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Determination of Prevalence and Antimicrobial Resistance Profiles of *Mycobacterium tuberculosis* Complex Isolates from HIV Positive Patients in Kisumu County, Kenya

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Abstract:
The purpose of this study was to determine the prevalence and antimicrobial resistance profiles of Mycobacterium tuberculosis complex isolates from HIV positive patients in Kisumu County. This is because the spread of Multidrug-Resistant Tuberculosis (MDR-TB) strains has become a challenge to the global TB control and prevention program. Moreover, researchers from neighboring countries like Ethiopia have reported that despite the existence of MDR-TB strains, very limited information on the strains exist especially among the rural people. A cross-sectional study was conducted between December 2013 and June 2014. The study engaged, 379 HIV positive patients suspected of TB infection who gave sputum samples. The sputum samples were then decontaminated, concentrated, liquefied and neutralized before being cultured in liquid media using Mycobacteria Growth Indicator (MGIT) 960 tubes. The culture positive MGIT tubes were sub cultured in Brain Heart Infusion Agar (BHIA) before microscopic examination of the culture using Ziehl-Neelsen (ZN) smear for Acid Fast Bacilli (AFB), and identified using Genotype MTBC. MGIT 960 tubes positively identified to have Mycobacterium tuberculosis were then subjected to drug sensitivity test using BACTEC 960 susceptibility. This was on the four first line drugs collectively referred to as SIRE (Streptomycin, Isoniazid, Rifampicin and Ethambutol). In the study, 130 (34.3 %) of the 379 suspected TB patients were diagnosed positive for pulmonary TB by MGIT culture. This study also reveals a higher prevalence rate of MDR- TB (18.46 %) among HIV/ TB patients in Kisumu. Further, the study revealed that, the overall resistance to the first line drugs was 6.9% while the individual TB drugs had the following resistance rates; 6.2 % Streptomycin, 10 % Isoniazid, 9.2 % Rifampicin and 3.8 % Ethambutol. Based on the results, the study concludes the following: that Rifampicin and Isoniazid had the high resistance rates 9.2 % and 10 % respectively; the study also reveals a high prevalence rate of MDR-TB (18.46 %) among HIV/TB patients in Kisumu County. Therefore, there is urgent need for the public health sector to incorporate drug susceptibility testing in the management and control of TB so as to detect antimicrobial early and then treated by enhancing capacities of the medical Laboratories in the county. Also, there in need to develop shorter TB drug schedules to enhance adherence to treatment.

Keywords: *Mycobacterium tuberculosis complex, Prevalence, Drug resistance, MDR-TB, Susceptibility, Kisumu-Kenya*

1. Introduction

Mycobacterium tuberculosis complex are rod shaped aerobic bacteria that do not form spores [3]. These micro-organisms are significant pathogens of humans since they cause tuberculosis. Tuberculosis is caused by different species in the *Mycobacterium tuberculosis* complex family. According to National Leprosy Tuberculosis Programme (NLTP) (2005), TB can be caused by *M tuberculosis*, *M. bovis* or *M. africanum*. Currently, tuberculosis disease is the most threatening infection globally (Shinghal *et al.*, 2012). According to World Health Organization (2015), a third of the world's population is infected with the TB bacillus.

Globally, TB incidence has been on the increase since the early 1990's (WHO, 2015). It is estimated that the disease kills approximately eight thousand people a day which translates to 2-3 million people each year (WHO, 2012). This death toll is the world's second highest for an infectious disease, after HIV/AIDS (WHO, 2013). HIV and TB form a deadly combination, since each speeds the other's progress. People living with HIV have a weakened immune system that makes them vulnerable to the devastating effects of TB (WHO, 2013). In 2009, The Indian Central TB Division (2009) reported that India had 3.5 million HIV patients of whom about 1.8 million were also infected with tuberculosis. According to this report, diagnosis of TB in HIV patients is more difficult than in people without HIV infection, thus the need for proper scrutiny. In Sub-Saharan Africa, TB is the major cause of mortality among people living with HIV (WHO, 2004).

The rise in new TB cases since the early 1990's is due to several factors that includes inability to accurately and rapidly diagnose active TB and the emergence of Multi-Drug Resistant TB (MDR TB) strains of the pathogen (CDC, 2011). The existence of MDR – TB is a major public health concern in several countries (Adane, 2015). Indeed, Multidrug-resistant TB (MDR-TB), defined as resistant to the four first line TB drugs collectively referred to as SIRE (Streptomycin, Isoniazid, Rifampicin and Ethambutol) is emerging as a major clinical and public health challenge in Southern Countries.

