Prime Journal of Microbiology Research (PJMR)
ISSN: 2251-127X. Vol. 5(1), pp. 190-197, June 24th, 2020
www.primejournal.org/PJMR

Full Length Research

Frequency of genetic mutations causing resistance to Rifampicin and Isoniazid in *Mycobacterium tuberculosis* from clinical specimens using molecular line probe assay in Kenya

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Accepted 16th June, 2020

Drug resistance in *Mycobacterium tuberculosis* is as a result of genetic mutation occurring spontaneously in highly conserved regions of *Mycobacterium* genome. This cross-sectional study was to evaluate frequencies of genetic mutations conferring resistance to rifampicin and Isoniazid using molecular line probe assay (LPA). A total of 429 sputum specimens were selected randomly from samples processed at the National Tuberculosis Reference laboratory, Kenya between 2016-2017. DNA was extracted and resistant genes detected using GenoType MTBDR*plus* ver. 2.0 (Hain Lifescience Gmbh) which identified *rpoB, KatG,* and *InhA* genes. The mutation frequencies patterns, sensitivity and specificity of rifampicin and Isoniazid were analyzed. Of 429 specimens, 20 (4.6%) were resistant only to rifampicin, 62 (14.4%) to Isoniazid and 38 (8.9%) MDR-TB. Overall, 58 (13.5%) and 100 (23.3%) were resistant to RIF, INH respectively. Of the 58 RIF resistant strains, failing of the *rpoB* Wild Type 8 probe with subsequent detection of a corresponding mutation probe MUT3 at Ser531Leu accounted for 26 (44.8%) of the mutations. 25 (25%) of the 100 INH resistant strains showed a failing *KatG* WT probe with a corresponding detection of MUT1 probe at Ser315Thr1. 17 (17%) showed a failing *inhA* WT1 and WT2 with corresponding substitution detection at Cys15Thr. The GenoType MTBDR*plus* ver. 2.0 in comparison to culture DST showed a sensitivity and specificity of 96.6% and 99.7% for RIF resistance and 99.0% and 100% for INH resistance detection. LPA can be useful in prevalence studies by mapping the distribution of mutant genes and also for routine clinical diagnosis of drug-resistant tuberculosis.

Keywords: Genetic mutation, *Mycobacterium tuberculosis,* Isoniazid, line probe assay (LPA)

INTRODUCTION

Globally it is estimated that there were 10 million new cases in 2018 and 1.5 million TB deaths, which included 1.2 million among HIV negative people, and 251,000 HIV positive associated TB deaths (WHO Global TB report 2019).

In Kenya the prevalence of tuberculosis stood at 426 per 100,000 population with an annual incidence of 169,000 cases according to the national tuberculosis prevalence survey report 2018. The current gold standard for *Mycobacterium tuberculosis* drug susceptibility testing (DST) is the BACTEC™ MGIT culture and it takes weeks to grow after inoculation. Moreover, even with the improved liquid culture identification methods, it takes an average of 14 days for diagnosis and additional 14 days to perform drug susceptibility testing (Siddiqi *et al*., 2012). However, these tests do not determine mutant

responsible for drug resistance hence it is difficult to elucidate the role of dominance in single gene mutations associated with resistance to rifampicin and isoniazid.

Over the past 10 years significant knowledge has been gained concerning the molecular mechanisms of mycobacterium drug resistance (Donald P.R *et al.,* 2011). These studies have established that chromosomal loci responsible for resistance to various drugs are not linked. Thus the polydrug or multi-drug resistance in *Mycobacterium tuberculosis* is not caused by a single genetic locus, but by an accumulation of multiple different mutations.

Molecular assays such as the GenoType® MTBDR*plus* Ver. 2.0 (Hain LifeScience GmbH, Nehren, Germany) can detect such mutations in genes associated with drug resistance for isoniazid and rifampicin and offer effective tools for determining drug resistance to TB with high sensitivities and specificities (Tassema *et al.,* 2012). The test can characterize mutations in the *rpoB* gene (D516V, H526Y/D, and S531L), *katG* gene (S315T) and *inhA* promoters for rifampicin and isoniazid respectively.

