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Research Article

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Detection of *katG* and *inhA* Genes Mutation in Rifampicin-Resistant *Mycobacterium Tuberculosis* Using Line Probe Assay (LPA) In Kebbi, Nigeria

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Abstrac

Elimination of tuberculosis remains a public health menace, due to multidrug resistant strains of Mycobacterium tuberculosis, that are resistant to the first-line anti-tubercular drugs. Consequently, detection of resistant strains depends on early diagnosis. Intervention of molecular techniques is essential to reduce mortality and morbidity rates. This study aimed to detect katG and inhA gene mutations among rifampicin resistant strain of Mycobacterium tuberculosis in Kebbi State. A cross-sectional study was conducted which covered all the five major health zones in Kebbi State. Two hundred and forty (240) acid fast bacilli confirmed sputum samples were recruited and subjected to Gene Xpert testing. Positive sputum samples were later subjected to Line Probe Assay technique. 14 sputum samples were resistant to rifampicin out of the 240 confirmed acid fast bacilli sputum samples while 226 were sensitive to rifampicin. Mutations were found at the frequency of 100% for katG gene (katG/S315T) in all the 14 rifampicin resistant samples (rpoB/S531L) at the different health zones in kebbi state using the line probe assay. The research has also detected inhA gene mutation at a frequency of 100% in all rifampicin resistant samples showing that mutation is associated with multi resistance to isoniazid and rifampicin. This study confirms that resistance due to katG and inhA mutation is a better surrogate of multidrug-resistant tuberculosis among TB patients in Kebbi State compared to the single-marker analysis. Hence, early molecular detection of rifampicin resistant strains will suffice in determining the management of multidrug resistant tuberculosis in Kebbi State.

Keywords: Tuberculosis, Rifampicin Resistant, Line Probe Assay, Sputum, Mutation

Background

Tuberculosis (TB) remains a crucial public health challenge globally with latent forms of *Mycobacterium tuberculosis* asymptomatically affecting about one third of the general populace [1]. It is an infectious bacterial disease caused by *Mycobacterium tuberculosis* (MTB), spread from one person to another through airborne droplets via coughs or sneezing [2, 3]. The most common mode of infection of these bacteria is inhalation. TB primarily affects the pulmonary system but it can also affect other bodily systems, including the lymphatic system, central nervous system, and circulatory system among others, resulting in extra pulmonary TB.

Various health awareness campaigns are targeted towards reducing the incidence of TB, but elimination faces a setback due to resistance most especially to rifampicin (RIF) and isoniazid (INH), the first line anti-tubercular drugs. Nigeria is among the high TB, and drug resistant-tuberculosis (DR-TB) affected countries by global rating signifying the relevance of TB surveillance system evaluation in improving performance and capacity of the pre-existing system. The country ranks 7th among the 30 high TB burden countries and 2nd in Africa, accounting for 4% of the estimated incidence cases globally [4].

The management and control of drug-resistant TB, and especially multidrug resistant tuberculosis (MDR-TB), is difficult due to the poor detection rate, financial constraints which can be partly attributed to lack of rapid and accurate diagnostic tests. MDR-TB needs long-term treatment regime with second-line anti-tubercular drugs which may incur high cost and undesirable side effects [5]. So, rapid detection of antibiotic resistance in TB patients is important in developing strategies aimed to control the spread of drug-resistant disease.

Major mutations in *kat*G and *inh*A genes is principled on two

mechanisms leading to INH resistance. *kat*G gene encodes an enzyme, catalase peroxidase which converts INH to its active form. 42-95% of resistance to INH is due to amino acid substitution especially at codon 315 of *kat*G. Another mutation in the promoter region of the *inh*A gene which encodes the enoyl-ACP- reductase, confers 6%–34% of INH resistance in MTB strains [6]. There is a variation in the prevalence of the *kat*G (AGC-ACC) mutation among MDR-TB strains globally, being low in areas with low TB incidence and high in areas with [7]. Most INH-resistant clinical isolates become resistant by losing or altering katG activity, nevertheless, not all observed INH resistance is as a result of *kat*G mutations. Gene markers responsible for MDRTB are S315T in katG, −15C/T (CGT-CAT) in the promoter region of *inh*A, and H526D and S531L in rpoB [8].

Advancements in molecular methods for MTB detection has impacted positively in the turnaround time, whereas diagnosis by conventional culture systems needs several weeks [9]. Diagnostic delay is burdensome to effective MDRTB care, in which development and application of molecular approaches for rapid detection of MDRTB, are needed for decreasing MDRTB burden.

Line probe assays (LPA) is a promising rapid diagnostic tool that allows rapid drug-susceptibility testing for rifampicin and isoniazid based on combination of the multiplex polymerase chain reaction (PCR) followed by the deoxyribonucleiacid (DNA) strip reverse hybridization assay [10].

