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Mycobacterium tuberculosis resistance to Isoniazid and Rifampicin in a HIV-1 endemic population in western Kenya in 2012-2014

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Abstract
Background: *Mono-resistant and multi-drug resistant tuberculosis (MDR-TB) has been enhanced by delays in the identification of resistant strains. However, resistance gene patterns and the extent of mono-resistant TB and MDR-TB in western Kenya is unknown. The objective of the study was to identify cases of mono-resistant TB and MDR-TB in western Kenya.*
Methods: *Early morning sputum samples were cultured on Mycobacteria growth indicator tubes (MGIT) and incubated at 37 °C. Drug susceptibility testing (DST) using the SIRE® kit was done on ZN smear positive MGIT tubes and line probe assay (LPA) performed to identify specific mutations on the rpo B, kat G and inh A genes. Mutations on discordant samples were confirmed by sequence analysis.*
Results: *The rpo B H526Y and the kat G S315T1 mutations were common in HIV positive patients (8 % and 18 % respectively) and that the S3I5T1 and S531L was the most common mutation in MDR-TB strains in both HIV positive and negative patients (5 % and 8 % respectively). Binary logistic regression, indicated that RMR TB is associated with HIV status (P = 0.025).*
Conclusions: *Our findings showed that there is a potential association between RMR TB and HIV-1 status in western Kenya.*
Keywords: *Tuberculosis, HIV, Drug susceptibility test, Line Probe Assay, MDR-TB, mono resistant TB.*

Background

In 2013, 480 000 new cases of multidrug-resistant tuberculosis (MDR-TB), defined as TB that is resistant to at least rifampicin (RIF) and isoniazid (INH), were diagnosed globally [(1)]. In sub-Saharan Africa (SSA), 2.4 % of new TB cases and 13 % of previously treated TB cases were estimated to have MDR-TB [(1)] and in Kenya, the percentage of TB cases with MDR-TB was 2.2 % and 14 % in new and retreatment TB cases respectively [(2)]. In 2013, an estimated 1.1 million of the 9.0 million people who developed TB worldwide were HIV-positive and SSA accounted for 78 % of the estimated number of HIV positive TB cases.

Treatment of drug resistant TB in Kenya is based on the Ministry of Public Health and Sanitation Guidelines [(3)] and RIF and INH are first-line drugs recommended for TB treatment [(7)]. Studies have

proved that high level and low level INH resistance occur as a result of mutations in the *kat* G gene and the promoter region of the *inh* A gene respectively. Patients with INH mono-resistant TB require longer treatment periods than those with INH-susceptible TB [4]. Rifampicin interacts with the ß subunit of RNA polymerase, encoded by the *rpo* B gene [5]. Studies in high burden TB countries have emphasized the importance of molecular characterization of mutations for the management of MDR-TB particularly in HIV infected patients [6]. Current data is however needed to study the prevalence and the role of specific RIF and INH mutations in MDR-TB. This will further provide information on whether or not these mutations are attributed to high or low level clinically relevant resistance in previously treated cases of TB. The current study aimed to establish the proportion of INH and RMR MTB and MDR-TB as well as the association of specific mutations with HIV status in western Kenya.

Methods

Study site

A total of 1666 sputum samples were processed from August 2012 to October 2014 from 415 Division of Leprosy, Tuberculosis and Lung Diseases (DLTLD) supported facilities in 14 counties.

Study population

Western Kenya has a population of 5 442 711 (Kenya National Bureau of Statistics, 2009 population census) which was projected to increase by 2.46 % by 2015. 39.2 % adolescents in the age group 12-18 years have suspected TB [7] and in a previous national survey, MDR-TB prevalence was between 0.0 % - 1.1 % [18]. The prevalence of HIV infection is 15.1 % in persons between 15-64 years of age and it represents the highest cases in Kenya and TB is the leading cause of death in people living with HIV [8].

Sample collection

Sputum

Sputum samples were collected at enrollment and transported to a referral laboratory for processing. This laboratory participates in External Quality Assurance (EQA) for the performance of both phenotypic and genotypic tests.

