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Assessment of Genetic Mutation Patterns Associated With Anti-Tuberculosis Drug Resistance in Tuberculosis Isolates From Uasin Gishu County, Western Kenya

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Abstract: **Drug resistant tuberculosis is becoming a major concern and has hindered the control of tuberculosis (TB) worldwide. What is the size of drug resistant-TB problem locally? Objective: To study mutation pattern of drug resistance tuberculosis isolates in Western Kenya. Methods: Sputum samples were obtained from TB patients and stained for acid fast bacilli and *M. tuberculosis* isolated from positive cases. Anti-tuberculous drugs resistance pattern and their genetic mutation patterns were screened by use of GenoType®MDRTB*plus* assay. Results: Among the 120 *M. tuberculosis* isolates from positive sputum smears, 102 (85%) isolates were sensitive to both rifampin (RIF) and isoniazid (INH) whereas 18 (15%) isolates were resistant to at least one of these drugs. Those that were resistance to RIF and INH were found respectively in 17/120 (14.2%) and 18/120 (15%) isolates whereas 17 (14.2%) were resistant to both RIF and INH. Genotypic multi-drug resistance was then confirmed in 14.2% (17/120) isolates. Genetic mutations in *rpoβ* 505-533, *katG*315 and *inh*-8 to -16 codons associated with resistance to RIF and INH in anti-tuberculous resistant isolates were found respectively in 17/18 (94.4%), 17/18 (94.4%), and 16 (88.9%). Conclusion: Findings from this study showed that the prevalence of anti-tuberculous drug gene mutation pattern among TB isolates was diverse with high prevalence in codons 505-533 and 315 of *rpoβ* and *katG* respectively.**

Keywords: **Anti-tuberculosis drugs, MTBDRplus, mutation patterns, inh gene, katG gene and rpoβ gene.**

1. INTRODUCTION

Tuberculosis remains a major public health problem and the high upsurge and drug resistant tuberculosis is becoming a major concern in the control of tuberculosis (TB) [1]. Multi-drug resistant TB (MDR-TB) and extensively drug-resistant (XDR) TB, defined as MDR-TB (resistance to rifampicin [RIF] and isoniazid [INH]) with additional resistance to a fluoroquinolone antibiotic and at least one of three injectable drugs used for MDR-TB treatment (capreomycin, kanamycin, and amikacin) [2], has heightened the challenge faced in controlling the epidemics of TB. Resistance of *Mycobacterium tuberculosis* (*M. tuberculosis*) to at least INH and RIF [4] is a major threat to the tuberculosis control program.

World Health Organization (WHO) has noted that the transmission of drug-resistant *M. tuberculosis* complex strains is increasing because of the growing burden of MDR-TB patients [5], [6], [7]. Vijdea, *et al.*, insinuate that rapid detection of MDR-TB allows the establishment of an effective treatment regimen; minimizes the risk of further resistance and limits spread of drug-resistant strains [8].

Conventionally, the diagnosis of MDR-TB and XDR-TB is based on mycobacterial culture and drug susceptibility testing (DST) on liquid or solid media, with results available in weeks to months. The capacity of these conventional methods is, however, severely limited, especially in resource-poor countries.

A number of commercial assays have been proposed for detection of mutation frequency and patterns associated with drug resistance [9], [10]. In response to the growing problem of MDR-TB and the threat of an epidemic of virtually incurable XDR-TB, new molecular diagnostic methods for TB drug susceptibility testing have been developed and validated [11]. Among them is the Genotype MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany) line probe assay (LPA), approved for use with specimens growing in culture media and also for use with smear-positive sputum samples. This test detects mutations in three genes: *rpoB,* which confers resistance to RIF; *KatG*, which confers high-level resistance to INH; and the *inhA* regulatory region, which confers low-level resistance to isoniazid [12].

