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Profiles of Isoniazid and Rifampicin drug resistance conferring mutations in *Mycobacterium tuberculosis* among new and previously treated pulmonary tuberculosis cases from Kisumu County, Kenya

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Abstract

Mycobacterium tuberculosis infection is one of the leading causes of mortality and morbidity in developing countries and drug resistance remains a challenge. Drug resistance is hastened by point mutations in the bacilli genome and their frequencies vary geographically hence the need to understand regional specific patterns for early detection of mutant strains. In this cross sectional study, Sputum samples from pulmonary tuberculosis clinical suspects were collected to detect mutations in *Mycobacterium tuberculosis* that confers resistance to Isoniazid and rifampicin anti-anti tuberculosis drugs. Detection of mutations was done using GenoType MTBDR*plus*. Out of a sample of 256 from Tuberculosis clinical suspected cases, 145 were *Mycobacterium tuberculosis* bacilli confirmed out of which 32 (22%) were new Tuberculosis (TB) cases and 113(78%) retreatment. The total for isoniazid resistance was 9(6.2%), out of which 2(6.3%) were in new cases and 7(6.3%) in retreatment cases. Multi Drug Resistance (MDR) was 2(1.8%) and all in retreatment cases. Rifampicin resistance was 7(4.8%), 1 (3.1%) in new case and 6(5.3%) in retreatment. The MDR among Rifampicin Resistance(RR) was 28.6%. Low Isoniazid resilient strains had changes in the *katG* gene resulting to nucleotide change from A G C to A C C. Four rifampicin resistant isolates showed mutations at the *rpoB* gene with nucleotide change from C A C to T A C and a single isolate displayed mutation at the *rpoB* gene with nucleotide change from T C G to T T G. Same nucleotide change A G C to A C C from different patients in the same facility might be an indication of local transmission of drug resistance and greater variability of mutations observed in HIV positive and retreatment cases are possibilities of mutations acquired during treatment courses by repeated administration of the same anti-TB drugs.

Keywords: *Multi-drug resistant TB; Mutation probe; Rifampicin Resistant, Wild-type*

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Introduction

Tuberculosis is caused by acid fast bacilli from family of *Mycobacteriaceae* and species *Mycobacterium tuberculosis (Sotgiu et al., 2013)*. The advent of tuberculosis drug resistant is global health concern as the existing cases of drug-resistant has increased substantially in the past 20 years making Tuberculosis (TB) a great contributor to deaths among people HIV infection and accounting for approximately 40% of deaths in this particular population (Gupta *et al.*, 2015; Smith *et al.*, 2008). The disease has always been associated with morbidity and mortality, especially in sub Saharan Africa and other resource limited regions and is often the first indicator of HIV infection (WHO, 2018a). According to World Health Organization (WHO), there were close to 9 million new Pulmonary Tuberculosis incidences globally in 2018 (WHO, 2018a), of which 56% were in the South Asia and Pacific, while 29 % were in Africa (Abebe *et al.*, 2012; Khan *et al.*, 2019). Rifampicin-resistant (RR) or multidrug-resistant (MDR) Tuberculosis arose to 3.6% of new Tuberculosis cases, 18 percent from previously treated Tuberculosis cases, and 5.6 percent across all the TB cases (UN, 2016). Misuse of anti-tuberculosis drugs during treatment among drug susceptible patients, sub-optimal treatment regimens and failure to complete treatment has been reported to be some sources of drug resistance (Kidenya *et al.*, 2014). Mutations resulting to drug resistance strains are frequently associated with elevated morbidity and mortality among those with Drug resistant tuberculosis, Multi-drug resistant tuberculosis, extensively drug resistant tuberculosis and total drug resistant tuberculosis (UN, 2019). Drug resistant strains pose significant threats to effective control and management of TB (WHO, 2017). Multidrug resistant Tuberculosis is caused by a strain that is resistant to the first-line anti-Tuberculosis drugs, rifampicin and isoniazid, while extensively drug-resistant strain is caused Multidrug resistant Tuberculosis that is resistant to a fluoroquinolone and any of the three second-line injectable drugs: kanamycin, capreomycin and amikacin (CDC, 2006). Drug resistant strains specifically are challenging to treat since they require higher drug combinations that are toxic, extensive, and costly (Manjelievskaia *et al.*, 2016). Tuberculosis management programs in high burden countries rely on prompt detection and early determination of mutation patterns which is essential in reduction of resistance and community transmission (Seung *et al.*, 2015). Routinely, detection of Tuberculosis drug-resistant starts from sputum culture complemented by drug resistance testing for the bacilli, in culture media however, these procedures are usually lengthy, relatively biohazardous, costly and usually limited in developing countries (Campbell *et al.*, 2011). The development of drug resistance resulting to failure in treatment is usually a common clinical indicator in Tuberculosis disease, presently scanty data from Sub-Saharan Africa has been documented, Kenya inclusive (Abhijeet *et al.*, 2020). Studies show that MDR-TB strains advances during management of fully drug vulnerable isolates and these resistance occurs due to disruption of the treatment regimen or unsuitable treatment options resulting to gene mutation and emergence of serotypes that are forbearing to the toxicity and bactericidal effects of the commonly available anti-mycobacterial agents (WHO, 2018a).

Kenya is among the 14 countries that are in the high burden list for Tuberculosis, Tuberculosis/HIV, Multi drug resistant-TB and fifth highest burden in Africa (WHO, 2020). The estimated incident for TB in Kenya is 348/100,000 population, translating to about 169,000 TB cases occurring annually, the mortality rate (excluding HIV+TB) is 60/100,000 population (Dheda *et al.*, 2017). The MDR-TB prevalence in Kenya was 1.3 percent in newly detected cases and 4.4 % in retreatment cases according to WHO TB global report of 2018 (WHO, 2018b). A study done on the "prevalence and detection of drug resistant mutations in *Mycobacterium tuberculosis* among drug naïve patients in Nairobi Kenya" from 2015 to 2017 found that out of 132 patients that were tested for drug resistance, two patients demonstrated resistance associated with first and second-line Tuberculosis drugs (Ogari *et al.*, 2019). Out of the 132 patients tested for the resistance to second-line anti TB drugs, one cross-resistance was found for aminoglycosides and fluoroquinolones (Ogari *et al.*, 2019). While comparing susceptibility between first-line and second-line drugs, it was noted that the