HIV testing

Patient HIV status was determined by the Uni-Gold HIV Rapid Test® (Trinity Biotech Plc, Bray, Co Wick low, Ireland) and Abbott Determine® HIV-1/2 test (Abbott Laboratories, Chicago, Illinois, USA) as per the national guidelines for HIV testing and counseling.

Sample processing

Culture of Mycobacterium tuberculosis

Sputum specimens were processed using the N-acetyl-L-cysteine (NALC) and sodium hydroxide (NaOH) decontamination and digestion procedure and inoculated on the *Mycobacteria* growth indicator tubes (MGIT) tubes and incubated at 37°C in the Bactec MGIT 960® instrument (BD Diagnostic systems, Baltimore, Maryland, USA) and monitored weekly for six weeks.

Drug susceptibility testing

Drug susceptibility testing (DST) was done using the BACTEC™ MGIT™ 960 SIRE kit (BD Diagnostic systems, Baltimore, Maryland, USA) for first line anti-tuberculosis drugs on samples that had *M. tuberculosis* after incubation in the Bactec MGIT 960® instrument (BD Diagnostic systems, Baltimore, Maryland, USA).

DNA extraction

Extraction of DNA was done using the GenoLyse® kit (Hain Life Sciences, Nehren, Germany).

Line Probe Assay

The Genotype MTBDR line probe assay (LPA) (Hain Lifescience, Nehren, Germany) was performed as per the manufacturer. Thereafter, samples with discordant LPA and DST results

were sequenced to confirm the presence of mutations.

Gene sequencing

Isolates with LPA and DST discordant results were sequenced using the BigDye terminator (Applied Biosystems Inc., Foster City, California, USA) method.

Statistical analysis

Multivariate and univariate analysis were used to study the effect of age, gender and HIV status and TB resistant genotypes. Binary logistic regression was used to evaluate the association between gene mutations and HIV status. Test performance was calculated using free online statistical calculators available at http://www.medcalc.org/calc/.

Ethical Considerations

Approval was obtained from the Ethical Review Committee (ERC) of the Kenya Medical Research Institute (KEMRI) Nairobi. (SSC number 2854).

Results

Clinical characteristics of patients

Majority of patients were aged between 25-44 years, 801 (58.5 %) (Table 2).

Treatment history

Patients reporting for TB retreatment were less (n=19, 1.1 %) than the patients seeking treatment for TB for the first time (n=1 647, 98.9 %), (Table 3).

Prevalence of drug resistant TB

A total of 62 (3.72 %) isolates had RIF, INH and multi-drug resistant TB (Table 4). Overall, 24 samples had discordant results and 38 samples had concordant results (Table 4). The LPA and conventional DST methods reported 13 (0.78 %) and 21 (1.26 %) samples with MDR-TB; 25 (1.50 %) and 27 (1.62 %) samples with INH mono-resistant TB; 12 (0.72 %) and 9 (0.54 %) samples with RIF mono-resistant (RMR) TB, respectively.

The performance characteristics for LPA as compared to DST were as follows; Sensitivity; RIF 66 % (49 % to 80 %) and INH 78 % (62 % to 89 %, 95 % CI), Specificity; RIF 88 % (68 % to 97 %) and INH 82 % (60 % to 95 %, 95 % CI).

Mutations in concordant samples

Overall, 38 (2.3 %) samples had concordant results. The LPA identified *rpo* B, *kat* G and *inh* A gene mutations in this samples. The 23 (1.38 %) discordant samples were sequenced to confirm the presence of mutations. 14 (58 %) samples were sequenced and 8 were resolved in agreement with DST. Therefore, using LPA and genetic sequencing, mutations were identified in 46 (2.76 %) samples. However, HIV status data was only available for 38 isolates (table 5). The *rpo* B S531L and the *kat* G S315T1 were the most common MDR-TB mutations in HIV negative patients, 3 (8 %). The *rpo* B H526Y rifampicin mutation was also common in the HIV positive patients, 3 (8 %). The *kat* G S315T1 was the most common INH mono-resistant mutation in HIV positive patients.