The aim of this research is to detect *Kat*G and *inh*A gene mutations from Rifampicin Resistant *Mycobacterium tuberculosis* samples in Kebbi State, Nigeria.

Methods

Study Area

Kebbi State is located in the North-Western geopolitical zone of Nigeria. It is mostly populated by Hausas and Fulanis. The main occupations of the peoples of the area are farming, fishing and trading it is bordered by Niger Republic, Zamfara State and Sokoto State. It is mostly populated by Hausas and Fulanis. According to the National Population Commission, population figures stand at 3,256,541 persons spread over an area of 36,800 square kilometer of land [11].

Study Population

The study was a descriptive cross sectional study conducted in Kebbi State, Nigeria. The research was conducted in some designated health zones in the Kebbi state which serve as reference laboratories for various local governments within the State, namely: Birnin-Kebbi, Kamba, Argungu, Zuru and Yauri. Study population includes all patients that have been suspected of having TB who visited the designated health zones, serving as reference laboratories.

Eligibility Criteria

Inclusion Criteria

All patient's samples that are positive for tuberculosis and showed rifampicin resistant within Kebbi State designated health centers were included for this research

Exclusion Criteria

Patient's that are negative for tuberculosis were excluded for this research.

Sample Collection and Processing

Sputum samples were collected in a wide-mouth, dry, clean, leak-proof container [12]. Trained National Tuberculosis and Leprosy Control Programme (NTBLCP) staff assisted in the collection of the sputum samples used during the research. The sputum sample was collected as an early morning on the spot specimen.

It was subjected to Gene Xpert assay to check for presence of *Mycobacterium tuberculosis* and rifampicin resistance. A positive result indicating resistance was recorded and then a molecular assay to assess mutation of the *kat*G and *inh*A gene were performed.

DNA Extraction: Using a sterile graduated pipette 0.5 ml (500 µl) of the decontaminated sputum sample was transferred into micro centrifuge tube. This was done for all the samples, after which the tubes were closed and centrifuged for 15 minutes at 10000xg. The supernatant was discarded and 100µl lysis buffer was added and re-suspended by overtaxing gently for 30 sec. The tubes were arranged in a floater inside the BSC II and incubated for 5minutes in a water bath at 950C. Then, 100µl neutralization buffer was added and vortexed for 30 seconds and the tubes were centrifuged at maximum speed (10,000xg). The heavier debris formed the pellet and the lighter DNA (free from impurities) was suspended in the supernatant which was transferred into clean labeled micro-centrifuge tubes for further use.

PCR amplification of the extracted DNA: The master mix preparation was done according to manufacturer's specification and World Health Organization (WHO). The master mix was made up of 10µl of the AM-A and 35µl of AM-B Reagent which was placed in a PCR tube labeled with sample number and mixed very well. This was prepared inside dead air box in a clean DNA free room. Then 5µl of each sample (containing the extracted DNA from above) was added to the corresponding tube containing the master mix and then mixed gently by pipetting up and down a few times. The PCR tubes were then placed in a 30 cycle (10 + 20) thermal cycler program for amplification. After amplification the DNA contained in the amplicons were denatured in the TwinCubator® which was pre-warmed to 45 oC and 20µl of denaturation solution (NaOH) was added to each labeled well of the TwinCubator® tray followed by the addition of 20µl of the amplicons respectively. The mixture was mixed gently by pipetting up and down five times and then incubated at room temperature for 5mins.

Hybridization and Detection: Hybridization and detection procedures were carried out according to according manufacturer's specification. After denaturation of the amplicons, 1ml of the pre-warmed hybridization buffer (HYB) was carefully added to the wells using a pipette and thoroughly mixed. The tray was placed on the TwinCubator® and labeled strips were added to each well ensuring that the strips were completely covered by the liquid and incubated at 45°C for 20mins. After incubation, the HYB buffer was aspirated completely from each well and 1ml of the pre-warmed red stringent wash buffer (STR) was then dispensed into the tray. After 10 minutes' incubation at 45 °C in the TwinCubator®, STR buffer was aspirated and was washed off with 1 ml of Rinse solution (RIN) for 1 minute. Then 1ml of the Conjugate solution was dispensed into each well and incubated for 20 minute on the TwinCubator®. The strips were washed twice with 1 ml of Rinse solution (RIN) for 1 minute in the TwinCubator®. Then sterile distilled water was added and a 1-minute wash performed on the TwinCubator® to wash off the RIN solution after which the distilled water was completely decanted. One (1) ml of the Substrate solution was then dispensed into each well and incubated for 10 minutes on the TwinCubator® after which the Substrate solution was aspirated and the strips washed twice with sterile distilled water. A pair of clean tweezers was used to remove the strips from the TwinCubator® tray and placed onto absorbent paper. The developed strips were partially dried and transferred to the GenoType® MTBDRplus score sheet for interpretation

Statistical Analysis

Results obtained from line probe assay were analyzed using Microsoft Excel 2016 and Statistical package for social sciences (SPSS).