multidrug resistance TB had an additional second-line drug resistance while the mono-resistant had no additional second-line anti TB drug resistance (Ogari *et al.*, 2019). In Western Kenya, anti-tuberculosis drug resistance is an emerging health problem especially in Kisumu County where cases of HIV and TB Co infection are predominant (Nyamogoba and Mbuthia, 2018). Studies show that of being infected with *Mycobacterium tuberculosis* is 16 to 27 times higher among people living with HIV/AIDS than in the general population (Ogari *et al.*, 2019). According to report from Kenya National Tuberculosis, Leprosy and Lung Disease program, Kisumu County had the third highest *Mycobacterium tuberculosis* and HIV co infection rate in Kenya at 59% after, Homa Bay 64% and Siaya 63% which is way above the national co-infection of 28% and the TB Prevalence was 379 out of 100,000 people which was higher than the average National TB prevalence of 223 (MOH, 2020). Studies on tuberculosis data assessment in key vulnerable populations showed that Kisumu county was more associated with high risk populations (prisoners, slum dwellers, diabetic patients and uniformed personnel) compared to Siaya and Homa Bay Counties, while information on drug resistance conferring mutations in the region is scanty (KELIN, 2018). Drug resistance is hastened by point mutations in the bacilli genome, their frequencies vary geographically and this data is limited in Kisumu county hence the need to understand regional specific patterns for early detection of mutant strains. Given the limited data in the region and the synergetic relationship between tuberculosis drug resistance and Tuberculosis modifiable factors, there was need to describe Isoniazid and Rifampicin drug resistance conferring mutations in *Mycobacterium tuberculosis* among new and previously treated pulmonary tuberculosis cases from Kisumu County, Kenya.

Materials and methods

Study Site

The current study was done in Kisumu County, Western Kenya. The County lies between latitude 0^0 20'S and 0^0 50'S and longitudes 33^0 20'E and 35^0 20'E. The county covers approximately 567 km^2 on water surface (Lake Victoria) and 2086km^2 land area, representing 0.36% of the total land area of Kenya's 580,367km^2 (County Government of Kisumu, 2018).

Kisumu County has a total population of 1,153,343; 489,392 between 0 to 15 years and 663,951 being 15 years or above (GOK, 2018). Administratively, the County has seven Sub-counties: Kisumu Central, Kisumu West, Nyakach, Kisumu East, Seme, Muhoroni and Nyando. (County Government of Kisumu, 2018). The TB prevalence rate in Kisumu is 379 out of 100,000 people which is higher than the average National TB prevalence of 223 and TB-HIV co infection rate of 59% (GOK, 2018).

Study Design

In this cross sectional study, sputum samples and data was collected from tuberculosis clinical suspects attending TB clinics and hospital facilities within Kisumu County. This study was conducted between November 2020 and October 2021.

Data collection

The study employed clinical case reports forms and laboratory test reports as the tools for collecting data. The case report forms contained all demographic and clinical data for each patient while the laboratory request form was used to record test results. Socio demographic characteristic of respondents were defined as Categorical nominal data and included variables Gender, Sub-County, TB case, HIV status, nucleotide changes and mutant probes and Numerical data included Age variable.

Sample collection and processing

This study recruited all clinically suspected Tuberculosis patients attending various health facilities in Kisumu County and saturated sampling was favoured in this study because TB Clinics and Hospital facilities within Kisumu County was quite few. Patient meeting the minimum inclusion criteria were consented and recruited into the study. They were then given sputum cups by the clinician or laboratory personnel in the recruiting facility to have their sputum samples taken. A pipette drop from the sample was used to bacteriology confirm the sample for acid fast bacilli at the facility and an aliquot of the sputum sample was then parked in screw cups with double biohazard bags inside a cooler box and transported to Kenya Medical

Research Institute (KEMRI) Microbiology Reference Laboratory in Kisian for further confirmatory staining, culturing and Molecular drug resistance testing. Sputum sample processing was done according to the requirement of Biosafety level 3 Containment level. Local specimen shipment was done as per the regulation recommended by the International Air Transport Association (http://www.iata.org/ ads/issa/htm).

At KEMRI Microbiology reference laboratory, collected sputum samples together with the lab request forms were received from health facilities within the County and checked for sample acceptance criteria (Packaging, leakages and labelling). Samples meeting the acceptance criteria were given laboratory study number and refrigerated at +4°C awaiting processing (Figure 1).