Drug resistant genotypes association with HIV status, age and gender

Multivariate analysis indicated that drug resistant mutations were potentially associated with the variables, HIV status, age and gender ($\lambda = 0.634$, $F(6, 64) = 2.735$, $P = 0.02$) table 6. Subsequent univariate analysis showed that age ($F(2, 33.422$, $P = 0.044$) and HIV status ($F(2, 34) = 3.827$, $P = 0.032$) is associated with gene mutations, table 6. Tukey *post hoc* analyses showed age had an association with INHMR and MDR mutations ($P = 0.044$), table 7. Games-Howell *post hoc* analyses also showed that HIV was associated with RMR and INHMR mutations ($P = 0.023$), table 7. Logistic regression analysis to investigate the association of HIV status with RMR TB, INHMR TB and MDR-TB mutations showed statistical significance indicating that the mutations were associated with HIV status. (Chi square = 7.305 $p < 0.026$, df = 2. RMR TB were associated with HIV status ($P = 0.025$).

Table 1: Primer sequences

Gene	Primer designation	Oligonucleotide sequence 5'- 3'	Size (bp)
rpo B	rpoB-1f	CTT GCA CGA GGG TCA GAC CA	543
	rpoB-2r	ATC TCG TCG CTA ACC ACG CC	
inh A	inhA-1f	TGC CCA GAA AGG GAT CCG TCA TG	455
	inhA-2r	ATG AGG AAT GCG TCC GCG GA	
kat G	katG-F5	AAC GAC GTC GAA ACA GCG GC	455
	katG-R6	GCG AAC TCG TCG GCC AAT TC	

bp = base pair; *rpo* B = rifampicin resistance conferring gene; *inh* A = Isoniazid resistance conferring gene; *kat* G = Isoniazid resistance conferring gene

Table 2. Characteristics of patients

Parameter	Category (n, %) Total
Age (years)	0-14 (38, 2.8) 15-24 (177, 12.9) 25-44 (801, 58.5) 45-64 (281, 20.5) 65+ (72, 5.3)
Gender	Male (808, 59.0) Female (561, 41.0)
HIV Status	Infected (835, 61.0) Uninfected (534, 39.0)

n, %, number and percentage

Table 3. TB treatment history

	TB treatment history, n, (%)		Xpert MTB/RIF test, n, (%)	
County	New cases 1647, (98.9)	Retreatment 19, (1.1)	MTB positive, 742, (44.5)	RIF resistance, 51, (6.9)[a]
Busia	7, (0.4)	-	5, (0.3)	-
Elgeyo Marakwet	4, (0.2)	-	3, (0.2)	-
Homabay	272, (16.3)	3, (0.2)	110, (6.6)	5, (0.7)
Kakamega	48, (2.9)	-	24, (1.4)	-
Kericho	13, (0.8)	-	12, (0.7)	-
Kisii	219, (13.2)	7, (0.4)	84, (5.0)	5, (0.7)
Kisumu	443, (26.6)	-	204, (12.2)	12, (1.6)
Migori	191, (11.5)	3, (0.2)	88, (5.3)	7, (0.9)
Nandi	7, (0.4)	-	3, (0.2)	-
Nyamira	38, (2.3)	3, (0.2)	21, (1.3)	4, (0.5)
Siaya	383, (23.0)	3, (0.2)	172, (10.3)	17, (2.3)
Tranzoia	8, (0.5)	-	8, (0.5)	1, (0.1)
UasinGishu	11, (0.7)	-	7, (0.4)	-
Westpokot	3, (0.2)	-	1, (0.1)	-

MTB = Mycobacterium
RIF = Rifampicin
[a]RIF resistance percentage calculated from the MTB positive cases

Table 4. Percentage of discordant and concordant (in bold) RIF mono-resistant, INH mono resistant and Multi-drug resistant (MDR) isolates estimated by the conventional drug susceptibility test (DST) and line probe assay (LPA) method in western Kenya in 2012-2014