Mutations within an 81-bp "core region" of the *rpoB* gene are responsible for approximately 95% of rifampicin resistance in *M. tuberculosis* strains. Three of these mutations at positions 516 (D→V), 526 (H→Y/D), and 531 (S→L) constitute the majority of mutations within this region (Daum *et al.,* 2012).

In Kenya, prevalence of genetic mutations for rifampicin and Isoniazid in *Mycobacterium tuberculosis* has not been fully documented due to limitations of molecular tools for the genetic identification of mutant genes and their distribution. In 2016, Kenya conducted a national wide TB prevalence survey on tuberculosis where findings indicated that 36% of TB cases go unnoticed and untreated. However, this survey did not indicate the distribution of genetic mutations conferring resistance to rifampicin and Isoniazid (Ogari *et al.,* 2019).

We evaluated the use of GenoType MTBDR*plus* ver.2.0 assay to provide new insights on the local resistance patterns for rifampicin and isoniazid and assess the sensitivity and specificity of the test for drug resistant TB detection.

MATERIALS AND METHODS

Study Design and Sample Collection

We carried out a cross-sectional study among new and retreatment TB patients with confirmed positive sputum specimens and culture isolates collected at the National Tuberculosis Reference laboratory, Kenya.

The National TB Reference laboratory is integrated within the national systems of laboratory services under the division of public health laboratories located in Nairobi, Kenya. The laboratory is mandated to provide TB laboratory referral services for culture and drug susceptibility testing to all the peripheral facilities and MDR-TB surveillance among previously treated TB patients. It also conducts follow-up tests among MDR-TB patients for treatment monitoring and management.

The laboratory is well equipped and has the capacity to perform Nucleic acid amplification tests (NAAT) including Line probe assay (LPA), GenXpert MTB/RIF assay (Cepheid Inc, CA. USA), and liquid culture using the BACTEC™ MGIT™ 960 system (Becton Dickinson, New Jersey, USA) for drug resistance susceptibility testing.

Purposive sampling technique was done where all the samples from TB patients with pulmonary TB and on TB retreatment cases were enrolled for the study consecutively until the desired number was attained. A total of 429 specimens were randomly selected and enrolled into the study between January 2016-December 2017. Approval of the study was obtained from Jaramogi Oginga Odinga Teaching and Referral Hospital Ethical Review Committee (JOOTRH).

Demographic Data

We collected and analyzed patient's demographical data which included Age, Sex, HIV status, type of patient and history of TB.

Sputum Processing

All patient specimens (sputa) were processed under biosafety cabinet class II (BSC II) using the NALC/NaOH method for digestion, decontamination and concentration according to the CDC recommendation ("Public Health mycobacteriology: A Guide for the level III Laboratory"). Equal volumes of 2.9% sterile sodium citrate (Sigma Aldrich, St. Louis, MO) and sterile sodium hydroxide (NaOH) 4 times the final concentration; (Mallinckrodt, hazelwood, MO) were mixed. N-acetyl-L-Cysteine NALC (Sigma Aldrich) was added on to the mixture and gave a final concentration of 0.5%. 5 ml of the NALC-NaOH mixture was added to the sputum sample and incubated for 15 minutes at room temperature. The Mixture was centrifuged at 3000rpm in a tabletop centrifuge at 4°C for 15 minutes and the supernatant discarded. 2 ml of phosphate buffered saline (PBS) was added on to the pellet for re-suspension. The solution was centrifuged again at 3000 rpm for 15 minutes at room temperature. The supernatant was decanted and the sediment re-suspended in PBS ready for phenotypic DST and molecular testing.

Direct Examination

The sputum was prepared for microscopic slide examination of *Mycobacterium tuberculosis* bacilli. Sputum was spread at the center of the microscope slide using an applicator wooden stick and left to air dry. The smear was heat fixed by flaming over a bunsen burner and thereafter subjected to Zeihl-Neelsen (ZN) staining. Specimens were considered positive once the ZN stained smears showed a pink rod shaped image under the microscope.