Shinghal *et al.* (2012) posits that although positive development has been made to reduce global incidence of TB, the emergence of Multi-Drug Resistant (MDR) TB threatens to erode these advances. This is because a greater number of patients who have completed TB treatment still develop tuberculosis within a year after treatment thereby raising the question of drug resistance (Nyamogoba *et al.*, 2012).

In Kenya, TB is the most common opportunistic infection affecting people living with HIV and contributes to the high morbidity and mortality rates (MoH, 2003). Kenya has witnessed a rise in new TB cases since the early 1990's, recording a nine-fold increase of reported TB cases (Ministry of Public Health and Sanitation, 2012). The method of diagnosis of TB used in most hospitals in Kisumu County, does not determine drug sensitivity (WHO, 2012) and thus poses a challenge in the proper treatment of tuberculosis. According to Shinghal *et al*. (2012) unsuitable treatment and poor patient compliance to treatment have led to drug resistance. A definitive and diagnosis can best be made by culturing *Mycobacterium tuberculosis* complex isolates from specimens of patients, use of GeneXpert or by use of PCR (WHO, 2012). Also, early detection of antimicrobial and multidrug resistant tuberculosis can help in proper treatment.

Data on antimicrobial susceptibility and multidrug resistant tuberculosis of people living with HIV is not available in most hospitals in Kenya. Most studies on TB/HIV are generalized and limited information is available on antimicrobial resistance of HIV patients in Kisumu County. Sanchez-Padilla *et al.* (2013) documents that, in the developing world much emphasis is put on treatment and curbing spread rather than on sensitivity to antimicrobials which may have resulted in MDR-TB. This study therefore sought to find out the levels of resistance of *Mycobacterium Tuberculosis* Complex isolates to the commonly used first line anti-tuberculosis drugs in Kisumu County.

2. Materials and Methods

2.1. Study Site

This study was carried out at KEMRI/CDC Laboratories on sputum samples collected by KEMRI/CDC from health facilities within Kisumu County. The County covers an area of 2085.9 Km^2 and has a population of 968,909. It neighbors Vihiga County to the North, Nandi County to the North East, Kericho County to the East, Nyamira County to the South, Homa Bay County to the South West and Siaya County to the West (Fig. 1). The County has one provincial referral hospital, three district hospitals, five sub-district hospitals, fifty-three dispensaries and six health centers.

2.2. Study Population

The study was a cross-sectional design on 379 people living with HIV, 15 years and above who presented symptoms of pulmonary tuberculosis in health facilities in Kisumu County. The sample size was determined using the Fisher *et al.* (1998) formula. Children below fifteen years of age with HIV presenting with symptoms for PTB were not included because they are not able to expectorate and provide specimen (Chakaya, 2005). HIV patients presenting with symptoms for extra pulmonary tuberculosis and HIV negative patients presenting with symptoms for pulmonary tuberculosis were also not included in the study.

2.3. Mycobacterium tuberculosis Resistance and Susceptibility Testing

Three sputum samples collected per patient were put in a cool box and transported to the laboratory at KEMRI-CDC for analysis. At the laboratory, the sputum samples were macroscopically examined for adequate quantity and quality. Decontamination and concentration of the specimens, was carried out in a biosafety cabinet at a Biosafety Level 3 (BSL 3) Laboratory at KEMRI-CDC. Briefly, all specimens were transferred into 50ml falcon tubes which were staggered in racks to prevent cross contamination. An equal amount of N-Acetyl-L-Cysteine-Sodium hydroxide (NALC-NaOH) solution was added at a time to each sample. The tubes were rotated and inverted to ensure that the mixture coated the entire interior surface and then vortexed for 15 seconds (Siddiqi and Rusch-Gerdes, 2006). To the specimens that were too mucoid to liquefy, a small amount of NALC powder (30 grams) was added to each directly and vortexed, then allowed to stand for 42 days until the culture flagged positive at 37 ^{0}C and monitored for increasing fluorescence. Neutralization was carried out by slowly adding a phosphate buffer pH 6.8 into the specimen to the 50-ml mark and the surface of the tubes wiped with disinfectant (5 % phenol). The tubes were inverted several times to mix thoroughly.