		Conventional DST				Total
		$RIF^R INH^R$	$RIF^S INH^R$	$RIF^R INH^S$	$RIF^S INH^S$	
LPA	$RIF^R INH^R$	**12 (0.72)**	1 (0.06)	-	-	13 (0.78)
	$RIF^S INH^R$	4 (0.24)	**18 (1.08)**	1 (0.06)	2 (0.12)	25 (1.50)
	$RIF^R INH^S$	1 (0.06)	-	**8 (0.48)**	3 (0.18)	12 (0.72)
	$RIF^S INH^S$	4 (0.24)	8 (0.48)	-	--	12 (0.72)
Total		21 (1.26)	27 (1.62)	9 (0.54)	5 (0.30)	62 (3.72)

RIF = Rifampicin
INH = Isoniazid
$RIF^R INH^R$ = MDR-TB
$RIF^R INH^S$ = RIF mono-resistant
$RIF^S INH^R$ = INH mono-resistant
$RIF^S INH^S$ = Sensitive to RIF and INH

Table 5. Rifampicin and isoniazid gene mutations in western Kenya in 2014

rpo B		*kat* G		*inh* A		HIV Status n, (%)	
Codon	Nucleotide change	Codon	Nucleotide change	Region	Nucleotide change	**Positive 25, (66)**	**Negative 13, [34]**
D516F	GAC-TTC	S315T1	AGC-ACC	?	?	?	1, [3]
D516V	GAC-GTC	S315T1	AGC-ACC	?	?	?	1, [3]
S531L	TCG-TTG	S315T1	AGC-ACC	?	?	2, [5]	3, [8]
S531L	TCG-TTG	?	?	-15	C - T	1, [3]	?
Missing wild type *	?	S315T1	AGC-ACC	?	?	1, [3]	2, [5]
D516V	GAC-GTC	?	-	?	?	1, [3]	?
H526D	CAC-GAC	?	?	?	?	2, [5]	?
H526R	CAC-CGC	?	?	?	?	?	1, [3]
H526Y	CAC-CCC	?	?	?	?	3, [8]	?
H526Y/H526D	CAC-CCC/CAC-GAC	?	?	?	?	1, [3]	?
H526Y/H526D/S531L	CAC-CCC/CAC-GAC/TCG-TTG	?	?	?	?	1, [3]	?
S531L	TCG-TTG	?	?	?	?	1, [3]	?
?	?	S315T1	AGC-ACC	?	?	7, [18]	2, [5]
?	?	S315T2	AGC-ACA	?	?	?	1, [3]
		S315N	AGC-AAC	?	?	1, [3]	?
?	?	?	?	-15	C – T	4, [11]	2, [5]

* missing wild type = absence of staining of the rifampicin wild type probe
Amino acid abbreviations: S, Ser; T, Thr; R, Arg; L, Leu; V, Val; H, His; D, Asp; Y, Tyr; F, Phe.
Nucleotide abbreviations: A, adenine; C, cytosine; G, guanine, T, thymidine.

Table 6 Multivariate and univariate analysis of TB drug resistant genotypes and Age, Gender and HIV status in western Kenya in 2012-2014

	Wilks' Lambda				Univariate analysis		
	Value	*F*	*P* value	Partial eta squared	*F*	*P* value	Partial eta squared
Age					3.422	0.044	0.168
Gender	0.634	2.735	0.02	0.204	1.201	0.313	0.066
HIV Status					3.827	0.032	0.184

Table 7. ***Post hoc*** **analysis of drug resistant TB genotypes and Age, Gender and HIV Status in western Kenya in 2012-2014**

	Genotype		*P* value [a]
Age	RMR	INHMR	0.618
		MDR	0.274
	INHMR	RMR	0.618
		MDR	0.04
	MDR	RMR	0.274
		INHMR	0.04

	Genotype		*P* value [a]
Gender	RMR	INHMR	0.289
		MDR	0.575
	INHMR	RMR	0.289
		MDR	0.777
	MDR	RMR	0.575
		INHMR	0.777