MGIT Drug Susceptibility Testing (DST)

MGIT antibiotic supplement PANTA was reconstituted into a 15ml of MGIT growth tube and 0.5ml of the

decontaminated sputum sediment were inoculated and mixed with 0.8ml of the PANTA growth supplement. The tubes were incubated in the BACTEC MGIT system at 37°C and the growth was continuously monitored automatically by the MGIT software for an average of 14 days (MGIT™ procedure manual 2006). Positive growth of *Mycobacterium tuberculosis* was detected by a red light indicator of the MGIT system. All positive samples were further inoculated with the MGIT SIRE kits for streptomycin, Isoniazid, rifampicin, and ethambutal phenotypic DST as stipulated in the manufacturers protocol. The critical concentration of rifampicin and Isoniazid drugs in the medium were 1.0 µg/ml and 0.1µg/ml respectively.

Molecular Assay - GenoType MTBDR*plus* Ver. 2.0

The Hain GenoType MTBDR*plus* ver. 2.0 is a molecular identification assay based on the DNA STRIP Technology for genetic identification of *Mycobacterium tuberculosis* complex (MTBC) and gene mutations conferring resistance to rifampicin and Isoniazid simultaneously.

DNA Extraction

Mycobacterium tuberculosis DNA was extracted directly from decontaminated clinical specimens using the GenoLyse® reagent which is a chemical lysis method. The decontaminated sputum specimens was centrifuged in table top micro-centrifuge at a speed of 10,000 x g relative centrifugal force (RCF) for 15 minutes. The resultant supernatant was discarded and the remaining pellet mixed with lysis buffer, heat inactivated at 95°C in a water bath to lyse the *Mycobacterium* cell walls. The lysed cells was thereafter subjected to a neutralization buffer to stop the lysis reaction. The suspension was centrifuged at top speed and the supernatant (DNA) was transferred in to a clean eppendorf tube and stored at -20^{0}C according to the manufacturers instructions (GenoLyse® instructions for use-IFU-51610-10).

Polymerase Chain Reaction (PCR)

Multiplex Polymerase chain reaction (PCR) amplification of the resistance-determining region (RDR) for the *rpoB, KatG* and *InhA* genes was performed using ready to use amplification mixes AM-A and AM-B containing primers-nucleotide mix (GenoType MTBDR*plus* ver. 2.0. instructions for use- IFU-304-06). 5µl of the extracted DNA was mixed with reconstituted 10µl of AM-A and 35µl of AM-B biotinylated primer-nucleotide mixes. The DNA primer solution was amplified in a thermal cycler using the following amplification profile; 95°C for 15minutes (1 cycler); 95°C for 30 seconds and 65°C for 2 minutes (20 Cycles); and 95°C for 25 seconds, 50°C for 40 seconds, 70°C for 40 seconds (30 cycles); final elongation at 70°C for 8 minutes. The PCR product was then refrigerated at -20°C.

Probe Hybridization

Labeled PCR products were hybridized with specific oligonucleotide probes immobilized on a strip using the reverse hybridization technique. Hybridization process was carried out in the TwinCubator® machine and following the manufacturers instructions for use (GenoType MTBDR*plus* ver. 2.0. instructions for use IFU-304A-06). Captured labeled hybrids were then detected by colorimetric development, enabling detection of the presence of *Mycobacterium tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance detection.

Evaluation of Results

Developed strips with visible bands were scanned using the automated reader and interpretation GenoScan software ver. 3.4 which is installed in a GenoScan reader machine and raw data stored as an excel sheet.

Statistical Analysis

The data was entered and analyzed using SPSS statistical package software ver. 16 (SPSS Inc., Chicago, IL, USA) to determine the prevalent gene mutations and their frequencies. The sensitivity, specificity and predictive values of GenoType MTBDR*plus* Ver. 2.0 and culture MGIT drug susceptibility testing was entered in an excel sheet and analyzed using cross tabulation method.

RESULTS

A total of 429 specimens were analyzed during the period of January 2016 to December 2017 and out of this 299 (69.6%) were male and 130 (30.4%) were female. The median age of the study subjects was 35.0 with a mean of 35.5. (Table 1). Demographic data showed 105 patients were new cases and 212 patients had a relapse retreatment profile, which accounted for 24.4% and 49.4% of the total retreatment cases respectively.