The tubes were then loaded into aerosol-free centrifuge cups and centrifuged at 3000 x g for 15-20 minutes to concentrate the specimen. The supernatant was discarded and the sediment re-suspended by adding 1-2ml of phosphate buffer pH 6.8. The tube contents were gently mixed and the suspension used for preparation of smears and microbiological processes (Siddiqi and Rusch-Gerdes, 2006).

Microscopic examination for acid-fast bacilli (AFB) was carried out on each sputum sample after staining each slide with the standard Zeihl Neelsen (ZN) staining method for *M. tuberculosis* (Siddiqi and Rusch-Gerdes, 2006). The AFB smears appeared either as serpentine cording, clumps or singly. Positive (*M. fortuitum*) and negative (*E. coli*) controls were included with each batch of slides for staining (WHO, 2012).

Mycobacteria Growth Indicator (MGIT) culture inoculation was carried out by carefully adding about 0.5 ml of the concentrated specimen suspension to the prepared BBL™ mycobacteria growth indicator tube using a pipette. The MGIT 960 tubes (Becton Dickinson and Co., Cockerysville, Md.) were tightly recapped and the contents mixed thoroughly. The inoculated tubes were left at room temperature for 30 minutes before loading into the MGIT system following manufacturer's instructions for incubation. The tubes were thereafter incubated at 37°C and monitored for increasing fluorescence. The incubation duration was 42 days until the culture flagged positive. Positive and negative controls were included with each set of specimens. The growth of *Mycobacterium tuberculosis* obtained was used for sensitivity testing (Siddiqi and Rusch-Gerdes, 2006). The inoculated cultures were then examined and identified according to the MGIT Procedure Manual (2007). All culture positive MGIT 960 tubes were then subjected to tests to rule out contamination before testing for drug sensitivity.

Specimen in the MGIT 960 tubes found to be positive for *Mycobacteria* were sub-cultured in brain heart infusion agar (BHIA). These sub-cultures were incubated in carbon (IV) oxide incubators for 48 hours. The plates examined after every 24 hours to rule out bacterial false positive. For quality control, an empty BHIA was incubated on the carbon (IV) oxide incubator for 48 hours alongside another BHIA with *E. coli* and *S. aureus*. The cultures were considered positive after they were confirmed to show acid fast bacteria by ZN microscopy and negative growth on BHIA. ZN positive and BHIA positive tubes were also considered but after re-decontamination. They were all then identified using Genotype MTBC. Microscopic examination was carried out using the Ziehl-Neelsen staining procedure described earlier on each sample.

Genotyping of *Mycobacterium tuberculosis* complex was based on the DNA-STRIP technology according to the manufacturer's instructions (Hain Life science GmbH, Nehren, Germany). Thereafter, DNA was extracted from bacteria cultured in MGIT 960 media and then one milliliter of bacteria growth was spanned into a pellet at 10,000 x g for 15 minutes in a standard table top centrifuge. The supernatant formed was discarded and the bacteria re-suspended in 100-300 µl of water by vortexing. The bacteria were then incubated for 20 minutes at 95 °C in a water bath. The bacteria were again incubated in an ultrasonic bath and once again span at full speed for 5 minutes. Five microliters of the supernatant were placed in new labelled tube and used for amplification.

Standard PCR Amplification was done using mixes A and B containing polymerase and primers (Hain Life Sciences, Nehren Germany). The resultant products from the PCR were subjected to further identification on strips as described by Siddiqi and Rusch-Gerdes, (2006). MGIT 960 tubes positively identified to have *Mycobacterium tuberculosis* were then subjected to drug sensitivity test using BACTEC 960 susceptibility (Siddiqi and Rüsch-Gerdes, 2006). This was on the four first line drugs collectively referred to as SIRE (Streptomycin, Isoniazid, Rifampicin and Ethambutol).

2.4. Data Analysis

Data was analyzed using both descriptive and inferential statistics with help of STATA version-10. Descriptive statistics was used to summarize data and chi- square (χ^2) test used to investigate differences between; sputum culture positive and negative and resistance to anti tuberculosis drugs used.