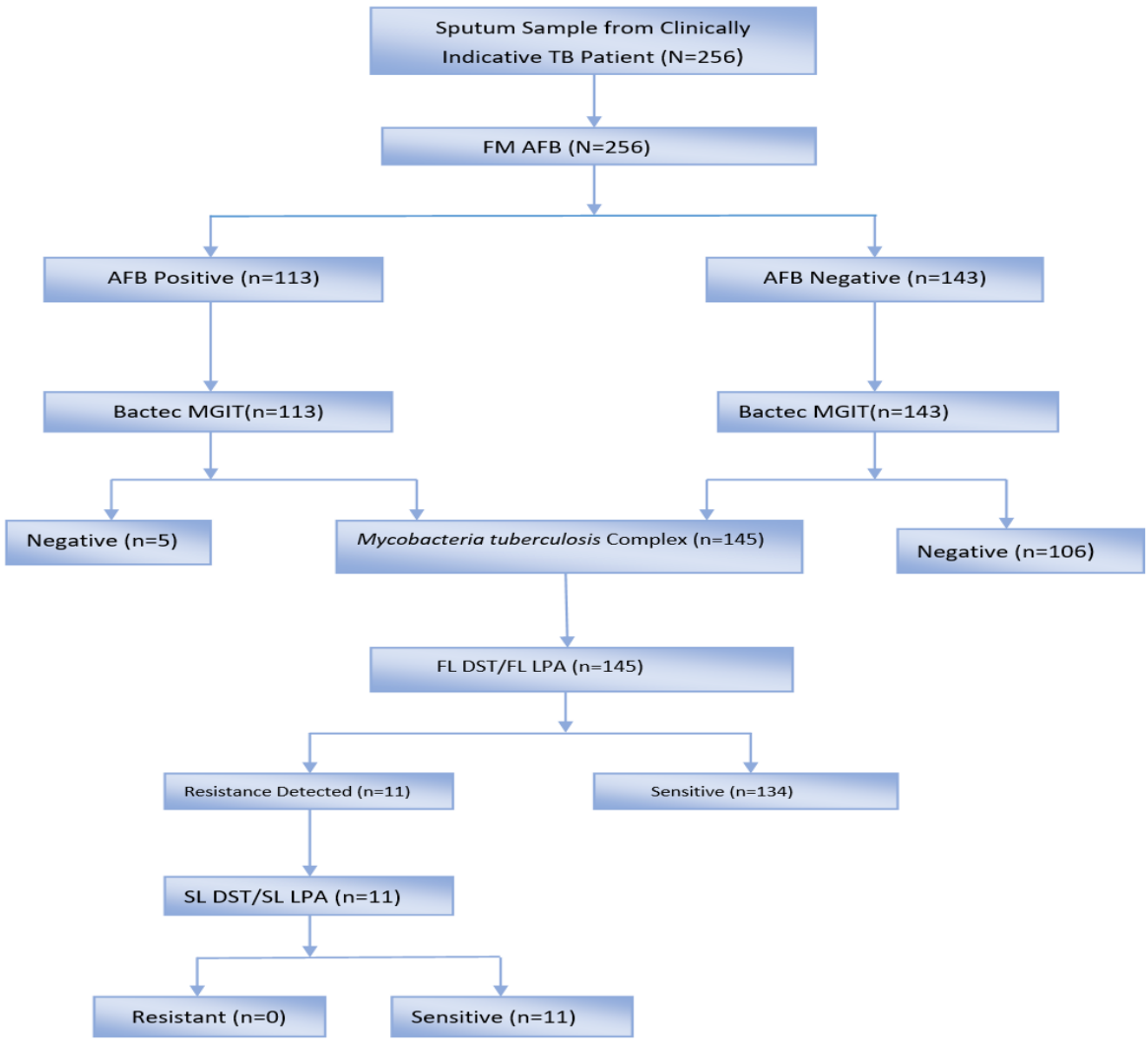


Figure 1. Flow Chart of Research Procedure

Sample Decontamination and microscopy

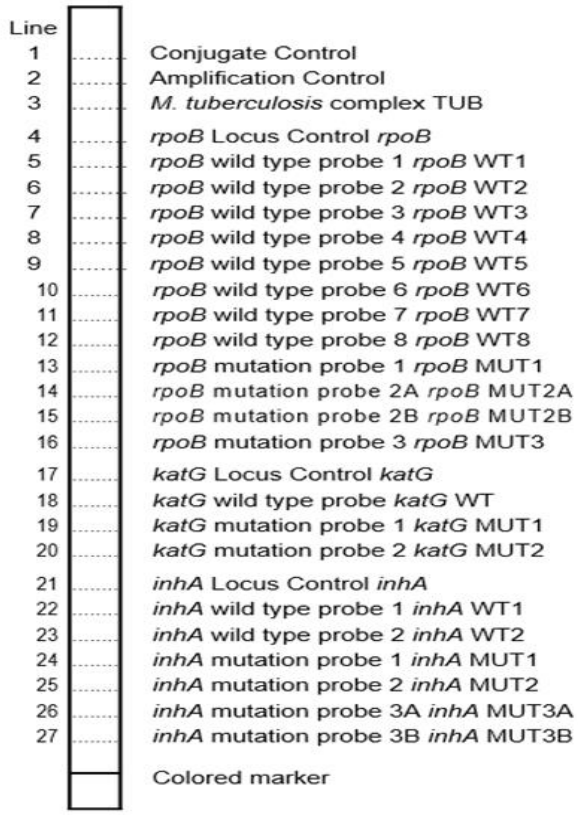
Decontamination of sputum specimen was done by the *N*-acetyl-l-cysteine-sodium citrate-NaOH method (WHO, 2015). Sputum sample was then centrifuged at 3000 g for 15 min to decant, and pellets re-suspended to make 3 ml using phosphate buffer solution. Four aliquots of 1.0 ml were made from the stock sample,1 aliquot was used for florescent microscopy, another for phenotypic DST, Line Probe Assay and the remaining stored at − 80 °C as back up. Staining, microscopy was done as follows; sputum smear was flooded with Carbol Fuchsin and heat-fixed. The slide was then washed with deionised water, decolourised with 3% acid alcohol, and flooded with malachite green and left for 2 minutes to stain. The stain was later washed with water, air dried and later observed microscopically using X100 oil immersion objective (Cheesbrough, 2006). Microscopy was done to all 256 Sputum samples.

Phenotypic drug resistance testing

Drug resistance testing for *Mycobacterium tuberculosis* to isoniazid and rifampicin anti tuberculosis drugs was done using BD BACTEC™ MGIT™ 960 system in the KEMRI Tuberculosis Microbiology Laboratory. After decantation of sediments to be used for culturing, one vial of mycobacteria growth indicator tube (MGIT) with lyophilized combination of antimicrobials was reconstituted together 15.0 ml of Mycobacteria Growth indicator enhancement. A micropipette was used to transfer, 0.8 ml of the combination to each MGIT tube to be inoculated with specimen negative and positive controls. Using a pipette, 0.5 ml of the sample was added to labelled MGIT tubes and inverted a couple times to get a uniform constitution. The MGIT tubes were later scanned and inserted into the BACTEC machine (Cheesbrough, 2006). The optimum growth temperature for M. tuberculosis was maintained at +37 °C Plus or minus -1 °C. Mycobacteria Growth Indicator tubes were incubated up to a maximum of 6weeks for the negative (no growth) while positive tubes were flagged on as soon as growth was detected. Positive tubes were removed and scanned on the Bactec instrument.

Line Probe Assay

GenoType® MTBDR*plus* assay for detection of isoniazid and rifampicin resistance mutations, was performed for 145 *Mycobacterium tuberculosis* confirmed samples by phenotypic method. Line probe assay was performed according to the manufacturer's recommendations (Hain Life Science GmbH, Nehren, Germany)(Hain Lifescience, 2015). Using multiplex PCR, GenoType® MTBDR*plus* assay was used to target specific mutations in the Rif-resistance determining region(RRD) of the *rpoB* gene (from codon 505 to 533) to detect rifampicin resistance and mutations in the *inhA* promoter (from -16 to – nucleotides upstream) and *katG* (Codon 315) regions for isoniazid resistance. These genes responsible for first line drug resistance such as *katG, inhA, rpoB* were amplified and the resulting biotin-labelled amplicons were hybridized to DNA probes bound to membrane probes. Briefly, for amplification 35µl of a primer nucleotide mixture, amplification buffer containing 5µl mM MgCl2, 2.5µl sterile water, 2.5µl (1 unit) Taq DNA polymerase (ROCHE, Mannheim, Germany), and 5 µl of DNA in a final volume of 50µl were used. This amplification protocol consisted of 15 min at 95°C for denaturation, followed by 10 cycles comprising 30s at 95°C and 2min at 58°C, an additional 20cycles comprising 25s at 95°C, 40s at 53°C, and 40s at 70°C, and a final extension at 70°C for 8min. Hybridization of the single stranded, biotin-labelled amplicons to membrane-bound probes on the strip followed by addition of conjugate, and substrate to detect visible band patterns on the strip was performed manually. The strips were allowed to dry and interpreted according to the instructions provided by the manufacturer. For each gene, GenoType® MTBDR*plus* tested for presence of wild type (WT) and mutant (MUT) probes (Figure 2).



Source: GenoType MTBDRplus test (Hain Life science GmbH, Nehren, Germany).