Results

Table 1: Distribution of Rifampicin-resistant TB across Kebbi Health Zones

Health Zones	Number of TB-positive Samples	Rifampicin-resistant	Rifampicin-sensitive
Birnin-Kebbi	108	6	102
Argungu	36	4	32
Zuru	48	2	46
Yauri	36	2	34
Kamba	12	0	12
Total	240	14	226

Table 1 above showed the distribution of rifampicin-resistant TB (RR/TB) across all Kebbi Health Zones. Out of 240 tuberculosis positive samples collected from different health registered zones in the state, 14 samples were reactive for rifampicin-resistant strains, and 226 samples were sensitive to rifampicin throughout all the health zones in the state.

Table 2: Prevalence of *KAT*G gene mutation among Rifampicin resistant strain across Kebbi Health zones

Health Zones	Rifampicin-resistant (*rpoB*) Gene	*kat*G Gene mutation
Birnin-Kebbi	6	6(100%)
Argungu	4	4(100%)
Zuru	2	2(100%)
Yauri	2	2(100%)
Kamba	0	0(0%)

Table 2 above shows the mutations associated with the rifampicin resistance. Every sample flagged with rifampicin-resistant TB in all the health zones exhibited mutations in the *kat*G gene (100%). These mutations were seen at the S315T codon for *kat*G and S531L for *rpo*B gene mutations as a result of the missing WT (wild type) and presence of MUT (mutation probe) for both genes. The six (6) rifampicin-resistant samples from Birnin-Kebbi, four (4) from Argungu, and two (2) from both Zuru and Yauri, all had *kat*G gene mutation. This indicates that there were mutations in the *rpo*B and *kat*G gene which may be suggestive of MDRTB.

Table 3: Non *RPO*B/ *KAT*G gene mutation associated with multidrug resistance

Health Zones	Rifampicin-resistant (*rpo*B) Gene	Other Gene (*inh*A)
Birnin-Kebbi	6	6(100%)
Argungu	4	4(100%)
Zuru	2	2(100%)
Yauri	2	2(100%)
Kamba	0	0(0%)

Table 3 above shows other non-*rpo*B gene mutations associated with the rifampicin resistance. Every sample (100%) with rifampicin-resistant TB in all the health zones exhibited mutations in the *inh*A gene. The *inh*A gene which is another less common gene responsible for drug resistance in TB treatment and management beside the most common two (*rpo*B and *kat*G genes) indicates the presence of resistance to the two major first line drugs in TB management (Rifampicin and Isonaizid). In essence, it shows that the six (6) rifampicin-resistant samples from Birnin-Kebbi, four (4) from Argungu, and two (2) from both Zuru and Yauri, all had *inh*A gene mutation.

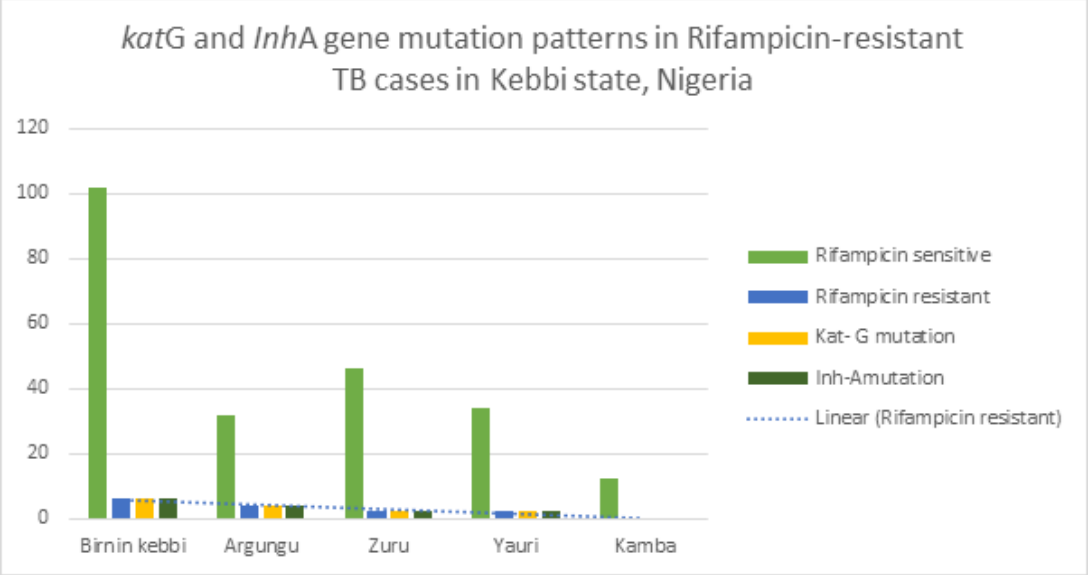

Figure 1: KatG and inhA gene mutation patterns in rifampicin-resistant tuberculosis in Kebbi State, Nigeria

Figure 1 above shows the mutation patterns of katG and inhA in rifampicin resistant tuberculosis in kebbi state, Nigeria with a linear regression in the pattern of mutation across the different health zones.