The Line probe assay detects relevant gene mutations directly from smear-positive sputum and the results have been obtained within 1 day [13]. It has been used in several countries [14], [15] with reliable results. It uses DNA based strip that is designed to simultaneously detect the most important *rpoβ* and *katG* gene mutations conferring RIF and high-level INH resistance in the clinical isolates [16], [17]. It is commercially available and is based on the principle of multiplex PCR in combination with reversed hybridization to identify *rpoβ* and *katG* amplicons to membrane-bound probes. The DNA strip covers eight *rpoβ* wild-type probes, four *rpoβ* mutant probes (with D516V, H526Y, H526D and S531L mutations), one *katG* wild-type probe and two *katG* mutant probes (with S315T1 and S315T2 mutations) [18]. The mutations that predominate in RIF-resistant *M. tuberculosis* isolates are located in an 81-bp "core region" of the *rpoβ* gene (95% of all RIF-resistant strains) [19]. Resistance to INH conferred by mutations in catalase-peroxidase enzyme gene (50-95% of INH-resistant strains) is targeted in codon 315 of the *katG* gene [20] and 20-35% contains mutations in the *inhA* regulatory region.

Continuous monitoring of drug resistance pattern especially of MDR-TB isolates is a crucial need for future TB control in Kenya. In this study we assessed the genetic mutation patterns associated with anti-tuberculosis drug resistance in tuberculosis isolates.

2. MATERIALS AND METHODS

Study design:

All smear positive isolates from re-treatment (failure at month, 5 or above, relapses, treatment after interruption) cases between June 2011 and June 2014 in the Uasin Gishu and Huruma sub-County Hospitals in Western Kenya were recruited. Before isolation, sputum smears were stained for acid fast bacilli and microscopic examination was performed in the site laboratory of each hospital, before transporting to Moi University TB Reference Laboratory for cultivation, susceptibility testing, and genetic mutation analysis.

Specimen processing:

Sputum specimens were decontaminated using the N-acetyl cysteine-sodium hydroxide (NALC-NAOH) method, as prescribed for Genotype® MTBDR*plu*s and BACTEC™ MGIT™ 960 culture (BD, Sparks, MD, USA) [21]. All of the decontaminated sputum samples were stained with Ziehl-Neelsen (ZN), microscopically examined and graded as negative, scanty, 1+, 2+ and 3+, according to International Union Against Tuberculosis and Lung Disease (IUATLD) guidelines [22]. A 0.5µl portion of the sediment was cultured using the BACTEC MGIT 960 system (BD Diagnostics Systems, Sparks, MD), including mycobacterial growth indicator tubes (MGITs) with polymixin, amphotericin B, and naladixic acid (PANTA) and oleic acid albumin, dextrose, and catalase (OADC). Positive cultures were confirmed as *M. tuberculosis* complex using ZN staining and streptomycin, isonizid, rifampicin and ethambutol (SIRE) drug susceptibility was performed. Sputum samples that were negative on microscopy were subjected to MGIT ™ culture and, if 'flagged' positive, cultures were subjected to indirect Genotype® MTBDR*plus.*

GenoType MTBDR*plus* test:

The GenoType MTBDR*plus* assay was performed according to the instructions provided by the manufacturer (Hain Lifescience GmbH, Nehren, Germany). Briefly, the amplification mixture contained 35 µl of the primer nucleotide mix, 5 µl of 10× polymerase incubation buffer, 5 µl of 25 mM $MgCl_2$, 1 µl of AmpliTaq Gold polymerase (5 U/µl; Applied