Figure 2. Configuration of GenoType MTBDRplus V2

Data Management and analysis

Clinical and Laboratory data collected was entered into Laboratory Information management system database. The Statistical Package for Social Sciences (SPSS) v23 (SPSS Software | IBM) was used for data analysis and it merged the clinical and the laboratory databases prior to analysis. To describe the general characteristic of the population, nominal categorical data such as Sub-counties were compared to tuberculosis test outcomes variables (*Mycobacterium tuberculosis* detected or not detected). This data was presented in numbers and percentages. Socio demographic characteristic of respondents were defined as binary categorical (sex, HIV Status, FM microscopy and MGIT BACTEC) were described in Frequencies and presented in numbers and proportions. Numerical variable such as age was described through measures of central tendency (mean and mode) that was used to present age of the respondents. Sub-County nominal categorical variables were compared with tuberculosis resistant cases and presented as numbers and proportions. Frequency tabulation was used to classify tuberculosis cases as new or retreatment while cross tabulation was used to compare tuberculosis cases among different sub counties. To describe the profiles of Isoniazid and Rifampicin drug resistance conferring mutations in *Mycobacterium tuberculosis*, demographic data such as Sex (categorical) and Age (numerical) were analysed using descriptive analysis. Mean was used to determine the mean Age among the tuberculosis cases while mode was used to determine the modal sex mostly affected by the tuberculosis drug resistance. Frequency tables and bar charts were used to present this data. Inferential statistics was used to analyse categorical test results such as new and previous TB cases, HIV status, bacteriological smear and culture MGIT test results. Multiple response LPA drug resistance results had the variables defined

and presented in frequency tables as numbers and proportions. Cross tabulation was used to explore first line drug resistance mutation patterns among new and previously treated cases and second line drug resistance mutations in new and previously treated TB cases. Chi-squared test was applied to assess factors associated with drug resistance TB in terms of the odds ratio at 95% confidence interval (CI). Associations were considered statistically significant when p-value less than or equal to 0.05.

Ethical Considerations and Approval

Linking of identifying information, such as patient name and birthdate, to the study identification number appeared only on a cover sheet of the patient data form. These data forms were kept in a locked Good Clinical Practice (GCP) compliant cabinet at KEMRI-CGHR, Kisumu and was only accessible by the investigation team and the sputum samples were labeled with the date of collection and patient's study identification number. Data entry was performed on site by the local investigators in a password protected computers and only the study investigators and data staff had access to this data. Scientific Ethical approval was granted by the Kenya Medical Research Institute, Scientific Ethical Review Unit (KEMRI/SERU/CGHR/002-02-330/4079) and National Commission for Science, Technology & Innovation (NACOSTI/P/21/10981).

Results

Socio-demographic Characteristics

The samples received were classified as new TB cases and previously treated cases as per the World Health Organization guidelines for surveillance of drug resistance in tuberculosis.

The study showed that, out of sample size of 256, 168 (65.6%) were male while the remaining 88 (34.4%) were female. Ages under 18 years were 5 (2%), while 18 years and above 251 (98%). All the patients had a mean age of 40 years with a standard deviation of ± 12.9 and a range of 13 to 77 years. Mycobacteria culture on BACTEC showed 145 (56.6%) *Mycobacterium tuberculosis* confirmed while 111 (43.4%) Mycobacteria not detected. Fluorescent microscopy showed 113 (44.1%) positive while 143 (55.9%) negative (Figure 3).

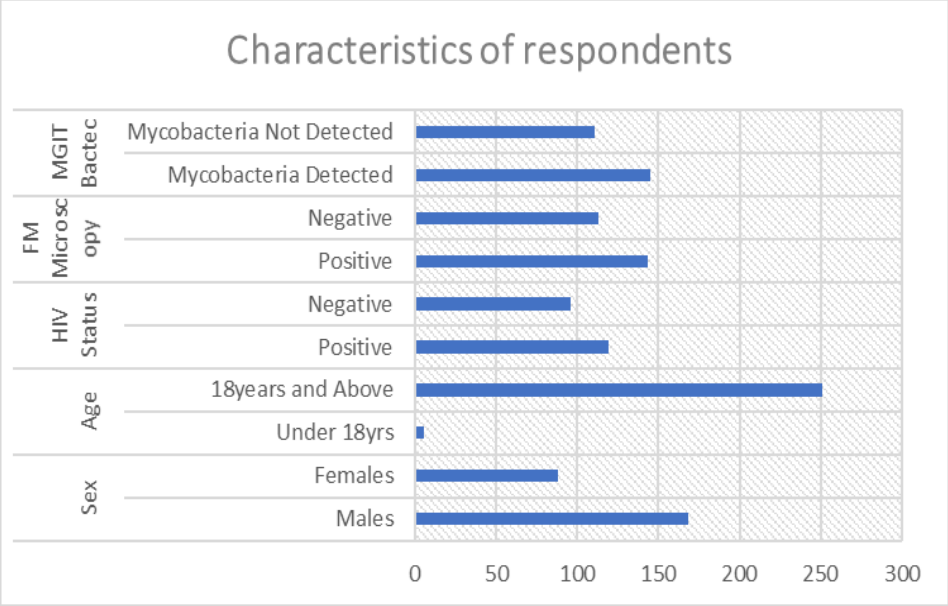


Figure 3. Socio-demographic characteristics of the respondents

Characteristic of TB Confirmed cases

Out of a total of 145 *Mycobacterium tuberculosis* confirmed cases on MGIT BACTEC from tuberculosis suspect cases 32 (22.1%) were from new TB cases and 113 (77.9%) retreatment. Males were 112 (77.2%) while females were 33 (22.8%). Ages under 18 years were 2 (1.4%), 18 years and above 143 (98.6%), while 75 (51.7%) were HIV positive and 46 (31.7%) were negative. None response for this variable was 24 (16.6%) (Figure 4).

Sub-County drug resistance profile

From a sample of 145 tuberculosis confirmed cases on phenotypic culture, rifampicin resistance was highest in Seme Sub-County 4 (2.8%) and isoniazid 3 (2.1%); followed by Kisumu Central rifampicin 3 (2.1%) and isoniazid 3 (2. 1%). No resistance to both isoniazid and rifampicin were experienced in Muhoroni and Nyakach Sub-counties. Out of the 4 (2.8%) MDR cases, 3 (2.1%) were from Seme, while 1 (0.7%) was from Kisumu Central Sub-county. All the 3 MDR cases from Seme were from the same facility (Body health Centre) and 1 case from Kisumu central was from Lumumba Health Centre (Figure 5).

