%) or retired (3.5%). For monthly income, 45.6% earned between ₦18,000 and <₦35,000, while 24.4% earned below ₦18,000. About 17.5% earned ₦35,000–<₦70,000, 10.5% earned ₦70,000–₦120,000, and only 2.0% earned above ₦120,000 Table 1.

Table 1: Socio-demographic characteristics of study participants

Age group (years)	Category	Frequency (n=401)	Percentage (%)
	5 – 14	12	3.0
	15 – 24	75	18.7
	25 – 34	142	35.4
	35 – 44	81	20.2
	45 – 54	36	9.0
	55 – 64	30	7.5
	≥65	25	6.2
	Mean ± SD	35.0 ± 14.8	—
Sex	Male	287	71.6
	Female	114	28.4
Marital status	Single*	165	41.1
	Married	236	58.9
Tribe	Hausa	396	98.8
	Yoruba	3	0.7
	Idoma	1	0.2
	Igbo	1	0.2
Educational attainment	No formal education	25	6.2
	Primary	31	7.7
	Secondary	141	35.2
	Tertiary	204	50.9
Occupation	Employed for wages	56	14.0
	Self-employed	183	45.6
	Retired	14	3.5
	Student	125	31.2
	Unemployed	23	5.7
Monthly income (₦)	< 18,000	98	24.4
	18,000 – < 35,000	183	45.6
	35,000 – < 70,000	70	17.5
	70,000 – 120,000	42	10.5

Age group (years)	Category	Frequency (n=401)	Percentage (%)
	> 120,000	8	2.0

Prevalence of MDR-TB in Kano State, Nigeria

Table 2 shows prevalence of MDR-TB in Kano State Nigeria. Out of a total of 401 tuberculosis (TB) patients evaluated using Lowenstein-Jensen (LJ) culture and drug susceptibility testing (DST), 41 patients (10.2%) were found to have multidrug-resistant TB (MDR-TB), defined as resistance to at least isoniazid (INH) and rifampicin (RIF).

A pronounced sex disparity was observed in MDR-TB prevalence. Males accounted for 85.4% (36/41) of the MDR-TB cases, while females represented only 14.6% (5/41). This suggests a significantly higher burden of MDR-TB among male patients, which may reflect differences in exposure risks, treatment adherence, or healthcare-seeking behavior.

For monoresistance patterns, a total of 6 patients (1.5%) had INH monoresistance, with 4 (66.7%) cases occurring in males and 2 (33.3%) in females. Similarly, 18 patients (4.5%) exhibited RIF monoresistance, with 12 (66.7%) among males and 6 (33.3%) among females.

No cases of ethambutol (ETH) monoresistance were recorded in either sex, indicating a continued effectiveness of this drug in the study population. Overall, these findings demonstrate a notable burden of MDR-TB among TB patients in Kano State, with a marked male predominance and a relatively low level of monoresistance to isoniazid and rifampicin when considered separately. The absence of ETH monoresistance is a positive indicator for its continued role in TB treatment regimens Table 2.

Table 2: Prevalence of MDR-TB among TB patients in Kano state by Sex using LJ culture and DST (%)

Sex	MDR-TB (%)	INH mono (%)	RIF mono (%)	ETH mono (%)
Males	36 (85.3)	4 (66.7)	12 (66.7)	0 (0.0)
Females	5 (14.7)	2 (33.3)	6 (33.3)	0 (0.0)
Total	41 (10.2)	6 (1.5)	18 (4.5)	0 (0.0)

Molecular characterization of drug-resistant tuberculosis in Kano State

The molecular characterization of drug-resistant *Mycobacterium tuberculosis* isolates was assessed by analyzing mutations in three key genes—*rpoB*, *katG*, and *inhA*—associated with resistance to rifampicin (RIF) and isoniazid (INH) as shown in Table 3. The analysis covered multidrug-resistant TB (MDR-TB), INH monoresistant, and RIF monoresistant strains among 41 MDR-TB, 6 INH monoresistant, and 26 RIF monoresistant isolates.

rpoB Gene (Rifampicin resistance): Mutations within the *rpoB* gene, especially in the RIF resistance-determining region (RRDR), were prevalent among both **MDR and RIF monoresistant isolates:** WT8 (codons 530–533) was the most frequently affected wild-type region, with 85.4% mutation loss in MDR-TB and 61.5% in RIF monoresistant cases. MUT3 (S531L), a well-documented mutation linked with RIF resistance, was detected in 41.5% of MDR-TB and 38.5% of RIF monoresistant isolates. Other affected wild-type probes included WT3 (513–517) and WT7 (526–529), with respective mutation frequencies of 12.2% and 7.3% among MDR cases, and notably 34.6% and 3.8% among RIF monoresistant isolates. The WT1 (506–509) region was less frequently mutated, occurring in 4.9% of MDR and 3.8% of RIF monoresistant isolates. No *rpoB* mutations were observed in INH monoresistant isolates, as expected.

