Assessment of Extremely Drug Resistant Tuberculosis (XDR-TB) prevalence among Multi-Drug Resistant Tuberculosis (MDR-TB) cases in Zambia using GenoType MTBDR Assay

BY

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ABSTRACT

Tuberculosis (TB) remains a global public health problem that claims millions of lives every year. The emergence of drug resistant tuberculosis has been recognised as the major threat to the control of TB. The first most important drug resistant TB is the Multi-drug resistant tuberculosis (MDR-TB).

We carried out a retrospective study of 113 MDR-TB isolates stored from 2008 to 2015 to determine the presence of Extremely Drug Resistant Tuberculosis (XDR-TB). These isolates were retrieved from the three TB culture laboratories of Zambia ((Tropical Diseases Research Centre (TDRC), University Teaching Hospital (UTH) and the Chest Diseases Laboratories (CDL)).

A total of 113 MDR-TB isolates were subjected to GenoType MTBDR*sl* assay to detect any mutations associated with resistance to fluoroquinolones, aminoglycosides-capreomycin and ethambutol.

Out of the 113 MDR-TB Isolates, one isolate had mutation at *gyrA* and rrs MUT1 with absence of rrs WT1. The isolate belonged to a 22 year old female MDR-TB patient who was attended to at a local clinic with resistance to Streptomycin, Isoniazid, Ethambutol and Rifampicin. One other isolate showed mutation in the *gyrA* region only. This was an MDR-TB male patient aged 38 years who was also earlier diagnosed with resistance to Streptomycin, Isoniazid, Ethambutol and Rifampicin. No mutations were observed in about 111 (98.2%) isolates.

Declaration

I, Mweemba Muvwimi Wilfred, hereby declare that the work on which this research is based is original (except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is been or is to be submitted for another degree at this or any other university.

Signed:…… Date:……

Signed:……Date:……

Supervisor

Certificate of Approval

This dissertation is approved as part of the fulfillment of the requirements for the award of the Masters of Science in Pathology (Clinical Pathology) degree at the University of Zambia.

Examiner 1:

Signature……Date……

Examiner 2:

Signature……Date……

Examiner 3:

Signature……Date……

Statement

I, Mweemba Muvwimi Wilfred, hereby certify that this study is entirely the result of my own independent investigation. The various sources to which I am indebted are clearly and gratefully acknowledged in the text and in the references.

Signed:……..

Candidate

Date:……

Dedication

This dissertation is dedicated to Late Ms. Ruth Bwalya Tembwe, without whose guidance I would not have reached this level of professional development. During her tenure as Head of the National Tuberculosis Reference Laboratory, Late Ms. Tembwe inspired me into believing in myself. She encouraged me to see the broader picture of the world of tuberculosis and helped me blend character and skill in improving Tuberculosis laboratory networks.

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First and foremost, I wish to give gratitude to the Lord for giving me life and for teaching me to interact with other fellow human being in a respectable manner. Further, I wish to give thanks to my supervisor, Professor Trevor Kaile and the Assistant Dean (Post Graduate Studies), Dr Selestine Nzala for seeing potential in me and encouraging me to complete my studies. I would fail in my life if I do not thank my wife, Mrs. Tracy Sijatwa Muvwimi who was always available to ensure that I am always doing something about my studies. My children (Namonga, Mzumi, Muleya and Mizila) provided invaluable support and allowed me to work without being disturbed. I wish to thank them too. Clive provided much needed financial support to see me through school as well as supporting the family.

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List of Abbreviations

AIDS	Acquired Immuno-deficiency Syndrome
ART	Antiretroviral Therapy
CDL	Chest Diseases Laboratory (National Tuberculosis Reference Laboratory)
DNA	Deoxyribonucleic Acid
DRS	Drug Resistance Survey
DST	Drug Susceptibility Testing
FLQ	Fluoroquinolones
HIV	Human Immuno-deficiency Virus
INH	Isoniazid
LJ	Lowenstein Jensen
LPA	Line Probe Assay
MDR	Multi-Drug Resistance
MGIT	Mycobacteria Growth Indicator Tube
MTBDR	Mycobacteria Tuberculosis Drug Resistance
NTCP	National Tuberculosis Control Program
PCR	Polymerise Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
SADC	Southern Africa Development Community
SOP	Standard Operating Procedure
TB	Tuberculosis
TDRC	Tropical Diseases Research Centre
UTH	University Teaching Hospital
UNZABREC	University of Zambia Biomedical Research Ethics Committee
WHO	World Health Organisation
XDR	Extremely Drug Resistant

CHAPTER 1: INTRODUCTION

1.1Background

Tuberculosis (TB) remains a global public health problem that claims approximately1.5 million lives. Of these 1.1 million are Human Immunodeficiency Virus (HIV) negative people while 0.4 million deaths from among HIV-positive people (Global TB Report, 2014).

It is caused by closely related intracellular bacterial species termed *Mycobacterium tuberculosis* complex which comprises *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canetti*, *Mycobacterium pinnipedii* and *Mycobacterium caprae.* The main cause of human disease is *mycobacterium tuberculosis* (Sandhu, 2011). The disease is mainly acquired through inhalation of aerosolised droplets containing the mycobacteria. It typically affects the lungs but can also affect other body sites such as the abdomen, spine, kidneys, bones, etc. (Sandhu, 2011). Generally, a relatively small proportion of people infected with *M. tuberculosis* will go on to develop TB. The probability of developing TB is much higher among people infected with the HIV (Corbett *et al*, 2003; Lawn, 2005; Ayles *et al*; 2008; Lawn *et al*, 2010). This is worrisome because it renders patients untreatable with available TB drugs (Bonnet, 2011).