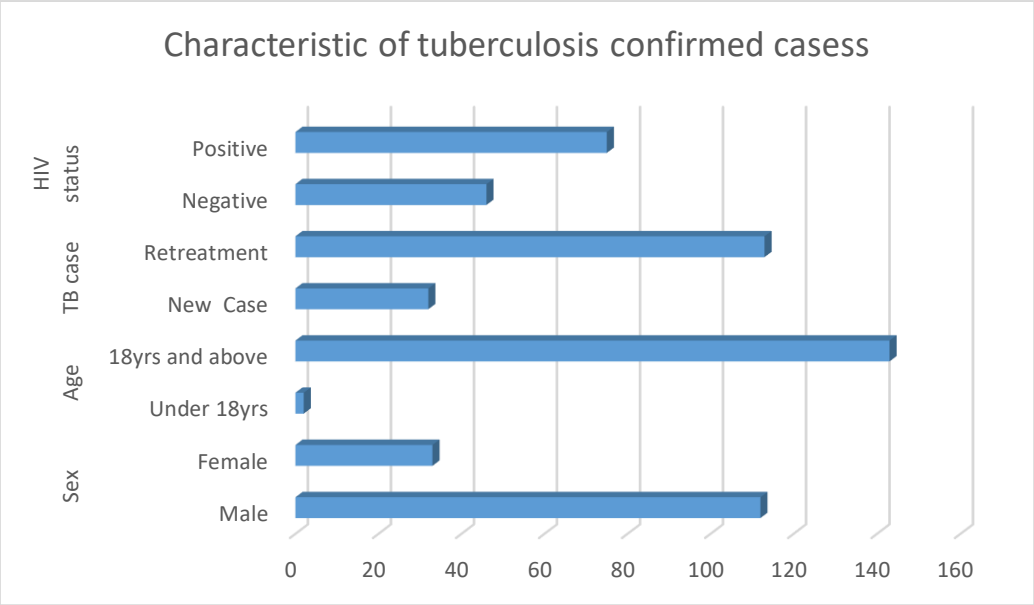


Figure 4. Characteristic of Mycobacterium Tuberculosis Confirmed cases

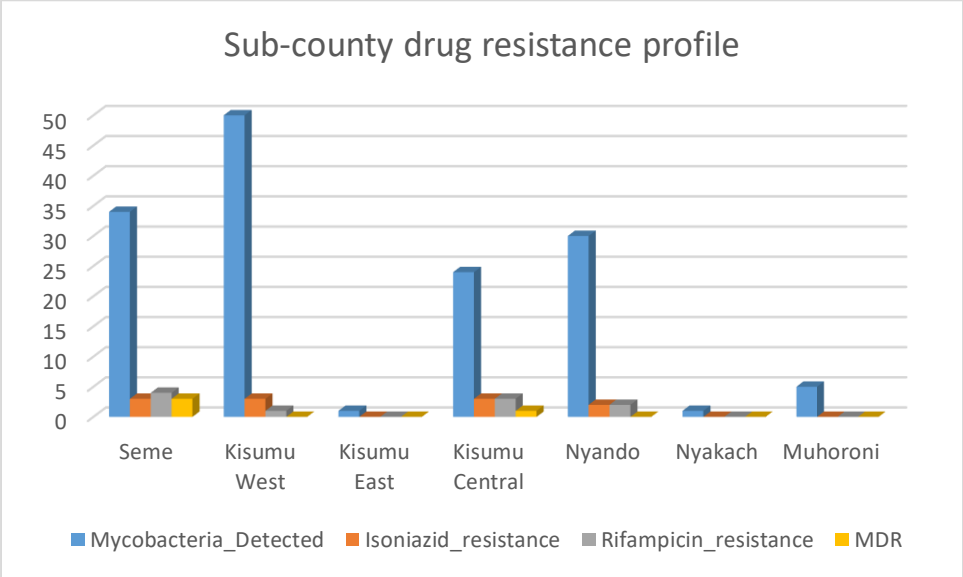


Figure 5. Sub-County Mycobacterium tuberculosis drug resistance profile

Factors Associated with Isoniazid Resistance

Out of a total of 2 children who were below 18 years, 1 (50.0%) showed isoniazid resistance whereas 10 (7.0%) out of a total 143 (98%) who were 18 years and above showed isoniazid resistance. Children under 18 years were more associated with isoniazid resistance compared to the adults over 18yrs, (OR=5.02,95%CI:0.788-32.095). Additionally, out of a male population of 112 (77.2%), 6 (5.4%) were isoniazid resistant and 5 (15.1%) out of a total female of 33 (34.4%) were resistance to isoniazid. Males were more likely to develop isoniazid resistance compared to females, (OR=1.60, 95%CI: (0.499-5.067). Fluorescence Microscopy for Acid Fast Bacilli showed positive test of 108 (74.5%) and Negative test of 37 (25. 9%). Out of the positive samples 6 (5.6%) were isoniazid resistance while 5 (13.5%) were isoniazid resistance from a total of 37 samples negative for Acid Fast Bacilli (AFB). Mycobacteria Growth Indicator Tube for BACTEC showed *Mycobacteria tuberculosis* detected 145 (56.6%) and *Mycobacteria* not detected 111 (43.4%) (Table 1).

Table 1. Factors Associated with Isoniazid Resistance

Factors Associated with Isoniazid Resistance					
		No of MTB Detected N=145	Isoniazid Resistance (%)	OR(95%CI)	P-Value[95 % CI)
Age Group	Children <18 Years	2(1.4)	1(50.0)	5.02(0.788-32.095)	0.08
	Adults> 18 Years	143(98.6)	10(7.0)		
Sex	Male	112(77.2)	6(5.4)	1.6(0.499-5.067)	0.429
	Female	33(22.8)	5(15.2)		
FM_AFB	Positive	108(74.5)	6(5.6)	0.66(0.206-2.102)	0.477
	Negative	37(25.5)	5(13.5)		

Factors associated with Rifampicin resistance

The study found out that, out of a total of 2 (2.0%) children who were below 18 years, 1 (50.0%) showed rifampicin resistance whereas 9 (6.3%) out of a total 143 (98.0%) who were 18 years and above showed rifampicin resistance. Children under 18 years were more likely associated with rifampicin resistance compared to adults over 18yrs, (OR=5.60, 95%CI:0.862-36.072). Additionally, out of a male population of 112 (77.2%) that showed detection for *Mycobacteria tuberculosis*, 4 (3.6%) were rifampicin resistance while 6(4.1%) out of a female of 33 (22.8%) that showed *Mycobacteria tuberculosis* detected, were resistant to rifampicin. Males were more likely to develop rifampicin resistance compared to females, (OR=1.60, 95%CI: (0.499-5.067). Out of the positive samples, 8 (7.4%) were rifampicin resistance while 2 (5.4%) were rifampicin resistance from a total of 37 samples negative for Acid fast bacilli. Chi- square test of association between Fluorescence Microscopy Acid fast bacilli and rifampicin resistance showed significant association between Fluorescence Microscopy Acid fast bacilli positive test and rifampicin resistance ($\chi 2=5.427$, df=1, p=0.02, 95%CI) (Table 2).