***rpoB, KatG,* and *inhA* gene mutations**

Out of the 429 subjects, 58 (13.5%), 100 (23.3%) and 38 (8.9%) showed resistance to rifampicin, isoniazid and MDR-TB respectively. Specimens that showed resistance to rifampicin and Isoniazid only were 20 (4.6%) and 62 (14.4%) respectively. Of the 429 clinical specimens analyzed, samples with results showing either MDR-TB, mono-resistant to RIF and mono-resistance to RIF were 120 (27.9%). Of the 58 RIF resistant strains, failing of the rpoB Wild Type 8 (WT 8) probe with subsequent detection of a corresponding mutation probe MUT3 at Serine531Leucine, accounted for 26 (44.8%) of the mutations. 25 (25%) of the 100 INH resistant strains showed a failing KatG WT probe with a corresponding detection of MUT1 probe at Serine315 Threonine while 17 (17%) showed a failing inhA WT1 and WT2 probes with corresponding substitution detection at Cysteine15Threonine position.

Of the 20 rifampicin mono-resistant cases, 8 (40.0%)

Table 1: Demographical Characteristics of subjects from New and TB Retreatment cases (N=429)

Variables		Number of Subjects	% Frequency
Sex	Male	299	69.60
	Female	130	30.40
Age Group	Median 35 IQR (28-44)		
	1-20	28	6.50
	21-30	139	32.40
	31-40	161	37.50
	> 40	101	23.50
HIV Status	Positive	103	24.0
	Negative	258	60.10
	Not Done	68	15.90
TB History	New Cases	105	24.50
	Retreatment Cases	324	75.50
Patient Type	New	105	24.40
	Loss to follow up	67	15.60
	Relapse	212	49.40
	Treatment failure	40	9.30
	Unknown	5	1.20
Drug Resistance	MDR-TB	38	8.9
	Rifampicin Resistant	58	13.50
	Isoniazid Resistant	100	23.30
	No resistance	233	54.30
Total		429	

MDR, multi-drug resistant; TB, tuberculosis; N, number

were due to Ser531Leu substitution and 2 (10.0%) to His526Asp substitution. In addition among the 62 Isoniazid mono-resistant cases, 32 (69.3%) were due to Ser315Thr1 substitution and 18 (29%) due to C15T substitution (Table 2).

GenoType MTBDR*plus* Ver. 2.0 comparison with phenotypic DST

The GenoType MTBDR*plus* ver. 2.0 in comparison to culture MGIT drug susceptibility test showed a sensitivity and specificity of 96.6% and 99.7% for rifampicin resistance; 99.0% and 100% for Isoniazid resistance detection. The positive predictive value and negative predictive value for rifampicin were 98.3 (88.9-99.8) and 99.5% (97.9-99.9) respectively; while for Isoniazid the values were 100% (93.4-99.9) and 99.7% (97.9-99.9) in a 95% Confidence Interval CI (Table 3).

DISCUSSION

In Kenya the prevalence of multi-drug resistant tuberculosis (MDR-TB) among previously treated and new cases stood at 2.1% and 0.7% respectively. Data from the tuberculosis prevalence survey in Kenya 2016, shows the prevalence of bacteriologically confirmed tuberculosis cases in Kenya stands at 558 (455-662) per 100,000 population which is significantly high compared to other countries within the East African region (Ministry of Health, Survey Report 2018). Higher tuberculosis incidences has been shown to be directly proportional to the rates of infection within a population (Mekonnen et al., 2015). Molecular diagnostics assay for multi-drug resistant tuberculosis (MDR-TB) detection have been adopted and implemented by the National TB reference laboratory in Kenya. The GenoType MTBDR*plus* ver. 2.0 and GenoType MTBDR*sl* ver. 2.0 assays (Hain Lifescience GmbH Nehren, Germany) are some of the molecular tests endorsed by the WHO for rapid detection of TB and drug resistant tuberculosis in clinical settings (WHO LPA policy statement, 2008).