The burden of TB has been exacerbated by the emergence of multi-drug (MDR-TB) and extremely resistant TB (XDR-TB) (Gandhi *et al*, 2010). Cases of totally drug resistant TB cases have been reported in Italy (Magliori *et al*, 2007), Iran (Velayati *et al*, 2009) and India (Rowland, 2012). These new strains of TB have even posed much greater challenge in the control of tuberculosis. Of the 480 000 cases of multidrug-resistant TB (MDR-TB) estimated to have occurred in 2014 globally, only about a quarter of these, 123 000 were detected and reported (Global TB Report, 2014). The number of TB deaths is unacceptably high; with a timely diagnosis and correct treatment, almost all people with TB can be cured (Global TB Report, 2014).Although MDR-TB can be treated successfully, it is usually difficult to manage than drug

sensitive TB. The drugs are much more expensive and toxic with a very long duration of treatment. MDR-TB has been reported in the former Soviet Union and Sub-Saharan African countries (Gandhi *et al*, 2012; Acosta *et al*, 2014). It is estimated that 8% of patients with MDR-TB develop XDR-TB and usually these cases result from primary transmission especially when the use of second-line drugs is unregulated (Zhao, *et al*, 2016). There is documented evidence that XDR-TB has also been reported in all continents (CDC, 2006). In Africa, South Africa is the most affected. XDR-tuberculosis has been detected in all provinces of South Africa and all of its neighbouring countries (Mlambo *et al*, 2008). Zambia is among the high TB burden countries in the Sub-Saharan Africa with prevalence rates of 638/100 000 population (Kapata *et al*, 2015). Cases of MDR-TB has been reported in Zambia (Kapata *et al*, 2015). Despite the presence of MDR-TB in Zambia, no XDR-TB case has been reported in Zambia due to unavailability of diagnostic tools for routine surveillance or testing for XDR-TB in the health system. However, it is clear that increased immigration and high HIV prevalence among Southern African countries serves as a conduit for the spread of tuberculosis, including XDR-TB, from affected countries to others including Zambia.

Extensively Drug Resistant tuberculosis cases have been reported in the Southern African Development Community (SADC) region (among others; South Africa, Mozambique and Botswana) since 2006 (Gandhi *et al*, 2006). Since Zambia shares borders with some of these countries and with the population frequently crossing borders, there is anxiety that some XDR-TB strains could easily be imported into Zambia. However, there have been no cases of XDR-TB reported so far. The major reason could be unavailability of a diagnostic tools for routine surveillance or testing for XDR-TB.

According to the Zambia National TB program, notification rates for 2014 stands at 42 716 cases (NTP Report, 2015) with MDR-TB rates for new cases at 1.1% (Kapata *et al*, 2015).

1.2 Statement of the Problem

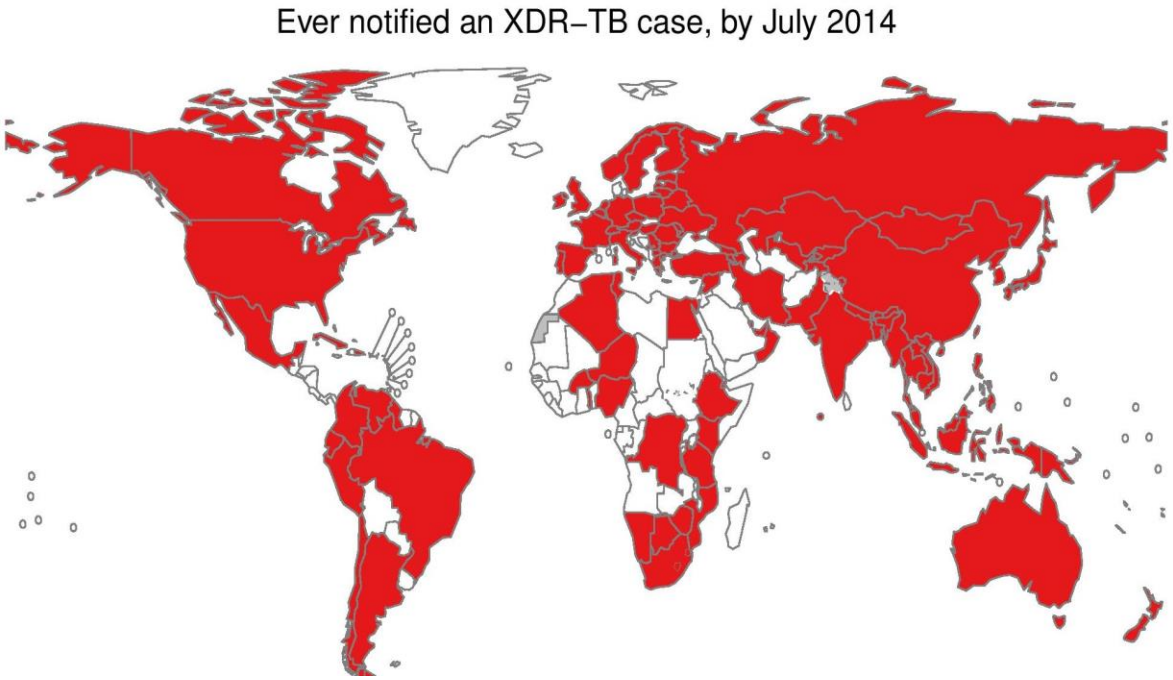
Drug resistance continues to be a major hindrance to the effective global TB control. Treatment for drug resistance is usually lengthy with poor outcomes (Jeon *et al,* 2011). National TB Control programs must therefore, aim at preventing the emergence of drug resistant TB.