3. Results

3.1. General Characteristics of Study Participants

Out of the 379 pulmonary TB suspects enrolled, 205 (54.1 %) were males while 174 (45.9 %) were females. Their ages ranged from 15 to 88 years although the mean age was 35.5 years with a standard deviation of 11.9. One hundred and thirty (34.3 %) of the three hundred and seventy-nine pulmonary TB suspects were diagnosed positive for tuberculosis by culture. Of the 130-culture positive, 122 (93.9 %) were smear-positive and 8 (6.1 %) smear-negative (Table 1). Male TB patients accounted for 57.7 % (75) of the patients while the females' were 42.3 % (55). There was no significant difference between the culture positive and culture negative groups in terms of gender and age distribution *p* - value of 0.309 (Table 1).

Characteristics	Positive sputum culture n (%)	Negative sputum culture n (%)	*P- value*
Gender			
Male	75 (57.7)		
Female	55 (42.3)		
Sputum microscopy		130 (52.2)	
Smear-positive	122 (93.9)		0.309
Smear-negative	8 (6.1)	119 (47.8)	
Age Categories (yrs)			
25 and below	75 (19.8)		
26-35	135 (35.6)		
36-45	102 (26.9)	27 (10.8)	
46-55	45 (11.9)		0.134
Above 55	22 (5.8)	222 (89.2)	

Table 1: General characteristics of participants enrolled in the study and their association with culture outcome as analyzed by the χ^2 test
n = frequency, % = percentage

3.2. Response to First Line anti-tuberculosis Drugs

Resistance cases from patients were stratified by the following categories: monoresistance, resistance to both Isoniazid and Rifampicin (multidrug resistance) or polyresistance. The results indicated varied resistance to the four drugs. Isoniazid contributed the highest resistance (10 %) compared to all the four drugs while Ethambutol had the lowest resistance (3.8 %). Rifampicin and Streptomycin each had a resistance of 9.2 % and 6.2 % respectively (Table 2). Result obtained from the study showed double resistance (multidrug resistance) to INH and RIF at 18.46 % (24 isolates). There was also a high resistance rate of the isolates to all the drugs used other than INH and RIF (poly resistance) of 6.9 % (9 isolates).

The study indicated high susceptibility of the isolates to all the four drugs used, the highest susceptibility was obtained with Ethambutol (96.2 %) and Streptomycin (93.8 %). Ninety-point eight percent of the isolates were susceptible to Rifampicin and 90.0 % susceptible to Isoniazid (Table 2).

	Resistance		**Susceptibility**	
Drug	**n**	**%**	**n**	**%**
INH	13	10.0	117	90.0
RIF	12	9.2	118	90.8
STR	8	6.2	122	93.8
ETH	5	3.8	125	96.2
Double resistance (INH and RIF)	24	18.46		
Polyresistance	9	6.9		

Table 2: Response to commonly used anti-TB drugs
n= frequency, %= percentage.
Key: INH; Isoniazid, RIF; rifampicin, STR; streptomycin, ETH; ethambutol.

3.3. Antimicrobial Resistance Profiles of TB Isolates to First Line Antimicrobials

The result of the drug sensitivity test indicated high susceptibility of the *Mycobacterium* to all the four drugs used. The most sensitive drug was Ethambutol, of the one hundred and thirty isolates subjected to DST one hundred and twenty-five were susceptible with only five isolates being resistant. Streptomycin was the second most sensitive drug with one hundred and twenty-two isolates susceptible to the drug out of the one hundred and thirty isolates. Isoniazid had one hundred and seventeen sensitive isolates out of the one hundred and thirty. Rifampicin had one hundred and eighteen sensitive isolates out of the one hundred and thirty (Figure 1). There was notably minimal resistance to Ethambutol drug compared to all the four drugs used in the study.