Discussion

Multidrug resistance in tuberculosis is one of the greatest health challenges still faced in today's world of advancing infectious disease diagnosis and treatment. Line probe assay is important in tuberculosis diagnosis and treatment due to the advantage it proffers to the treating clinicians and the patient. Observing the mutations associated with this manifestation of drug-resistance, three genes have always been implicated in mutations associated with manifestation of drug resistance. They are katG, rpoB and inhA. Mutation in the rpoB gene is responsible for majority of RIF resistance in MTB [16]. From the study conducted, every sample (100%) with rifampicin-resistant tuberculosis had mutations in all three genes. This is in similarities with results obtained from a similar study, which shows a 75% of multidrug resistance in all rifampicin-resistant tuberculosis [16, 17]. In this study, although inhA mutations were seen in all rifampicin-resistant MTB, no Non-rpoB/katG mutation was exclusively responsible for drug resistance of any kind. A study in Kano state, Nigeria reported 100% rifampicin resistance [13]. However, the results obtained from Brazil were different, with katG mutations of 41.9%, 25.6% for inhA. For katG, the most common pattern was seen as MUT1 (Pos) and WT (Neg) where point mutations have occurred in codon 315. This is similar to a study that reported 90% katG resistance among rifampicin resistant isolates, and higher in a previous study with 44.9% [12]. Among the inhA resistance pattern, MUT1 (Pos) and WT1 (Neg) were predominant. This was higher in previous studies with a recorded prevalence of 67.3% [14]. It is known that inhA confers low resistance towards Isoniazid compared to katG and accounts for 21% of multidrug resistance cases [15].

The frequency of these genetic mutations varies geographically. katG mutations tend to be more frequent (42–95% of isolates), while inhA mutations occur in 6–43% of isolates. 10% of Myobacterium tuberculosis clinical isolates have both mutations [20, 21]. Recognition of INH resistance patterns and the frequency of katG and inhA mutations in different geographic areas may help to guide decision making about standardization of treatment regimens or personalized medicine, mainly in the case of MDR or XDR-TB, due to limited number of effective available drugs.

Conclusions

Sequel to the results and other observations during the research, it shows that resistance due to katG and inhA mutation is a better surrogate of multidrug-resistant tuberculosis among confirmed TB patients in Kebbi State compared to the single-marker analysis. Recommendations that will benefit patients with tuberculosis in Kebbi State specifically as well as for the general populace should be considered such as investing better into tuberculosis drug research within the state, appropriate intervention strategies, procurement of more Gene Xpert machines and Line probe assays for all general hospitals within the State to facilitate prompt diagnosis

of resistant strains of MTB. Drug compliance should be strictly adhered to regardless of the HIV status of the patients so as to help eliminate unnecessary costs and hence, reduce the morbidity and mortality associated with resistance. More research in tuberculosis antimicrobial resistance pattern using line probe assay in Nigeria is essential to validate findings.

List of Abbreviations

DNA: Deoxyribonucleic acid
DR-TB: Drug resistant Tuberculosis
HIV: Human immunodeficiency virus
HYB: Hybridization buffer
INH: Isoniazid
LPA: Line probe assay
MDR-TB: Multidrug resistant Tuberculosis
MTB: Mycobacterium tuberculosis
MUT: Mutation probe
NTBLCP: National Tuberculosis and Leprosy Control Programme
PCR: Polymerase chain reaction
RIF: Rifampicin
RIN: Rinse solution
RR-TB: Rifampicin resistant Tuberculosis
SPSS: Statistical package for social sciences
STR: Stringent
TB: Tuberculosis
WHO: World Health Organization
WT: Wild type

Statement and Declarations

Competing Interest

The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate

Approval for this study was granted by the Health Research Ethical Committee of the Ministry of Health, Kebbi State, Nigeria with Health Research Ethics committee assigned number KSHREC: 106: 10/2021. Written and oral consent was obtained from participants before questionnaire survey and sample collection.

Consent for Publication
Not Applicable

Availability of Data and Materials

This article contains all data used in the study.

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Author's Contributions

VOO conceived the main idea, performed the data collection, writing and submission, SBM and YKD supervised and revised the work, AIA analyzed the data, wrote the manuscript and final editing, ACU helped in sample collection, practical's and analyzed the data. All authors approved the final manuscript.

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