Since drug resistant TB is transmitted in the same way as drug-susceptible TB, it is important to ensure that intensified case finding is done for drug resistant TB too. In 2014, there were an estimated 480 000 new cases of MDR-TB worldwide. Approximately 190 000 of these died from MDR-TB. Among those who were notified, 300 000 of them had MDR-TB (Global TB Report, 2015).

Zambia has also demonstrated that the problem of MDR-TB continues to be an emerging challenge. Kapata and colleagues reported cases of MDR-TB cases over a period of 11 years (Kapata *et al,* 2013). Subsequent National Drug Resistance survey of 2008 also reviewed presence of MDR-TB (Kapata *et al*, 2015).

On average, an estimated 8% of people with MDR-TB have XDR-TB. Access and adherence to treatment for MDR-TB is very essential as progression to XDR-TB can occur. So far extensively drug-resistant TB (XDR-TB) has been reported by 105 countries.

Below is a map highlighting the worldwide occurrence of XDR-TB since 2014.



South Africa and all its neighbours have reported at least one case of XDR-TB (Mlambo *et al*, 2008). Despite progress in responding to the challenge of drug-resistant TB in Zambia, routine surveillance of XDR-TB has not been developed. Therefore, efforts to respond to these challenges are urgently needed (Global TB Report, 2015).

The emergency of XDR-TB creates a new challenge to TB control. Tuberculosis contributes greatly to the decline in worker productivity. The time spend for an individual away from work during treatment is long, usually 24 months or more. This period is even much longer when treating drug resistant tuberculosis. The cost of treating drug resistant tuberculosis is higher and this is an economic burden on the health system. For example, the cost of hospitalization of an XDR-TB patient in the United States of America is estimated on average at $483,000, approximately twice the cost for MDR TB patients (Kim *et al*, 2007). Extremely Drug Resistant tuberculosis is associated with poor prognosis especially if accompanied with MDR-TB and HIV (Migliori *et al*, 2007; Gandhi *et al*, 2006 and Kim *et al*, 2007). It is clear that XDR-TB would

be a major public health problem in a poor resource setting like Zambia if it is not promptly detected and measures instituted to prevent its spread.

1.3 Study Justification

Extremely Drug Resistant Tuberculosis (XDR-TB) has been reported in many parts of the world (CDC, 2006). However, no case has been reported in Zambia even when it is well known that 8% of patients with Multi-Drug Resistant Tuberculosis (MDR-TB) develop XDR-TB (Zhao *et al*, 2016). According to the 2008 Drug Resistance Survey, about 1.1% of new and 8.1% of retreatment cases respectively, are MDR-TB in Zambia. The information from this study will provide baseline data which will serve as basis for developing Zambia's capacity to test for XDR-TB

1.4 Research Question

1.4.1 What is the estimated prevalence of XDR-TB in Zambia?

1.5 Objectives

1.5.1 Main objective

To assess the prevalence of XDR-TB among MDR-TB archival isolates in Zambia.

1.5.2 Specific Objectives

1. To identify MDR-TB cases from isolates archived at UTH, TDRC and CDL
2. To detect XDR-TB among MDR-TB isolates using GenoType MTBDR line probe assay.

CHAPTER 2: LITERATURE REVIEW

Tuberculosis (TB) is a worldwide pandemic and has been in existence for over 17, 000 years (Hirsch *et al*, 2004). According to World Health Organization (WHO), millions of people have continued to suffer and die despite numerous efforts to diagnose and treat tuberculosis. It is the leading cause of death among people living with HIV and among the top 10 killer infectious diseases (WHO, 2010). Drug resistance is recognised as a significant threat in the control of tuberculosis. In his research, Isaakidis and colleagues discovered that that burden of drug resistant TB was highest in HIV-infected patients attending Antiretroviral Therapy (ART) clinics in Mumbai, India (Isaakidis *et al*, 2014). The data highlighted the need to promptly diagnose TB drug-resistance among people living with HIV/AIDS. In Eastern Europe, the problem of drug resistance is enormous (Acosta *et al*, 2014). The problem is quiet common in countries where the tuberculosis control programs are poorly managed. Usually there are challenges in accessing appropriate treatment regimens and poor laboratory capacity to rapidly diagnose TB and to perform Drug Susceptibility Test (DST). Human resources and TB financing are other additional challenges (Acosta *et al*, 2014). The uncontrolled purchase and distribution of anti-TB drugs also plays a very important role in the development of drug resistant TB especially where there is no proper quality control mechanisms (Marahatta, 2009). Treatment for TB must be guided by periodic DST results and this should be assured for the high-risk TB patients for the prevention and control of drug resistant TB (Xu *et al*, 2014).

Multi-Drug Resistant tuberculosis (MDR-TB) is defined as resistance to Isoniazid and Rifampicin (Gandhi *et al*, 2012). These are the two most potent first line drugs in the treatment of tuberculosis. Globally, 3.3% of new TB cases and 20% of previously treated cases have MDR-TB. About 190 000 people died of MDR-TB in 2014. Not all presumptive TB cases were tested for MDR-TB in 2014. Assuming that all were tested, it is believed that an estimated 300 000 would have been found to have MDR-TB, with more than half of them occurring in China, India

and Russia (WHO, 2015). In South Africa, poor infection control, delayed diagnosis and a high HIV prevalence facilitates transmission of MDR-TB (Gandhi *et al*, 2012).

Only 50% of MDR-TB patients were successfully treated globally (Global TB Report, 2015). To successfully treat MDR-TB, a patient is required to complete a treatment course of two years. The long duration of treating MDR-TB presses additional economical costs to the health system as well as to the patient. This is coupled with social isolation, loss of employment, and long-term socioeconomic and psychological effects (Booker, 1996). Mortality is usually high compared to those infected with drug sensitive TB (Ormerod, 2007).