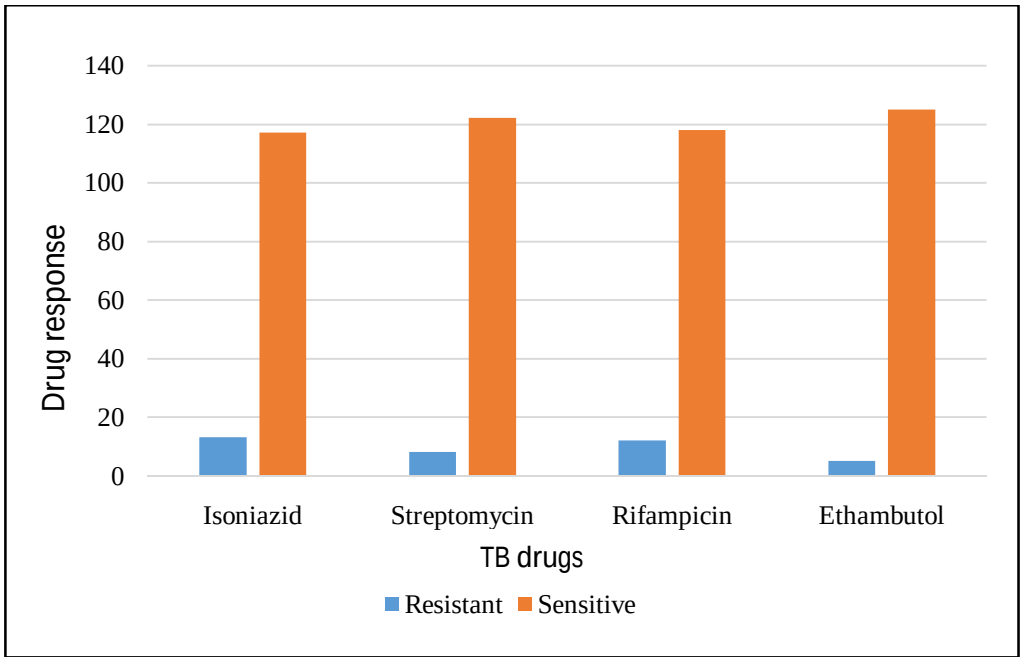

Figure 1: Response to commonly used anti-TB drugs.

3.4. Antimicrobial Resistance Profile of Rifampicin by Gender and Age

The result of the study indicated that, more male patients (66.7 %) than female patients (33.3 %) had TB resistant to Rifampicin. Chi-square test done to establish relationship between gender and TB resistance to rifampicin showed that there was no significant relationship (Table 3).

Resistance to Rifampicin was higher in the isolates from patients in the ages of 26 – 35 years. Chi-square test was done to establish relationship between the patients ages (years) and TB resistance to Rifampicin, the result showed that there was no significant relationship ($x^2 = 1.16$, P value =0.884).

Demographic	Item	frequency	Percent	P-Value
Gender	Female	4	33.3	0.776
	Male	8	66.7	
Age Category	Below 25	1	8.3	0.884
	26 – 35	6	50.0	
	36 – 45	3	25.0	
	46 – 55	1	8.3	
	Above 55	1	8.3	

Table 3: Resistance to rifampicin by gender and age

4. Discusion

The overall resistance to all drugs tested was 6.9 % (Table 2). This figure was much lower compared to that reported in earlier studies in Kenya (18.3 %) (GitHub *et al*., 2000); (18.8%) (Nyangau *et al*., 2015). A comparably similar trend was observed in studies carried out in South Africa where total resistance to drugs tested was 7.3 % (Churchyard *et al*., 2000). The resistance to isoniazid (INH) in this study was 10.0 % (Table 2) lower than that obtained in earlier studies carried out in Nairobi, Kenya 12.9 % (Ndungu *et al*., 2012). Resistance to the same drug in this study was comparatively higher than that reported in Ethiopia where resistance to INH was reported as 6.74 % (Adane *et al.,* 2015). In 2008, the WHO, reported a worldwide resistance rate to INH of 5.9 % (WHO, 2008). According to WHO, INH resistance rates higher than 10 % can predict the development of multi drug resistant tuberculosis.

Resistance to rifampicin (RIF) in this study was 9.2 % (Table 2) comparably similar to that obtained in studies done in Kenya where resistance to rifampicin was reported as 9.8 % (Nyangau *et al*., 2015). The resistance to rifampicin in this study, was higher than that observed in earlier studies in Pakistan and Bangladesh where resistance to rifampicin was 0.3 % and 0.5 % respectively (Taha *et al*., 2009; Magana *et al.,* 2009). RIF has several adverse effects such as nausea, vomiting, rashes, hepatitis, gastrointestinal upsets, flu-like symptoms, fever and jaundice, which could result in patient's non-adherence and hence may lead to the selection of resistant strains (Ndungu *et al*., 2011).