Biosystems), and 5 µl of the supernatant of the cell lysate, for a final volume of 50 µl. The amplification protocol consisted of 5 minutes of denaturation at 95°C, followed by 10 cycles comprising 30 s at 95°C and 2 minutes at 58°C, an additional 20 cycles comprising 25 s at 95°C, 40 s at 53°C, and 40 s at 70°C, and then a final extension at 70°C for 8 min. Hybridization and detection were performed with a TwinCubator (Hain Lifescience GmbH, Nehren, Germany). The hybridization procedure included the following steps: chemical denaturation of the amplification products at room temperature for 5 min, hybridization of the single-stranded biotin-labeled amplicons to membrane-bound probes at 45°C for 30 min, stringent washes, addition of a streptavidin-alkaline phosphatase (AP) conjugate at room temperature for 30 min, and an AP staining reaction to detect colorimetric bands. To detect RIF resistance, eight wild-type (WT) *rpoβ* probes encoding amino acids 505 to 533 and four probes for common mutations were utilized. Probes used for INH resistance detection were designed to recognize a WT S315 region, with two mutant probes for the highly resistant *katG* gene and two probes specific for WT regions, as well as four mutant probes for the *inhA* gene, which demonstrates low-level resistance. When all WT probes showed positive staining for an isolate and mutant probes demonstrated no staining, the isolate was considered susceptible. In contrast, the isolate was considered resistant when either any one of the WT probes was absent or any one of the mutant probes was present.

The GenoType® MTBDR*plus* strip contains 17 probes, including amplification and hybridization controls to verify the test procedures. For the detection of RIF resistance, eight *rpoβ* wild-type probes (probes WT1 to WT8) encompass the region of the *rpoβ* gene encoding amino acids 505 to 533. Four probes (probes *rpoβ* MUT D516V, *rpoβ* MUT H526Y, *rpoβ* MUT H526D and *rpoβ* MUT S531L) specifically target the most common mutations conferring resistance to RIF. For the detection of INH resistance, one probe cover the wild-type S315 region of *katG*, while two others (probes *katG* MUT1 and MUT2) are designed to assess the AGC-to-ACC (S315T) and the AGC-to-ACA (S315T) mutations. The promoter region of the *inhA* gene encompasses the regions from positions -15 to -16 for the *inhA* WT1 probe, and positions -8 for the *inhA* WT2 probe. Four mutations (-15C/T, -16A/G, -8T/C and -8T/A) can be targeted with the *inhA* MUT1, MUT2, MUT3A and MUT3B probes. Again, the absence of one or more wild-type probe (s) or the presence/staining of a mutant probes were indicative of resistant strain.

Each strip consists of 27 reaction zones (bands), including six controls (conjugate, amplification, *M. tuberculosis* complex, *rpoβ, katG*, and *inhA* controls), eight *rpoβ* wild-type (WT) and four mutant (MUT) probes, one *kat*G wild-type and two mutant probes, and two *inh*A wild-type and four mutant probes.

Interpretation of results:

The results were interpreted according to the manufacturer's instructions. A strain was interpreted as 'resistant' if it showed absence of one or more wild-type (⌂WT) bands and/or presence of any mutant band (MUT). An isolate that revealed hybridization to mutant as well as to the corresponding (WT) probe was considered hetero-resistant.

Statistical analysis:

All the relevant clinical details and results obtained using microscopy, culture and mutation analysis from GenoType® MTBDR*plus* were entered into Database in form of Microsoft Excel spread sheets; after compilation, the data were analyzed using SPSS, version 21.0 (Statistical Package for the Social Sciences; IBM Corp, Armonk, NY, USA). Data were expressed as percentages for categorical variables. $P < 0.05$ was considered significant. The prevalence of mutations in the *rpoβ*, *inh*A and *kat*G genes, as detected using GenoType® MTBDR*plus*, were estimated from the compiled data.

Ethical approval:

This study was approved by MUSOM/MTRH research and ethics committee (Approval Number: 000657; FAN: IREC 000657).

3. RESULTS

Isolates from 120 smear positive TB cases were analyzed, of whom 62 were from males and 58 were from females. The mean age of females and males was, respectively, 11.5 years and 15.2 years. Among isolates, 90, 29, and 1 were retreatment cases, relapse after treatment and drug defaulters, respectively. There was statistically no significant difference in the ages of the patients (Table 1).