Extremely Drug Resistant Tuberculosis (XDR-TB) by definition is TB that is resistant to isoniazid and rifampicin, plus any fluoroquinolones and at least one of the three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin)" (Jassal, 2009, WHO, 2006). In 2010 WHO reported alarming numbers of MDR and XDR TB cases. By 2015, extensively drug-resistant TB (XDR-TB) had been reported by 105 countries with an estimation that 9.7% of patients with MDR-TB have XDR-TB. At around 2006, the Centers for Diseases Control and Prevention (CDC) reported that XDR-TB was present in all continents with 17 countries that had at least one case of XDR-TB. The USA reported that 4% of the MDR-TB isolates between 1993 and 2004 were XDR-TB (CDC, 2006). In South Korea, 15% of the MDR-TB strains isolated in 2004 were also XDR-TB. There were several other countries which detected XDR-TB in MDR-TB isolates in Asia such Iran (twelve of the 113 MDR-TB isolates were XDR-TB) and Hong Kong (nine of the 75 MDR-TB strains had XDR-TB) (CDC, 2006; Masjedi *et al*, 2006). Europe also reported significant numbers of XDR-TB from MDR-TB isolates. For example, Latvia reported 19% cases of XDR-TB from MDR-TB isolated between 2000 and 2002. Russia and Norway has also reported cases of XDR-TB (Dahle, 2006).

In Africa, South Africa has the highest cases of XDR-TB. In Msinga sub-district of KwaZulu Natal, 53 possible XDR-TB cases were detected from 221 MDR-TB isolates in 2006. Out of these 53 cases, 52 died (Gandhi, 2006). By the year 2008, Extremely Drug Resistant Tuberculosis has since been detected in all provinces of South Africa and all of its neighbouring countries (Mlambo *et al*, 2008). In 2012, Gandhi and colleagues reported about 516 cases over a period of four years in Tugela Ferry, KwaZulu Natal in South Africa (Gandhi *et al*, 2012). The compounding factor of XDR-TB control is that XDR-TB is virtually untreatable. Patients with XDR-TB have also subtle immunity making them vulnerable to infection with other bacteria (Gandhi *et al*, 2010; Kim, *et al*, 2007). Furthermore, clinical features of tuberculosis sometimes are quiet similar to those of bacterial pneumonia and radiological features cannot be differentiated unless by bacteriological means (Shen *et al*, 2012).

Zambia is among the high TB burden countries in the Sub-Saharan Africa. During 2013-14 National TB Prevalence Survey, bacteriologically confirmed prevalence of TB in Zambia was 638/100 000 population (Kapata *et al*, 2015). Multi-drug Resistant tuberculosis has not been a serious public health problem in Zambia. In 2001, the first National Drug Resistance Survey (DRS) was conducted according to WHO guidelines. The prevalence of MDR-TB was estimated at 1.8% for both new and retreatment cases (Kapata *et al*, 2013). A second Drug Resistant Survey was conducted in 2008 and MDR-TB prevalence was estimated to be at 1.1% (Kapata *et al*, 2015). With the understanding that MDR-TB can progress into extensively drug resistant tuberculosis, an XDR-TB snap survey was conducted alongside the DRS of 2008. MDR-TB isolates were tested to determine resistance to second-line drugs using GenoType MTBDR*sl* Assay (Hain Lifesciences, Nehren, Germany). No XDR-TB was detected during this survey (Kapata *et al*, 2015). Zambia shares borders with countries that have reported cases of XDR-TB in the SADC region. There is also regular international travel

among these countries, hence a possibility that XDR-TB strains can be moved easily into Zambia.

2.1 Tuberculosis Drug Resistance

Drug Resistance refers to accumulation, on resistance plasmids or transposons, of genes, which codes for resistance to a specific agent, and/or by action of drug efflux pumps which can pump out one or more drug type (Nakaido, 2009).

2.2 Multi-Drug Resistant Tuberculosis (MDR-TB)

By definition, MDR-TB is associated with resistance to Isoniazid (INH) and Rifampicin. Isoniazid Preventive Therapy (IPT) has been used in preventing tuberculosis in HIV-Positive patients (Mwinga *et al*, 1998; Churchyard *et al*, 2007). Because Isoniazid is the most commonly used antituberculosis drug, resistance occurs most frequently (Karakousis, 2009). Mutations in INH-resistant isolates are detected in the *katG* gene resulting in the reduction of the catalase-peroxidase to activate the INH pro-drug. Resistance may also arise in the *inhA* region (Kolyva, & Karakousis, 2012).

Resistance to rifampicin is usually accompanied by resistance to INH in 90% of cases. Hence rifampicin resistance has been used as a surrogate marker for MDR-TB. This occurs as a point mutaion in the *rpoB* gene which encodes the β–subunit of the Ribonucleic Acid (RNA) polymerase (Kolyva, & Karakousis, 2012).

2.3 Extremely Drug Resistant Tuberculosis (XDR-TB)

When MDR-TB (resistant to isoniazid and rifampicin) become resistant to Fluoroquinolones and any one of the second line injectable drugs (capreomycin, amikacin or kanamycin), it is defined as XDR-TB (WHO, 2006).

Fluoroquinolones (moxifloxacin, gatifloxacin, sparfloxacin, levofloxacin and ofloxacin) is commonly associated with mutations in the *gyrA* and *gyrB* regions. High level resistance usually require multiple mutations in

the *gyrA* or concurrent *gyrA* and *gyrB* regions (Kocagoz *et al*, 1996). Resistance to the (Kanamycin and Capreomycin) is associated with mutations in the rrs gene coding for 16S rRNA (ribosomal RNA).