This study addressed the identification and prevalence of drug resistant mutations in *Mycobacterium tuberculosis* from the National tuberculosis reference laboratory in Kenya. In this study the GenoType MTBDR*plus* ver. 2.0. which detects the *rpoB, KatG* and *InhA* genes, shows a sensitivity of 96.6% and specificity of 99.7% for rifampicin resistance detection and a sensitivity of 99.0% and specificity of 100% for INH resistance detection. Further, Hain GenoType MTBDR*plus* ver. 2.0 assay showed multi-drug resistance tuberculosis (MDR-TB) cases were 38 (8.9%) from the overall sample size of 429.

Overall, mono-resistance to rifampicin and Isoniazid accounted for 58 (13.5%) and 100 (23.3%) respectively.

Table 2: Frequency and patterns of *rpoB, KatG* and *InhA* genetic mutations in *Mycobacterium tuberculosis* by GenoType MTBDR*plus* Ver.2.0 assay.

rpoB gene			***katG gene***			***inhA gene***			
Failing WT probe	**Mutation**	**Location of codons of WT/mutant/gene**	**Failing WT probe**	**Mutation**	**Location of codons of WT/mutant/gene**	**Failing WT probe**	**Mutation**	**Analyzed nucleic acid position**	**n (%)**
MDR *(n =38)*									
No	MUT3	S531L	No	MUT2	S315T2	No	No		1(0.83)
No	MUT3	S531L	No	MUT1	S315T1	No	No		6(5.00)
No	MUT3	S531L	WT	MUT1	S315T1	No	No		2(1.67)
No	MUT2A	H526Y	No	No		WT1	MUT1	C15T	1(0.83)
WT3	No	514-515	WT	MUT1	S315T1	No	No		1(0.83)
WT8	MUT3	S531L	No	No		WT1	MUT1	C15T	2(1.67)
WT8	MUT3	S531L	No	No		No	MUT1	C15T	2(1.67)
WT8	MUT3	S531L	No	MUT1	S315T1	No	No		8(6.67)
WT8	Unknown	531-533	WT	MUT1	S315T1	WT1	MUT1	C15T	1(0.83)
WT8	Unknown	531-533	No	MUT1	S315T1	No	No		2(1.67)
WT8	MUT3	S531L	WT	MUT1	S315T1	No	No		9(7.50)
WT8	Unknown	531-533	WT	MUT1	S315T1	No	No		3(2.5)
RIF Only (*n = 20*)									
No	MUT3	S531L	No	No		No	No		2(1.67)
No	MUT2A	H526Y	No	No		No	No		2(1.67)
No	MUT2B, MUT3	H526D; S531L	No	No		No	No		1(0.83)
WT1	No	505-509	No	No		No	No		3(2.50)
WT2, 3	Unknown	510-517	No	No		No	No		1(0.83)
WT3	No	514-515	No	No		No	No		1(0.83)
WT3, 4	MUT1	D516V	No	No		No	No		1(0.83)
WT8	No	531-533	No	No		No	No		3(2.50)
WT8	MUT3	S531L	No	No		No	No		5(4.17)
WT8	MUT2B	H526D	No	No		No	No		1(0.83)
INH Only (*n = 62*)									
No	No		No	No		WT1; WT2	MUT1	C15T	1(0.83)
No	No		No	No		WT1; WT2	No	Unknown	1(0.83)
No	No		No	No		WT1	MUT1	C15T	12(10)
No	No		No	MUT1	S315T1	WT1	No		2(1.67)
No	No		No	No		No	MUT1	C15T	5(4.17)
No	No		No	MUT1	S315T1	No	No		32(26.67)
No	No		WT	MUT1	S315T1	No	No		9(7.50)

rpoB, RNA polymerase beta subunit; *KatG*, catalase peroxidase; *InhA*, enoyl-acyl-carrier protein reductase; MDR, multi- drug resistant; WT, wild-type; MUT, mutant; LPA, line probe assay; C, cysteine; D, aspartate; H, histidine; L, Leucine; S, serine; T, threonine; V, valine; Y, tyrosine; No=No failing of wildtype gene and no appearance of mutant gene; Unknown= Unknown mutant genes detected but the LPA probes could identify.