Table 1: Characteristics of the TB patients enrolled

Isolates	Male (n=62)	Female (n=58)	Statistical tested (n=120)
Mean age ± SD	15.2±2.3 Min: 5, max: 16	11.5±2.5 Min: 5, max:18	0.891
Patients' category			
Retreatment	47	43	90 (75.0%)
Relapse	15	14	29 (24.2%)
Drug defaulters/Interrupters	0	1	1 (0.8%)
TOTAL	62	58	120 (100%)

Min = minimum; max = maximum; SD: standard deviation;

Among the 120 *M. tuberculosis* isolates from positive sputum smears, 102 (85%) isolates were sensitive to both rifampicin and isoniazid whereas 18 (15%) isolates were resistant to at least one of these drugs. Those that were resistance to rifampin (RIF) and isoniazid (INH) were found respectively in 17/120 (14.2%) and 18/120 (15%) isolates whereas 17 (14.2%) were resistant to both rifampicin and isoniazid. Genotypic multi-drug resistance was then confirmed in 14.2% (17/120) isolates (Table 2).

Table 2: Analysis of anti-tuberculous mutation patterns by MDRTB*plus* and MGIT 960 systems

No. of isolates (N=18)	MGIT 960 DST		MDRTB*plus* assay			Result
	RIF	INH	RIF Pattern	INH Pattern		
			rpoβ gene	*katG* gene	*inhA* gene	
5	R	R	MUT1 (D516V)	MUT1 (S315T1)	MUT3A (T8C)	MDR
2	R	R	MUT2A (H526Y)	MUT1 (S315T1)	MUT2 (A16G)	MDR
3	R	R	MUT2B (H526D)	MUT1 (S315T1)	MUT2 (A16G)	MDR
5	R	R	MUT3 (S531L)	MUT1 (S315T2)	MUT1 (C15T)	MDR
1	R	R	MUT 1 (D516V)	⌂WT	MUT3A (T8C)	MDR
1	R	R	MUT3 (S531L)	MUT1 (S315T1)	⌂WT	MDR
1	S	R	⌂WT	MUT1 (S315T1)	⌂WT	monR
102	S	S	⌂WT	⌂WT	⌂WT	SAD

Key: ⌂: Absence of hybridization signal with wild-type probes; INH: isoniazid; MDR: multi-drug resistant; monR: mono-resistant to *katG* gene; MUT: mutant; WT: wild-type; R: resistant; RIF: rifampicin; SAD: susceptible to all drugs

Genetic mutations in *rpoβ* 505-533, *katG* 315 and *inh*-8 to -16 codons associated with resistance to RIF and INH were found respectively in 17/18 (94.4%), 17/18 (94.4%), and 16 (88.9%) isolates (Table 3). The mutations at codon 315 of *katG* gene and at codons 505-533 of *rpoβ* gene are frequently found in MDR-TB isolates which confirm their strong implication in the development of multidrug resistant tuberculosis.

Table 3: Patterns of gene mutation among the anti-tuberculous drug resistance isolates

Resistance gene, n=18	Mutation probe	Codon analysis	Number (%)
rpoβ	MUT1 (D516V) MUT2A (H526Y) MUT2B (H526D) MUT3 (S531L)	505 – 533	17 (94.4%)
¥ *katG*	MUT1 (S315T1) MUT1 (S315T2)	315	17 (94.4%)
¥ *inhA*	MUT1 (C15T) MUT2 (A16G) MUT3A (T8C)	-8 to -16	16 (88.9%)

Key: ¥ Excluding the missing of one wild-type (WT) in the *kat*G and the *inh*A gene

Among the MDR-TB isolates, the most common mutation identified as RIF resistance mutation genes were seen in codons D516V and S531L (33.3%) and the least identified mutations in RIF resistance was in gene codon H526Y (11.1%). Among the MDR-TB isolates the most common mutants identified as INH-resistant mutations were 12 (66.7%) in the *kat*G gene and 5 (27.8%) in both *inh*A (C15T) and A16G. One strain harbored unknown mutation gene probe in the *inh*A gene and its mutation pattern could not be identified (Table 4).