Table 2. Factors associated with Rifampicin resistance

		No of Patient (%) n=145	**Rifampicin resistance N (%)**	**OR(95%CI)**	**P-Value**
Age Group	Children <18 Years	2(1.4)	1(50.0)	5.6(0.862-36.072)	0.061
	Adults> 18 Years	143(98.6)	9(6.3)		
Sex	Male	112(77.2)	4(3.6)	1.6(0.499-5.067)	0.429
	Female	33(22.8)	6(18.2)		
FM_AFB	Positive	108(74.5)	8(7.4)	0.196(0.043-0.912)	0.02
	Negative	37(25.5)	2(5.4)		

Isoniazid and rifampicin drug resistance conferring mutations in Mycobacterium tuberculosis

Out of one hundred and forty-five tuberculosis confirmed cases, MGIT BACTEC showed 7 (4.8%) isoniazid monoresistance, 6 (4.1%) showed rifampicin monoresistance resistance while 4 (2.8%) and phenotypic MDR was 4 (2.8%). Out of the 4 (2.8%) MDR cases, 3 (2.1%) were from Seme, while 1 (0.7%) was from Kisumu Central Sub-county. All the 3 MDR cases from Seme were from the same facility (Body health Centre) and 1 case from Kisumu central was from Lumumba Health Centre. For Genotype MTBDR*plus*, 7 (4.8%) showed Isoniazid monoresistance, 5 (3.4%) rifampicin monoresistance, while molecular MDR was 2 (1. 4%). GenoType MTBDR*plus* showed that out of the Seven isoniazid resistance, 5 (3.4%) had mutations in the *katG MUT1* showing high level isoniazid resistance, while 2 (1.4%) *inhA MUT1* showing low level of isoniazid resistance. Mutations associated with rifampicin resistance was detected at probes *rpoB MUT2A* 4 (2.8%) and *rpoB MUT3* 1 (0. 7%). Molecular MDR sowed hereto resistance to isoniazid and rifampicin, 1 sample showed mutations in the *rpoB MUT3, katG MUT1* probes while the other deletions in the *rpoB WT7*,

katG WT. Two MDR cases were from the same facility Body health Centre in Seme sub county and had the same mutant gene region and the same amino acid change S315T1. There were wild type gene deletions detected at the Rifampicin resistance determining region of *rpoB WT7* 1 (0.7%), Isoniazid Wild type gene deletion at *katG WT* 5 (3.4%), *inhA WT1* 2 (1.4%) and *inhAWT2* 1 (0.7%). No resistance to second line anti-tuberculosis drugs was detected in this study (Table 3).

Mycobacterium tuberculosis Nucleotide changes detected by MUT probes

Highest rifampicin resistance was experienced in genes *rpoB*, which had mutant probes *rpoB MUT2* in the codons 526 to 529.These mutations resulted into the change of amino acid H526Y, changing the nucleotide from CAC to TAC. Additional rifampicin resistance was associated with probe roB MUT3 in the codons 530 to 533, this resulted into the change of amino acid S531L, changing the nucleotide from TCG to TTG. Multidrug resistance showed mutations in the genes *roB* and *katG*. Gene *rpoB* mutations was detected by mutant probes *rpoB* MUT3, katG MUT1 codons 530 to 533. These mutations resulted into amino acid changes S531L, S315T1 resulting in specific nucleotide changes TCG to TTG, AGC to ACC and CAC to TAC, AGC to ACC. The *katG* gene was detected by mutant probe *katG MUT1*, in codons 526 to 529,315 resulting into changes in amino acid H526R, S315T1 and nucleotide changes from CAC to TAC, AGC to ACC.

High level isoniazid resistance was expressed through mutation in the gene *inhA*, which was detected by the mutant probe *inhA MUT1*, in codon -15, resulting to amino acid change C15T.

Low level Isoniazid resistance was shown by gene *katG*, which was detected by mutant probe katG MUT1, codon 315, resulting to change in amino acid S315T1 and nucleotide change from AGC to ACC in the amino acid S315T1 (Table 4).

Table 3. Percentages for phenotypic and molecular drug resistance

		Resistance Detected Frequency (%) n =145	**Resistance Not Detected Frequency (%) n=145**
MGIT BACTEC	INH Monoresistance	7(4.8)	138(95.2)
	RIF Monoresistance	6(4.1)	139(94.9)
	MDR	4(2.8)	141(97.2)
GenoType MTBDR*plus*	INH Monoresistance	7(4.8)	138(95.2)
	RIF Monoresistance	5(3.4)	140(141.6)
	MDR	2(1.4)	143(98.6)

Table 4. Specific nucleotide changes of Mycobacterium tuberculosis detected by MUT probes

	Mutant gene	***Mutant Probe***	***n=14***	***codons***	***Amino_acid_ Change***	***Nucleotide Change***
RMP resistance	*rpoB*	*rpoB MUT2A*	4	526 to 529	H526Y	cac> tac
	rpoB	*rpoB MUT3*	1	530 to 533	S531L	tcg>ttg
MDR	*rpoB*	*rpoB MUT3,katG MUT1*	1	530 to 533	S531L,S315T1	tcg>ttg, agc>acc
	katG	*katG MUT1*	1	526 to 529,315	H526R,S315T1	cac> tac,agc>acc
	inhA	*inhA MUT1*	3	-15	C15T	

INH resistance	*katG*	*katG MUT1*	4	315	S315T1	agc>acc

Mycobacterium tuberculosis Drug resistance in TB cases

Out of one hundred and forty-five Tuberculosis confirmed cases, 11 (7.6%) showed resistance detected for Phenotypic DST, of which was 2 (6.3%) were in new TB case and 9 (7.9%) in retreatment case. Chi square for Phenotypic DST Isoniazid resistance and TB cases was ($\chi2$ =0.13, df=1, p =0.908), OR=1.096, 95CI:0.279-5.240. The study found out that, Phenotypic DST for rifampicin resistance was 10 (6.9%), of which was 1 (3.1%) was in new TB case and 9 (7.9%) in retreatment case. Chi square for Phenotypic DST for rifampicin resistance and TB Cases was, ($\chi2$ =0.602, df=1, p =0.438), OR 2.239,95CI (0.277-18.09). LPA for isoniazid resistance detected was 9 (6.2%) of which 2 (6.3%) was in new TB case and 7 (6.2%) in retreatment case. Chi square for LPA Isoniazid and TB cases was, ($\chi2$ =0.043, df=1, p =0.836) OR 0.844,95CI (0.170-4.193). Out of 145 samples Tuberculosis confirmed cases, LPA for rifampicin resistance detected was 7 (4.8%) of which 1 (3.1%) was in new TB case and 6 (5.1%) in retreatment case. Chi square for LPA Rifampicin and TB cases was, ($\chi2$ =0.126, df=1, p =0.723), OR 1.47, 95CI (0.173-12.496). GenoType MTBDRplus showed that isoniazid and rifampicin Monoresistance was 7 (4.8%) and 6 (4.1%) respectively while isoniazid and rifampicin MDR was 2 (1.4%) (Table 5).