2.4 Drug Susceptibility testing (DST)

Drug Susceptibility Testing aims at finding out if a patient has got drug resistant tuberculosis. It is used for the selection of effective regimens to successfully treat tuberculosis patients as well as a drug resistance surveillance tool used for evaluation of TB control programs (Kim, 2005).

Conventional methods involving culture of mycobacteria has been used for a long time. Both solid and liquid media methods are technically demanding and have very long turnaround of approximately 2-8 weeks. There is need for rapid molecular tests that detects both tuberculosis and mutations associated with drug resistance. (Patricia *et al*, 2011). The GenoType MTBDR*sl* Assay is a rapid diagnostic method that enables the detection of mutations involved in resistance to fluoroquinolones and injectable drugs. This method can also detect resistance to second line drugs. It can be performed on direct sputum and culture isolates (Said et *al*, 2012)

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Design

This was a qualitative analytical study which assessed the presence of XDR-TB in MDR-TB isolates. It involved identification of isolates and extraction of DNA from MDR-TB isolates archived at the three Tuberculosis culture laboratories of Zambia (Tropical Diseases Research Centre, University Teaching Hospital and the Chest Diseases Laboratories and testing them for susceptibility/resistance to second-line drugs using GenoType MTBDR*sl* assays (Hain Lifesciences, Nehren, Germany). These laboratories were purposely selected as they are the only laboratories performing culture and drug susceptibility testing for mycobacteria tuberculosis.

3.2 Study site

The laboratory analysis was carried out at the Chest Diseases Laboratory in Lusaka. The Chest Diseases Laboratory is the National Tuberculosis Reference Laboratory (NTRL) for Zambia.

3.3 Target Population

The primary study population were MDR-TB isolates archived at TDRC, UTH and CDL. These isolates were classified as MDR-TB using Lowenstein Jensen proportional absolute concentration conventional drug susceptibility method during routine testing for drug resistance.

3.4 Sample Size

Records showed that there were 189 MDR-TB isolates archived at TDRC, UTH and CDL from 2008 to 2015 (CDL Routine isolates archives, TDRC TB archives, UTH TB LAB_Cryobank). However, only 113 isolates were correctly identified and analysed in this study.

Below is table showing MDR-TB isolates (resistant to Isoniazid and Rifampicin) used in this study. The Multi-Drug Resistant Tuberculosis isolates were detected during routine Drug Susceptibility Testing using BD Mycobacteria Growth Indicator Tube (MGIT) liquid media and Lowenstein Jensen (LJ) solid media.

Table 1. **Drug Resistance Characteristics of the sample**

Drug Resistance Characteristics				
STREP	INH	RIF	EMB	No. of Isolates (n = 113)
R	R	R	R	35
S	R	R	R	15
R	R	R	S	30
S	R	R	S	33

STREP, Streptomycin; INH, Isoniazid; RIF, Rifampicin; EMB, Ethambutol
R, Resistant; S,Susceptible

CHAPTER 4: LABORATORY PROCEDURES

All laboratory procedures were based on the Chest Diseases Laboratory Standard Operating Procedure (CDL-TECH 23, version 1, 2015) as well as GenoType MTBDRsl Assay manufacturer instructions.

4.1 Procedures at the MDR-TB isolates collection sites.

Colonies of MDR-TB isolates were scraped from solid media or aliquoted from the liquid media into Eppendorf tubes containing phosphate buffer. These were heated on a dry heating block at 95^0C for 15 minutes to kill the mycobacteria. The inactivated mycobacteria were transported to CDL for Deoxyribonucleic Acid (DNA) extraction and testing on second-line drugs using the GenoType MTBDR*sl* assay (Hain Lifesciences, Nehren, Germany).

All data concerning the isolates was collected and recorded on a worksheet.

4.2 Procedures at the Study Site.

Multi-drug Resistant tuberculosis isolates were received in either liquid or solid media at the Chest Diseases Laboratory (CDL). The isolates were given unique identification numbers and then subjected to GenoType MTBDRsl assay according to manufacturer instructions and the Standard Operating procedure of the CDL with the following steps.

4.2.1 DNA Extraction.

DNA was extracted from either liquid or solid media depending on the nature of the archived isolate by lysing the bacterial cell wall. For the isolates from the liquid media, 1000µl was pipetted into well labelled screw capped micro centrifuge tubes of about 1.5 ml capacity. Solid culture isolates were scooped from the media into a 1.5 ml screw capped micro centrifuge tube filled in with 300µl of sterile grade water. Care was taken not to scoop media as this contain inhibitors for the enzymes used in the amplification process. The tubes were brought into the micro centrifuge and centrifuged for 15 minutes at 10,000 x *g*. The

supernatant was discarded into a discard jar and pellets re-suspended in 100µl of Lysis Buffer (A-LYS) by vortexing. The sample was then incubated for 5 minutes in a dry heating block with temperature maintained at 95^0C. The samples were briefly spun down at 10,000 x g for 2 minutes. The sample mixture was neutralised by adding 100µl of Neutralization Buffer (A-NB). Further centrifugation was done for 5 minutes at a full speed of 13,000 x *g* in a table top centrifuge. About 5µl of DNA supernatant was used for PCR.