Specimens that showed resistance to Rifampicin and Isoniazid only were 20 (4.6%) and 62 (14.4%) respectively. Previous studies in Malaysia and Uganda (Zaw MT *et al*., 2018, Mboowa G *et al*., 2014) have shown 95% rifampicin resistance is associated with the *ropB* gene and occurs within the 81bp rifampicin resistance-determining region (RRDR).

In this study the prevalence of gene mutations in *rpoB* gene among RMP resistant isolates was 44.8% at position Ser531Leu, 3.4% at position His526Tyr and

Table 3: Comparison of GenoType MTBDRplus with convectional drug susceptibility testing (DST) MGIT culture.

GenoType™ MTBDRplus V. 2.0		BACTEC™ MGIT DST		Sensitivity	Specificity	PPV	NPV
		Sensitive	Resistant	%	%	%	%
Rifampicin	**Sensitive**	368	2	96.6	99.7	98.3	99.5
	Resistant	1	57			(88.9-99.8)	(97.9-99.9)
Isoniazid	**Sensitive**	328	1	99.0	100	100	99.7
	Resistant	0	100			(93.4-99.9)	(97.9-99.9)

DST, drug susceptibility testing; NPV; negative predictive value; PPV, positive predictive value. Values in parenthesis are within 95% confidence intervals

1.7% at position His526Asp. The remaining 50.1% mutations within the r*poB* gene were detected by the wild type (WT) probe and this has been classified as resistance inferred due to absence of a wild type probe without having a corresponding substitution with a mutant (MUT) probe. This is in agreement with most previous studies done in Ethiopia, China, Turkey and Sweden (Mekonnen *et al*., 2015; Yue *et al.,* 2003; Aslan *et al.,* 2008; Chryssanthou *et* al., 2012) which showed a prevalence between 46% - 79% at position S531L. However, lower *rpoB* gene prevalence values at codon 531 have been reported in India and Nepal (Tolani *et al.,* 2012; Poudel *et al.,* 2012) and could be attributed to differences in strains types and geographical distributions.

Both *katG* and *inhA* genes confer higher level and lower level resistances to INH respectively and the drug functions as a pro-drug in TB management. The mutation frequency of *katG* gene with a failing wild type probe and a corresponding mutation (MUT 1) detected at position 315 was 25%. Among the 100 INH resistance strains, *katG* gene S315T1 and S315T2 mutations accounted for 76 (76%) of the total INH mutations (Table 2). This finding is in agreement with previous studies in Ethiopia and Colombia, (Tessema *et al*., 2012; Hazbón *et al.,* 2006) indicating a frequency between 70-90% of mutations occurring at position 315 of the *katG* gene. The promoter region of the *inhA* gene which codes for the low-level drug resistance for INH has been reported to cause 8% to 43% of INH resistances in China (Zhang *et al.,* 2009). In this study, the proportion of *InhA* gene mutations stood at 27% of the total INH resistance cases, which is within the range reported in other countries. In addition, the prevalence of *InhA* gene mutations at position C15T stood at 17% *(n=17)* and all these samples showed a failing *inhA* WT1 and *inhA* WT2 probes with corresponding substitution mutation probe being detected.

CONCLUSION

This study showed the prevalence of mutations conferring resistance to rifampicin and isoniazid from both new and retreatment cases in Kenya. The most dominant mutant genes observed from this study are at codon 531 in the *rpoB* gene for rifampicin and at codon 315 in the *katG* gene for Isoniazid. The genotypic assay GenoType MTBDR*plus* ver. 2.0 had a high sensitivity and specificity for the detection of MDR-TB and mono-resistance to rifampicin and Isoniazid. In comparison to the phenotypic gold standard BACTEC MGIT system, the GenoType MTBDR*plus* ver. 2.0 showed over 95% concordance for *Mycobacterium tuberculosis* and multi-drug resistant detection. We recommend the use of this genotypic assay for routine clinical diagnosis of tuberculosis and drug resistant TB. The assay can also be useful in TB prevalence studies.

Acknowledgements

The author acknowledges the support and funding by Hain Lifescience GmbH, Nehren - Germany and the National Tuberculosis Reference laboratory staff for their technical assistance.

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