The *katG* gene, particularly the S315T1 (MUT1) mutation, was the dominant mutation associated with INH resistance: MUT1 (S315T1) was identified in 73.2% of MDR-TB and 33.3% of INH monoresistant isolates. Correspondingly, loss of wild-type (WT) *katG* signal at codon 315 was observed in 68.3% of MDR-TB and 33.3% of INH monoresistant isolates. As expected, *katG* mutations were absent in RIF monoresistant strains. *inhA* Promoter Region (Low-level INH Resistance): Mutations in the *inhA* promoter region were observed at a lower frequency: The most common *inhA* mutation among MDR-TB and INH monoresistant isolates was MUT1 (C15T), occurring in 17.1% and 33.3%, respectively. Other *inhA* mutations such as MUT2 (A16G), MUT3A (T8C), and MUT3B (T8A) were each present in 2.4% of MDR-TB isolates only. Mutation losses in the WT1 (-15/-16) region were detected in 19.5% of MDR and 33.3% of INH monoresistant cases. No *inhA* mutations were found among RIF monoresistant isolates Table 3.

Table 3: Detection of *rpoB*, *katG*, and *inhA* mutations in drug-resistant tuberculosis

Gene	Probe/Mutation	MDR (n=41)	INH Mono-resistant (n=6)	RIF Mono-resistant (n=26)
rpoB	WT1 (506-509)	4.9%	0.0%	3.8%
	WT3 (513-517)	12.2%	0.0%	3.8%
	WT7 (526-529)	7.3%	0.0%	34.6%
	WT8 (530-533)	85.4%	0.0%	61.5%
	MUT3 (S531L)	41.5%	0.0%	38.5%
katG	WT (315)	68.3%	33.3%	0.0%
	MUT1 (S315T1)	73.2%	33.3%	0.0%
inhA	WT1 (-15/-16)	19.5%	33.3%	0.0%
	MUT1 (C15T)	17.1%	33.3%	0.0%
	MUT2 (A16G)	2.4%	0.0%	0.0%
	MUT3A (T8C)	2.4%	0.0%	0.0%
	MUT3B (T8A)	2.4%	0.0%	0.0%

DISCUSSION

In this study, socio-demographic and clinical risk factors, including male sex, previous TB treatment, HIV co-infection, and poor adherence to therapy, have been consistently linked with rifampicin resistance and MDR-TB [9–12]. Our findings corroborate these associations, with significant predictors identified in relation to treatment interruption, multiple pulmonary TB (PTB) episodes, and exposure to MDR-TB patients. The majority of participants in this study were within the productive age group (25–44 years), with a mean age of 35.0 ± 14.8 years, consistent with global TB epidemiology showing TB predominantly affects economically active populations [30]. The male predominance (71.6%) mirrors findings from other Nigerian [31, 32] and sub-Saharan African studies [33], possibly linked to occupational exposure, mobility, and delayed health-seeking.

A higher proportion of patients had secondary (35.2%) and tertiary (50.9%) education compared to studies from other Nigerian regions, where illiteracy was more common among TB patients [34]. Economically, most participants were self-employed (45.6%), and nearly 70% earned below ₦35,000 per month, indicating vulnerability to poor treatment adherence and adverse outcomes [35].

The MDR-TB prevalence of 8.5% observed here is higher than the 4.3% national estimate reported by the Nigeria TB Burden Survey [36] and other reports from northern Nigeria [37, 38]. This may reflect improved case detection through expanded DST and molecular diagnostics. The male predominance (85.3%) among MDR-TB cases is consistent with risk patterns where smoking, incomplete prior treatment, and

diagnostic delays are more common among men [39]. Importantly, no ethambutol mono-resistance was detected, supporting its continued role in first-line therapy in this region.

Molecular analysis confirmed global patterns of resistance mutations. The *rpoB* S531L mutation was the most frequent, detected in 41.5% of MDR-TB and 38.5% of RIF-mono-resistant isolates. This finding aligns with Nigerian [40, 41] and international studies [42, 43] that identify S531L as the predominant rifampicin-resistance mutation. Similarly, *katG* S315T1 was identified in 73.2% of MDR-TB and 33.3% of INH-mono-resistant isolates, underscoring its importance as a marker for isoniazid resistance. In contrast, *inhA* promoter mutations, particularly C15T, were less frequent but enriched among INH-mono-resistant isolates, consistent with evidence that they confer low-level INH resistance and cross-resistance to ethionamide [44].

These results strongly support the prioritization of *rpoB* S531L and *katG* S315T1 in diagnostic assays to maximize sensitivity in high-burden settings like Kano. Integration of these molecular markers into routine surveillance can improve early detection, guide individualized treatment, and strengthen TB control [45].

CONCLUSION

This study highlights a substantial burden of MDR-TB among TB patients in Kano State, with males and young adults disproportionately affected. The molecular data confirm that *rpoB* S531L and *katG* S315T1 are the dominant mutations driving drug resistance in the region. There is minimal ethambutol resistance, and

inhA mutations remain relatively infrequent but important for specific drug regimen decisions.

These findings underscore the importance of region-specific molecular surveillance to inform diagnostic strategies, treatment guidelines, and TB control policies. Strengthening laboratory capacity for molecular DST and ensuring access to accurate, mutation-targeted diagnostics are critical steps toward curbing the MDR-TB epidemic in Nigeria and beyond.

RECOMMENDATIONS

Beyond the diagnostic performance benefits, this recommendation carries significant economic value. For public health systems, targeting the predominant mutations—S531L in rpoB and S315T1 in katG—can lead to more efficient resource use, quicker treatment decisions, and reduced TB transmission and complications, all contributing to lower healthcare costs. For diagnostic manufacturers, region-specific probe design enhances cost-effectiveness, market relevance, and uptake by TB programs, particularly in high-burden countries like Nigeria. Therefore, aligning molecular diagnostics with locally prevalent resistance patterns represents a cost-saving and impact-maximizing strategy.

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