4.2.2 Master Mix Preparation

This process was done in the **pre-amplification room (**clean room). With the room surface and laminar flow hood cleaned with 1% sodium hypochlorite, HotStart DNA polymerase, 10X PCR buffer, $MgCl_2$ solution, Molecular grade PCR water was removed from the freezer, Primer Nucleotide Mix for second line drugs (PNM*sl)* for Mycobaterium tuberculosis drug resistance for second line drug (MTBDR*sl*) as needed from the refrigerator to thaw. These were kept outside the hood. The PCR tubes and micro centrifuge tube were retrieved using aseptic techniques, closed and labeled. The PNM, 10X PCR buffer, $MgCl_2$ solution, molecular grade PCR water and HotStart DNA polymerase were aliquot in that order into the micro centrifuge tube.

Table 2. Sample Preparation matrix. The table below illustrates the different volume mixtures for a single test.

Reagents (Master Mix)	**Volumes per sample**
PNM*sl* (µl)	35
PCR Buffer (µl)	5
$MgCl_2$ (µl)	2
H_2O (µl)	3
Taq polymerase (µl)	0.2

The Master Mix reagents were mixed without creating bubbles. For optimal handling of the preparation, the Master Mix were aliquoted in 45µl into each labeled PCR tube. The rack containing the PCR tubes were covered with absorbent paper. All equipment were wiped with 1% sodium hypochlorite and Personal Protective clothing used were removed prior to transferring the test material to the processing laboratory where the 5µl of template DNA was to be added.

4.2.3 Amplification

DNA released into solution was multiplied or amplified to million copies using real-time polymerised chain reaction (RT-PCR) so that it could be quantified and detected.

All steps were done in the **Post-Amplification Room**. The PCR tubes were transfered to the thermocycler where a program for at least 40 cycles (10 + 30 cycles) for specimens (MTBDR Hot 40) was set. The program parameters were checked and a menu as shown below was followed to start the appropriate program.

Table 3. Amplification Profile:

Minutes/Seconds	**Temperature (^{0}C)**	**No. of Cycles**
15 Minutes	95	1
30 Seconds	95	10
2 Minutes	58	
25 Seconds	95	20
40 Seconds	53	
40 Seconds	70	
8 Minutes	70	1

Heating rate $\leq 2.2^{\circ}$C/sec

4.2.4 Hybridization

This was a process of binding the ssDNA to the specific probes on the strip (Reverse Hybridization) which was carried out in the Post-Amplification Room. The HYB and STR solutions were pre-warmed to 45^0C in a water bath for a minimum of 15 minutes followed by prewarming of the TwinCubator to 45^0C. The other remaining reagents were warmed to room temperature in exception of CON-C and SUB-C. In each well, 20l of DEN solution was pipetted and mixed with 20µl of corresponding amplified DNA sample in each well, pipetting up and down several times. This was followed by incubation at room temperature for 5 minutes. After 5 minutes, the DNA strips were removed from the tubes and labelled. About 1 ml of HYB solution was added to each well and gently shaken to homogenize the solution. The strips were put into each well with the coloured marker facing up. These were then placed in the TwinCubator to incubate for 30 minutes at 45^0C. After the 30 minutes incubation, the HYB solution was poured off carefully into the sink and the remaining solution removed by forcefully tapping the tray against a paper tower on a bench top. All condensation that formed on lids before incubation step was wiped off. Then 1 ml of STR buffer was added in each well and incubated for 15 minutes in a TwinCubator at 45^0C. While this was happening, diluted conjugate solution was prepared in 15 ml centrifuge/conical tube by adding 10µl of CON-C into 1000µl of CON-D for each sample. These was prepared fresh for each run. Following this incubation, the solution was removed and wash with 1 ml RIN for 1 minute per well on TwinCubator. The RIN solution was poured out and washing repeated for a further 1 minute. The last RIN solution was removed and 1 ml distilled water added per well on TwinCubator. Once the distilled water was removed, 1 ml of substrate was added in each well and the preparation placed under Aluminium foil in the TwinCubator for 2-10 minutes. A colour reaction indicated reaction completion after 4-5 minutes. After reaction was completed, two washes with 1 ml of distilled water for 1 minute was done and the strips were then transferred to result sheet.

4.2.5 GenoType® MTBDR*sl* Assay Results Interpretation

Interpretation of results for second line drugs was done using the GenoType® MTBDR*sl* DNA strips.The *gyrA* and *rss* each had a control band (locus control) which was to be present on the strip in order to interpret the results for each gene (See figure 1).The *gyrA* detected fluoroquinolone (FLQ) resistance, *rss* detected resistance to aminoglycosides/cyclic peptides (AG/CP) and *emb*B was allowed to draw a conclusion about a resistance to ethambutol for the strain tested.In order for a batch of results to be valid, the negative control strip had a CC and AC band present, but no other bands was to be visible.