Table 5. Drug resistance in TB cases

	New TB Cases N (%) n=32	**Retreatment N (%) n=113**	**Total Resistance N (%) n=145**	**P Value [95%CI]**	**OR[95%CI]**
DST Isoniazid	2(6.3)	9(7.9)	11(7.6)	0.908	1.096(0.279-5.240)
DST Rifampicin	1(3.1)	9(7.9)	10(6.9)	0.438	2.239(0.277-18.09)
LPA Isoniazid	2(6.3)	7(6.2)	9(6.2)	0.836	0.844(0.170-4.193)
LPA Rifampicin	1(3.1)	6(5.3)	7(4.8)	0.723	1.47(0.173-12.496)

Mycobacterium tuberculosis Mutation Pattern by TB cases

Codon analysed in new TB cases were Codon *-15* 1 (0.7%), codon *315* 2 (1.4%), codon *526 to 529* 2 (1.4%), While in retreatment cases were codon *-15* 2 (1.4%), codon *315* 3 (2.0%), codon *526 to 529*, 4 (2.6%) and codon *530 to 533* 2(1. 4%).Amino acid change among the New Tb Cases were, *C15T* 1 (0.7), *H526Y* 1 (0.7%), *S315T1* 1 (0. 7%).While in the retreatment was *C15T* 1 (0.7%), *H526R/S315T1* 1 (0.7%), *H526Y* 3 (2.0%), *S315T1* 3 (2.0%), *S531L* 1 (0.7%), *S531L/S315T1* 1 (0. 7%). Out of as ample of 256, 145 samples were tuberculosis confirmed cases of which 32 (22.1%) were from new TB cases and 113 (77.9%) retreatment. The total for isoniazid resistance was 7 (4.8%), out of which 2 (6.25%) were in new cases and 5 (4.4%) in retreatment cases. Molecular MDR cases was 2 (1.4%) and all in retreatment cases. Rifampicin resistance was 5 (3.4%), 1 (3.1%) in new case and 4 (3.5%) in retreatment (Figure 6).

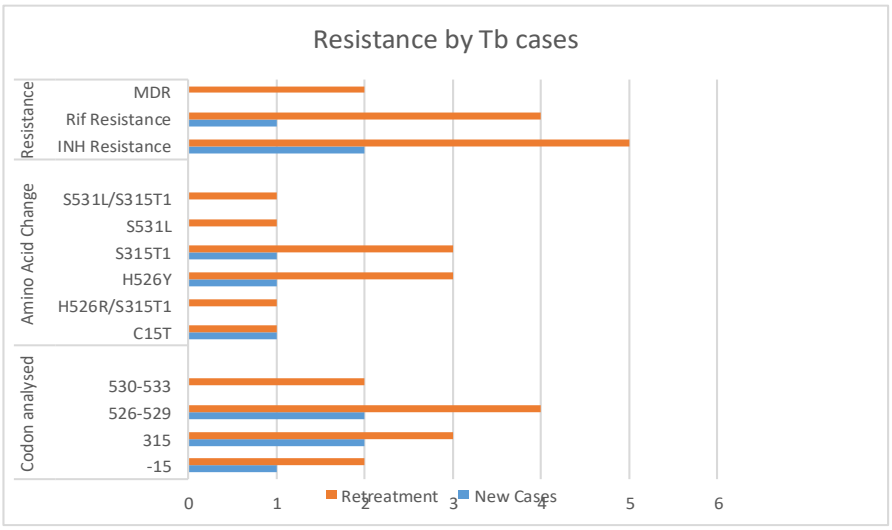


Figure 6. Mycobacterium tuberculosis Mutation Pattern by TB cases

Mycobacterium tuberculosis Mutant and Wild Type probes by TB cases

Mutant probes in New TB cases were *inhA MUT1* 1 (3.1%), *katG MUT1* 1 (3.1%), *rpoB MUT2A* 1 (3.1%), while mutant probes in retreatment cases were *inhA MUT1* 1 (0.9%), *katG MUT1* 4 (3.5%), *rpoB MUT2A* 3 (2.7%), *rpoB MUT3* 1 (0.9%), *rpoB MUT3/katG MUT1* 1 (0. 9%).Wild type gene probes in the new TB Cases were, *inhA WT1* 1 (3.1%), *katG WT* 1 (3.1%), while in the retreatment cases were, *inhA WT1/inhAWT2* 1 (0.9%), *katG WT* 3 (2.7%), *rpoB WT7/katG WT* 1 (0.9%) (Figure 7).

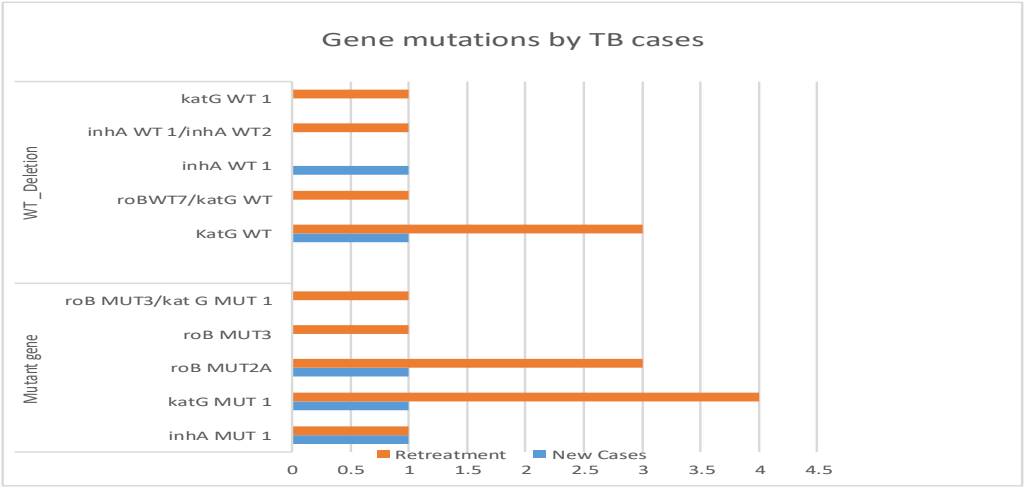


Figure 7. *Mycobacterium tuberculosis* Mutant and Wild Type probes by TB cases

Discussion

From the sample size, 145 (56.6%) were confirmed *Mycobacterium tuberculosis* cases while 111(43.4%) were negative for *M. tuberculosis.* Confirmed cases showed more males 112 (77.2%) compared to females 33 (22.8%), which is in agreement with the WHO report that relatively more males than females are exposed to Tuberculosis which may be attributed to the difference between the two sex groups in biological, societal role and access to health facilities (WHO, 2020). Among Tuberculosis confirmed cases, majority of participants were