	Genotype		*P* value [b]
HIV	RMR	INHMR	0.023
		MDR	0.239
	INHMR	RMR	0.023
		MDR	0.376
	MDR	RMR	0.239
		INHMR	0.376

[a] Tukey HSD statistic
[b] Games-Howell statistic
RMR = Rifampicin mono-resistant
INHMR = Isoniazid mono-resistant
MDR = Multi drug resistant
HIV = Human immuno deficiency virus

Table 8 Association of HIV status and TB drug resistant genotypes in western Kenya in 2012-2014

	HIV Status, n, (%)			
Genotype	Positive, 25, (66)	Negative, 13, [34]	*p* value	OR
RMR	9, 23.76	1, 2.60	0.025	15.75
INHMR	12, 31.68	5, 13.10	0.081	4.2
MDR	4, 10.56	7, 18.30	0.372	0.571

RMR = Rifampicin mono-resistant
INHMR = Isoniazid mono-resistant
MDR = Multi drug resistant

Discussion

In this study, a higher prevalence of INH resistance compared to RMR was reported (Table 4). A study in Uganda, also reported INH and RMR resistance prevalence rates of 5 % and 1.9 % respectively. We found a 1.26 % and 0.78 % prevalence of MDR-TB (Table 4), a previous study reported a slightly higher prevalence of 1.4 % [9]. This low prevalence is attributed to the longer treatment regimen recommended by the Ministry of Public Health and Sanitation.

The test performance characteristics were lower than what was reported by other studies. A recent study in India reported a sensitivity of 72 % for INH resistance [10]. These variations are depended on geographic and genetic distribution of drug resistant strains, bacillary load and routine diagnostic algorithms [11].

Consistent with previous studies [4, 5], we confirmed that MDR isolates had the *rpo* B S531L and the *kat* G S315T as the frequent combination of 5 % and 8 %, respectively. Studies have shown that MDR isolates with the *kat* G S315T mutation are associated with increased levels of INH resistance during treatment [11]. In HIV positive patients, the *rpo* B H526Y mutation was common, 8 %. Studies have shown an increased rate of drug resistant TB among HIV infected individuals [16]. Multivariate analysis showed that age, gender and HIV status had an effect on TB drug resistance genotypes (P = 0.02), (Table 6). Univariate analysis confirmed that age (P = 0.044) and HIV status (P = 0.032) independently had an effect on the TB resistance genotypes. Tukey *post hoc* analysis suggested that age significantly affected the INHMR and MDR-TB genotypes (P = 0.04)

and Games-Howell analysis suggested that HIV status had a significant effect on RMR and INHMR TB genotypes (P = 0.023), (Table 7). A study in Ethiopia reported that the age group 25-34 was associated with drug resistant TB [33]. In this study, the finding that age is associated with drug resistant TB could be due to alcoholism and health seeking behavior of patients which interfere with treatment.

A previous study found MDR-TB to be significantly frequent among previously untreated patients with TB and HIV co-infection than among those without HIV infection [17], however, using binary logistic regression, our study showed that RMR TB was associated with HIV status (P = 0.025). In a previous study in California, patients with RMR TB were seven times more likely than drug-susceptible TB patients to be co-infected with HIV [18]. The strong association of RMR TB with HIV status in our study site could be attributable to poor TB drug absorption and immunosuppression due to HIV which leads to high disease burden. Ridzon *et al* found out that the association of RMR TB with HIV status could be due to the use of Rifabutin in the treatment of *M. avium* [20]. In the present study there was no association between INHMR and MDR-TB with HIV status (P = 0.081, P = 0.472). Similar phenotypic studies in Ethiopia and Uganda reported no association between HIV status and drug resistance to first-line TB drugs [9].

Conclusion

Mono resistant TB was associated with HIV status.

Consent to publish

Informed consent was obtained from patients on sharing findings from this research through publications.

Competing interests

The authors declare that they have no competing interest.

Author's contribution

CS designed the study, collected and analyzed data and wrote the manuscript, CO and JMV revised the manuscript, JK and WM collected data and revised the manuscript, SM and AO revised the manuscript.

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