Table 4: Frequency of mutation in codons of anti-tuberculous drug resistant isolates

Resistant gene	Mutation probe	Codon analysis	Frequency
rpoβ	MUT1 (D516V) ⌂WT¾	513 – 519	6 (33.3%)
	MUT2A (H526Y) ⌂WT7	526 – 529	2 (11.1%)
	MUT2B (H526D) ⌂WT7	526 – 529	3 (16.7%)
	MUT3 (S531L) ⌂WT8	530 – 533	6 (33.3%)
katG	MUT1 (S315T1) ⌂WT	315	12 (66.6%)
	MUT1 (S315T2) ⌂WT	315	5 (27.8%)
*inh*A	MUT1 (C15T) ⌂WT1	-15	5 (27.8%)
	MUT2 (A16G) ⌂WT1	-16	5 (27.8%)
	MUT3A (T8C) ⌂WT2	- 8	6 (33.3%)
	Unknown	-	1 (5.5%)

Key: ⌂: Absence of hybridization signal with wild-type probes

4. DISCUSSION

Our study showed that the GenoType® MTBDR*plus* test correlated very highly with conventional culture and with SIRE MGIT 960 DST. It also showed a high incidence of laboratory-confirmed MDR-TB (12.5%) among isolates from retreatment TB cases in Uasin Gishu County. The findings from this region, 0.83% (1/120) of isolates had INH monoresistance. According to Colijn, *et al*, monoresistant cases may progress to MDR-TB or polyresistance and it is important to identify these cases early for prompt management [23]. In the present study, we observed that most retreatment cases were male, which is probably due to the fact that these individuals are more likely to have unreliable working hours, making them more likely to miss taking medication.

Mutations in the *rpoβ* gene were responsible for the majority of RIF resistance in *M. tuberculosis* [24], [25]. Our study finding showed that RIF resistance was associated with MUT3 (S531L) mutation in the region 530-533 of *rpoβ* gene. This finding was similar to those found by Rufai, *et al* who while using the line-probe assay found that RIF resistance was most commonly associated with S531L mutation in the region 530–533 of *rpoβ* [26]. This mutation has been more frequently found in MDR than RIF mono-resistant strains. The presence of WT8 region (⌂WT8) was observed in 40% (6/15) MDR-TB samples. These findings contrasted with findings from those of high-prevalence countries such as Ethiopia [27].

Mutations involving D516V, H526Y and H526D (MUT 1, MUT2A and MUT2B respectively) in the *rpoβ* gene were infrequent in our study (respectively 26.7%, 13.3% and 20.0% in MDR-TB). This finding is consistent with other studies from Pakistan [28], however, it sharply contrasts with the higher rates reported from studies in Italy [15].

Mutations involving codon 315 have been shown to be responsible for the majority of resistance to INH conferred by mutations in catalase-peroxidase enzyme gene (50-95% of INH-resistant strains) of the *katG* gene [18]. Our study found similar results, that majority, 66.7%, of INH resistance was associated with mutation in codon S315T1 of *katG* gene followed by mutation in codon S315T2. This confirmed that mutations involving codon 315 of *katG* were most commonly seen among INH-resistant strains, a finding which found by a similar study in China [29]. Mutant probes for the low resistant *katG* gene involving codons -15 and -16 were also found to be common among INH-resistant strains in our study.

5. CONCLUSION

The GenoType® MTBDR*plus* assay was found to be sensitive, specific and reliable rapid screening test for the detection and identification of different mutational patterns conferring resistance to MDR-TB cases. The most frequency resistance in this region was found to be associated with mutation in codon S315T1 of *katG* gene.

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