Resistance to streptomycin (STR) in this study was 6.2 % (Table 2) which was higher compared to the resistance of 1.8 % reported in a study carried out in North-Eastern Kenya (Githui., *et al*., 1993), but lower than that reported in Ethiopia, Cameroon and Shri Lanka which recorded 26 %, 20.5 % and 9.9 % respectively (Kassu *et al*., 2008; Assam *et al*., 2011., Nunes *et al*., 2008). The result of this study compares with those of studies conducted in Nairobi Kenya where resistance was recorded at 5.2 % (Ndungu *et al*., 2012).

Resistance to ethambutol (ETH) (Table 2) in this study was 3.8 % which was higher compared to that reported in Ethiopia of (2.7 %) (Kassu *et al*., 2008). However, it was lower than that of the studies carried out in Sri Lanka, Kenya and Ethiopia where ETH resistance was reported as 14.5 %, 8.0 % and 6.74 % respectively (Nunes *et al*., 2008; Nyangau *et al*.,2014; Adane *et al.,* 2015). Ethambutol enhances the effect of many drugs including beta lactams to different *Mycobacterium* species and can be used to develop a regimen for MDR-TB (Abate *et al*., 1998).

In this study, a higher number of patients (10.0 %) with TB showed isoniazid resistance yet were susceptible to all other tested drugs. The high rate of INH resistance is significant since it is a first-line drug which is used throughout the course of tuberculosis treatment (Ngungu *et al*., 2011).

The results of this study may serve as an indicator of a high probability of development of MDR- TB in the future since it has been observed that MDR often develops from initial INH mono-resistant strains. The high level of INH resistance among the study population is also an indicator that this drug will be completely useless for chemotherapy in Kenya (Ndungu *et al.,* 2012). Yuen *et al.* (1999) further argues that rifampicin is the most potent sterilizing antibiotic used for the treatment of TB. Rifampicin and isoniazid are the most active of the first line ant-TB drugs and *Mycobacterium tuberculosis* strains that are resistant to both these drugs are considered multidrug resistant.

The risk of drug resistant tuberculosis is higher among people living with HIV this is because of decreased immunity (Nyangau *et al.,* 2014). Tuberculosis drug resistance is usually related to non-adherence to therapy, severe immunodeficiency, diarrhea and concurrent antifungal therapy (Gillespie, 2008).

5. Conclusion

Based on the study results, Rifampicin and Isoniazid had high resistance rates (9.2 % and 10.0 % respectively). Further, the study also revealed a high prevalence rate of MDR-TB (18.46 %) among HIV/TB patients in Kisumu County. Therefore, there is urgent need for the public health sector to incorporate drug susceptibility testing in the management and control of TB so as to detect and treat antimicrobial early. Also, there in need to develop shorter TB drug schedules to enhance adherence to treatment.

6. Abbreviations

AFB	Acid fast bacteria
AIDS	Acquired Immunodeficiency Syndrome
AM-A	Amplification mix A

AM-B	Amplification mix B
BHIA	Brain heart infusion agar
BSL3	Biosafety laboratory level 3
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Short course
DST	Drug sensitivity test
EPTB	Extra-pulmonary Tuberculosis
ETH	Ethambutol
Genotype MTBC	Test system for the differentiation of Mycobacterium Tuberculosis Complex
GC	Growth control
HIV	Human Immunodeficiency Virus
INH	Isoniazid
JOOTRH	Jaramogi Oginga Odinga Teaching and Referral Hospital
KEMRI	Kenya Medical Research Institute
MDGs	Millennium Development Goals
MDR-TB	Multidrug Resistant Tuberculosis
MGIT	Mycobacteria Growth Indicator
MOTT	Mycobacteria other than M. tuberculosis
MoDP	Ministry of Devolution and Planning
MoH	Ministry of Health
MoPHS	Ministry of Public Health and Sanitation
MTBC	Mycobacterium tuberculosis complex
NACL	N-Acetyl-L-Cysteine
NaOH	Sodium Hydroxide
NASCOP	National AIDS and STI Control Programme
PCR	Polymerase Chain Reaction
PTB	Pulmonary tuberculosis
RIF	Rifampicin
SIRE	Streptomycin, Isoniazid, Rifampicin, Ethambutol
STR	Streptomycin
TB	Tuberculosis
WHO	World Health Organization
XDR-TB	Extensively drug resistant tuberculosis
XPERT MTB	Expert Mycobacterium tuberculosis
ZN	Ziehl-Neelsen

7. Competing interests

The authors would like to declare that there is no competing interests.

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