Ages 18 years and above 143 (98.6%), while the remaining 2 (1.4%), were under 18 years. All the participants had a mean age of 40 years with a standard deviation of ± 12.9 and a range of 13 to 77 years. The current study is in good agreement with a study reporting that the 31–40 years age group was the most predominant group for isolation of DR-TB and the male population was at the highest risk (Mukati *et al.*, 2019). According Ahmed et al..2018, in a study that was conducted in India which is one of the high burden Tuberculosis countries, it was found that 17.2% of samples were from new cases, and 82.8% were from previously treated samples (Ahmed *et al.*, 2018). These findings are consistent with the current study that found out that majority of TB cases were retreatment 113 (77.9%) while new Tuberculosis cases were 32 (22.1%). Children under the age of 18 years were more likey to be associated with isoniazid resistance (OR=5.02,95%CI:0.788-32.095) and resistance to rifampicin (OR=5.60, 95%CI:0.862-36.072), compared to adults 18 years and above. The study showed that males were more likely to develop isoniazid and rifampicin resistance (OR=1.60, 95%CI: (0.499-5.067) compared to females. The study further found out that out of 11 that developed rifampicin resistance 4 (36.4%) of the isolates were MD and, out of the 4 (2.8%) MDR cases, 3 (2.1%) were from Seme-Sub-county, while 1 (0.7%) was from Kisumu Central Sub-county. All the 3 MDR cases from Seme were from the same facility (Body health Centre) and 1 case from Kisumu central was from Lumumba Health Centre. Low MDR resistance in Kisumu County was consistent with other studies in high burden countries which encountered the same low levels (1.8%) for MDR and Ethiopia with 1.1% (Seyoum *et al.*, 2014) as well as India recording the same percentage (Ombura *et al.*, 2016). Kidenya et al in 2014 also reported the MDR prevalence for the past 15 years in Tanzania ranging from 0.4–2.1%, in addition to East Africa at 0.4–4.4% (Kidenya *et al.*, 2014). High INH resistant strains had mutations in the promoter region of *inhA* gene at codon *-15* with amino acid change of *S315T1*, a similar high prevalence (85%) of S531L mutation in rifampicin resistant isolates was reported in a study at Cameroon (Abanda *et al.*, 2017), while low INH resistant strains had mutations in the *katG* gene at codon *315.* Among rifampicin resistant strains, four isolates displayed mutations at codon *526 to 529* in the *rpoB* gene with amino acid change of *H526Y* and one isolate displayed mutation at codon *530 to 533* in the *rpoB* gene with amino acid change of *S531L* while the MDR strains had mutations in the *rpoB* and *katG* genes. The *rpoB* gene displayed mutations at codons *530 to 533* with amino acid changes *of S531L and S315T1*, while *katG* had mutations at codon *526 to 529* and *315* with amino acid changes of H526R and *S315T1.* These mutations have already been reported and are in concordance with previous published studies for strains from other parts of the world, which reflect a global pattern (Eddabra and Mounsef, 2020). The presence of a high frequency of *S531L* mutation around the globe might be due to low fitness cost which impacts its strong selection in environment and transmissibility (Gagneux, 2009). In a similar systematic review done by Eddabra and Mounsef, (2020) in Morocco, it was found that mutations in codons *516* (8.26%), *526* (8.05%), and *531* (70.33%) are the most associated mutations with rifampicin resistance (Eddabra &Mounsef, 2020). In the current study, isoniazid (INH) resistance was shown by high mutations in the *inhA* promoter showing high level resistance and in the *katG* showing low level isoniazid resistance. Most reports suggest that resistance of *Mycobacterium tuberculosis* to INH showing mutation at codon *315* (Fantahun *et al.*, 2013). Findings from this study were similar showing 100% of all isolates had mutations at S315T1, attributed to high level of drug resistance to INH. Highest proportion of rifampicin resistance was experienced in probes *rpoB MUT2A* which had mutations in the codons *526 to 529.* This mutation resulted into the change of amino acid *H526Y*, changing the nucleotide from CAC to TAC. Additional rifampicin resistance was associated with gene *roB* detected by probes *rpoB MUT3* which had mutations in the codons *530 to 533*, this resulted into the change of amino acid *S531L*, changing the nucleotide from TCG to TTG. Multidrug resistance showed mutations in the genes *rpoB,* which was detected by probes *rpoB MUT3*, *katG MUT1*, in codons *530* to *533,526* to *529* and codon *315.* A significant number of reports imply that resistance of *M. tuberculosis* to INH show mutation at codon *315* (Abhijeet *et al.*, 2020), these mutations resulted into amino acid changes *S531L,S315T1* resulting in specific

nucleotide changes TCG to TTG, AGC to ACC and CAC to TAC,AGC to ACC. In the current study, the RIF resistant isolate had the mutation S531 L; the most often recorded resistance mutation in various countries (Ogari *et al*., 2019). Studies show that *katG* is the most common region targeted with a bulk of mutations occurring in codon 315 in 30–90% of INH resistant strains, in the current study, low level isoniazid resistance was exhibited in the gene loci *inhA MUT1* and high level Isoniazid resistance was shown in loci *katG MUT1*. There was no nucleotide change for amino acid *C15T* whereas there was nucleotide change from AGC to ACC in the amino acid *S315T1*. Two MDR cases detected from the same facility Body health Centre in Seme sub-county had the same mutant gene *katGMUT1* region and the same amino acid change *S315T1* might be an indication local MDR transmission. Greater variability was observed in amino acid changes in retreatment cases compared to new cases. This may be an indication that such mutations might be acquired during treatment courses by repeated administration of the same anti-TB drugs.

Conclusion

Greater variability that was observed in amino acid changes for isoniazid and rifampicin in retreatment cases compared to new cases may be an indication that such mutations might be acquired during treatment courses by repeated administration of the same anti-TB drugs in Kisumu County. Additionally, two MDR cases that were detected from the same facility (Body health Centre) in Seme Sub-County indicated same mutant gene *katGMUT1* region and the same amino acid change S315T1 with nucleotide change A G C to A C C might be a sign of local MDR transmission. Continuous sentinel surveillance on *Mycobacterium tuberculosis* drug resistance conferring mutations should be done in Kisumu County to routinely monitor the development and spread of MDR and XDR strains.

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