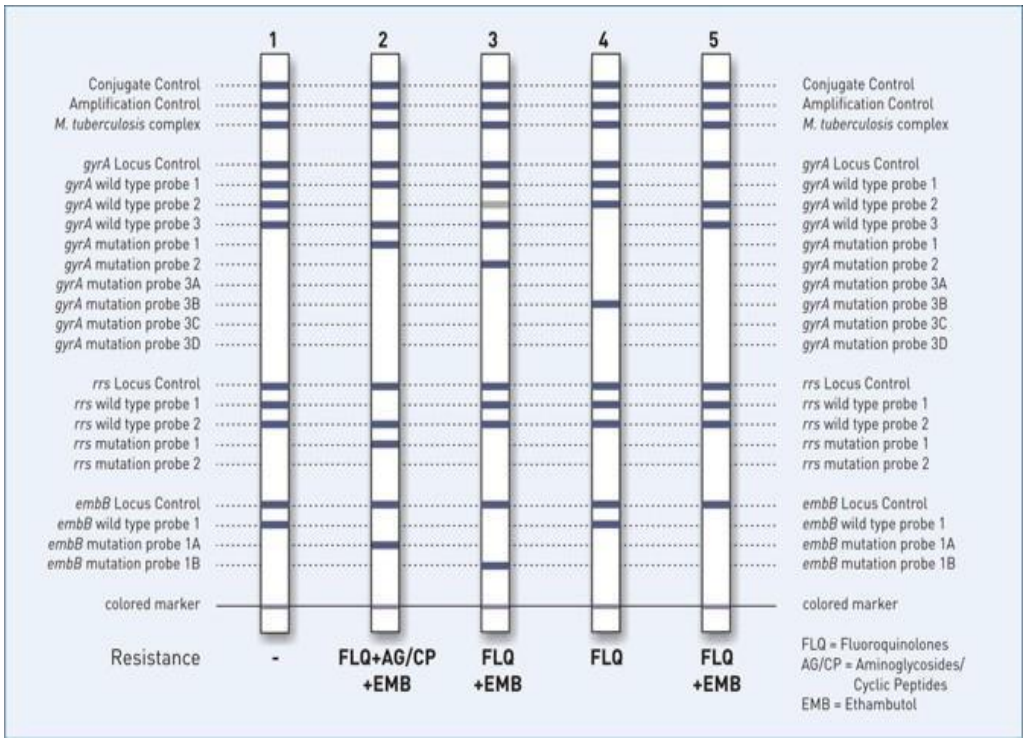


Figure 1. Example of GenoType MTBDR Line Probe Assay Results

4.2.6 Data collection and analysis.

A total of 113 MDR-TB isolates were collected from UTH, CDL and TDRC laboratories. The GenoType MTBDRR*sl* assay (Hain test) was used to identify mutations that codes for XDR-TB. Demographic data were correlated to ascertain specific characteristics of XDR-TB. Analysis of the data was done using SPSS statistical package version 22.0 to determine proportion of occurrence.

4.2.7 Ethical Consideration

Permission for the use of MDR-TB isolates was obtained from the management of the University Teaching Hospital, Tropical Diseases Research Centre and Chest Diseases Laboratory. Ethical clearance was sought from the University of Zambia Biomedical Research Ethics Committee (UNZABREC). However, no consent was obtained as the study used laboratory bacterial isolates only. Numerical identification was used to identify the samples and safeguard patient identity.

CHAPTER 5: RESULTS

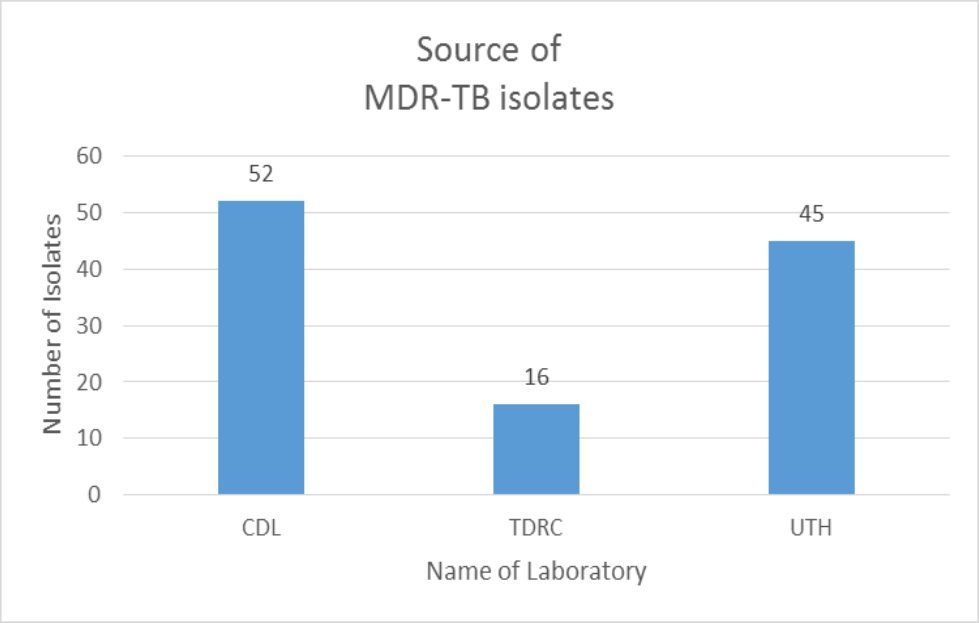
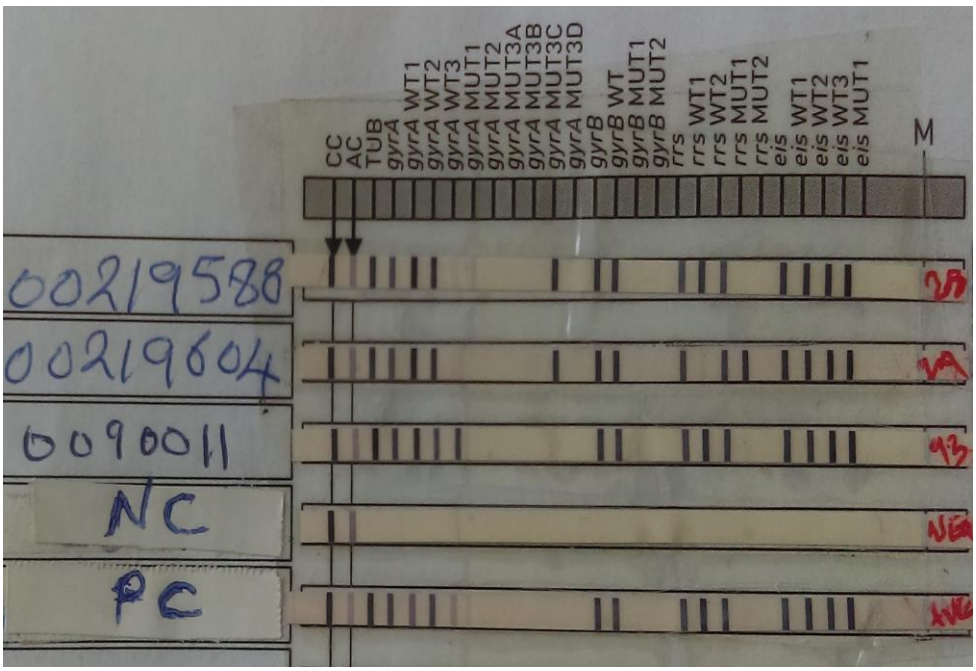


Figure 2: Origin of MDR-TB Isolates. A total of 113 MDR-TB isolates were tested (n = 16, TDRC; n = 52, CDL; n = 45, UTH)

These isolates were referred to the culture facilities for determination of drug resistant patterns using minimum inhibition concentration methods.



NC = Negative Control; PC = Positive Control

Figure 3. **GenoType MTBDR*sl* v2.0 assay results.**

The strip number 219588 show resistance to fluoroquinolones only with mutation at *gyrA* while strip number 219604 showed resistance to Fluoroquinolones and Kanamycin/Capreomycin/Viomycin with mutation at *gyrA* and at rrs MUT1 with absence of *rrs* WT1. Strip number 219604 is a characteristic XDR-TB strain.

The rest of the strips did not show any mutation bands at *gyrA*, rrs and *eis* regions and all Wild types were present. All the tests were run together with both negative and positive controls

CHAPTER 6: DISCUSSIONS

This is the first study to assess the presence of XDR-TB in Zambia. In order to identify XDR-TB, we utilised the GenoType MTBDR*sl* Assay to detect Mycobacteria tuberculosis and mutations associated with resistance to fluoroquinolones, aminoglycosides-capreomycin and ethambutol. The *gyrA* detected fluoroquinolone (FLQ) resistance, *rrs* detected resistance to aminoglycosides/cyclic peptides (AG/CP) and *emb*B. Determination of FLQ resistance occurs when there is mutation in the quinolone resistance-determining regions (QRDR) of the *gyrA* gene (coding for the A-subunit of the DNA gyrase). Resistance to AG/CP is detected by targeting a region of the 16S rRNA gene (encoded by the *rrs* gene), whereas identification of mutations in the embB gene enable detection of ethambutol resistance (Tagliani *et al*, 2015).

Our study identified mutations in the *gyrA*; *gyrA* and the *rrs* regions for two isolates respectively. The sample which had mutations associated with resistance to Fluoroquinolones (*gyrA*) and Kanamycin/Capreomycin/Viomycin (*rrs*) with absence of *rrs* WT1 was confirmed as an XDR-TB strain. The isolate belonged to a 22 year old female MDR-TB patient who was attended to at a local clinic in Lusaka with resistance to streptomycin, isoniazid, ethambutol and rifampicin. Information on her treatment for MDR-TB was not available. She died in 2012. The other isolate showed mutation in the *gyrA* region which was an indication that it was resistant to fluoroquinolones (FLQ) only. This was an MDR-TB male patient aged 38 years who was also earlier diagnosed with resistance to Streptomycin, Isoniazid, Ethambutol and Rifampicin. He was referred for MDR-TB treatment at the University Teaching Hospital and died in 2012 as well.

The age range of the subjects included in the study subject was between 14 – 70 years revealing that there is no specific age group for tuberculosis disease. This is consistent with other studies which confirmed that 70% of TB disease occur between the ages of 15 – 54 years (Phelamei, 2013). More males (58.4%) than females (41.6%) were found to have TB in this study. This is comparable to Neyrolles and

colleague who stated that of the 10% that get infected with mycobacteria tuberculosis regardless of sex, males are 70% more likely going to develop the disease than females. Males are much exposed to predisposing factors such as outdoor dust, smoking, alcohol and overcrowding places than females.

By detecting a case of XDR-TB in Zambia, this study serves as a wake-up call to the Ministry of Health to invest in diagnostic capacities for identification of XDR-TB before it becomes a serious public health problem. There is likely that a lot more cases are undiagnosed due to lack of laboratory capacity to perform second-line drug susceptibility testing. Implementation of comprehensive strategies which emphasises timely diagnosis through quality assured bacteriology must be prioritised if Zambia has to prevent the emergence of extremely drug resistant tuberculosis.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

Our study found one isolate (0.9%) with Extremely Drug-Resistant Tuberculosis (XDR-TB) among the 113 Multi-Drug Resistant Tuberculosis (MDR-TB) cases tested. It also revealed the presence of fluoroquinolone mono-resistant strains of TB.

7.2 Recommendations

This is the first study to report on the presence of Extremely Drug Resistant Tuberculosis (XDR-TB) in Zambia. It is therefore recommended that:

1. Zambia should introduce routine testing of XDR-TB among cases of MDR-TB by building capacity for second-line drug susceptibility testing.
2. Government should invest in the development and strengthening of drug resistant TB surveillance program.
3. Government should invest in rapid diagnostic tools which will respond to the challenges of drug resistant tuberculosis promptly.

7.3 Limitation of the Study

Time and resource constraints could not allow the researcher to carry out comparative studies using different test methods.

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Appendix

1. Information Data Sheet
2. Graduate Proposal Presentation Forum letter
3. Authority letters to conduct the study
4. Submission of Proposal to UNZABREC
5. UNZABREC Clearance Letter