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Evaluation of Diagnostic Algorithms, Drug Resistance, Molecular Typing, and Geospatial Clustering of Tuberculosis in Addis Ababa, Ethiopia

A PhD Dissertation submitted to Microbial, Cellular and Molecular Biology Department in fulfillment of the requirement for the degree of Doctor of Philosophy

By

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Declaration

I declare that the thesis has been composed by myself and has not been submitted for any other degree or professional qualification. Except where states otherwise by reference or acknowledgment, the work presented is an original report of my research.

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Abbreviations / acronyms

A	adenine
AAHRL	Addis Ababa Health research Laboratory
ACER	average cost-effectiveness ratio
AFB	Acid-fast bacillus
AHRI	Armauer Hansen Research Institute
Ala	alanine
AM	aminoglycocides
Arg	arginine
ART	antiretroviral therapy
Asn	asparagine
Asp	aspartic acid
C	cytosine
CHEERS	Consolidated Health Economic Evaluation Reporting Standards
CM	cyclic peptides
CrI	credible interval
DALYs	disability adjusted life years
DES	discrete-event simulation
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide
DOT	Directly Observed Treatment
DR	direct repeat
DR-TB	drug resistance TB
DST	drug susceptibility testing

DVR	direct variable repeats
EMB	Ethambutol
FLQ	Fluoroquinolones
FM	Fluorescence microscopy
G	guanine
GC	guanine-cytosine
GDP	gross domestic product
Gln	glutamine
Gly	glycine
HGI	Hunter-Gaston discriminatory index
His	histidine
ICER	incremental cost-effectiveness ratio
Ile	isoleucine
INH	Isoniazid
IS	Insertion sequences
LED	light emitting diode
Leu	leucine
LJ	Lowenstein-Jensen
LTFU	lost to follow-up
MDR	multidrug resistant
Met	methionine
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units-Variable Number tandem repeats
MPTR	Major polymorphic tandem repeats
MTBC	Mycobacterium tuberculosis complex
MUT	mutant

NALC	N-Acetyl-L-Cysteine
NTP	national tuberculosis program
PCR	polymerase chain reaction
PGRS	GC-rich repetitive sequence
Phe	phenylalanine
Pro	proline
PZA	Pyrazinamide
RFLP	restriction fragment length polymorphism
RIF/RMP	Rifampicin
Rpm	revolution per minute
RR	Rifampicin-resistant
rRNA	Ribosomal ribonucleic acid
RTI	recent transmission index
Ser	serine
SITs	shared types
SMS	spot-morning-spot
SNPs	Single nucleotide polymorphism
STM	Streptomycin
T	thymine
TB	tuberculosis
TFC	treatment follow up center
Thr	threonine
TIC	treatment initiating center
TRAC/FMOH	Tuberculosis Research Advisory Committee of Federal Ministry of Health
Trp	tryptophan

Tyr	tyrosine
US	United State
USAID	United States Agency for International Development
USD	United State dollars
UV	ultraviolet radiation
Val	valine
WGS	Whole genome sequencing
WHO	World Health Organization
WT	Wild type
XDR	extensively drug-resistant
ZN	Ziehl-Neelsen

Abstract

Ethiopia is one of the 30 highest burdens of TB, TB/HIV co-infection, and multidrug resistance TB countries. The objective of the paper was to determine the impact of alternative laboratory diagnostic algorithms on the patient, health system and population level using virtual implementation approach; to determine drug resistance, molecular, and spatial pattern of tuberculosis transmission; and to identify factors associated with the transmission in Addis Ababa, Ethiopia.

A mathematical modeling was used for assessing the impact of alternative diagnostic algorithms. BACTEC^{TM} MGIT 960^{TM} TB system (first-line anti-TB drugs) and GenoType MTBDRsl (second-line anti-TB drugs) were utilized for assessing drug susceptibility pattern. IS6110 Restriction Fragment Length Polymorphism, spoligotyping, and mycobacterial interspersed repetitive unit -variable number tandem repeat 24-loci typing (MIRU-VNTR) were used to analyze DNA fingerprinting. SaTScan and ArcMap were used for a purely spatial and space-time cluster analysis and mapping. Isolates were collected from pulmonary high MDR-TB risk group patients.

Three of the modeled diagnostic algorithms are cost-effective. The full roll-out of Xpert MTB/RIF as the primary test for all presumptive TB cases would avert 91170 DALYs (95% credible interval [CrI] 54888 – 127448) with an additional health system cost of US$ 11.6 million over the next 10 years. The incremental cost-effectiveness ratio (ICER) is $370 per DALY averted. Same day LED fluorescence microscopy for all presumptive TB cases combined with Xpert MTB/RIF targeted to HIV-positive and High MDR-TB risk groups

would avert 73600 DALYs (95% CrI 48373 - 99214) with an additional cost of US$5.1 million over the next 10 years. The ICER is $169per DALY averted. Same-day LED fluorescence microscopy for all presumptive TB cases (and no Xpert MTB/RIF) would avert 43580 DALYs with a reduction cost of US$ 0.2 million over the next 10years. The ICER is $13 per DALY averted.

About 70% of the isolates were resistance to one or more of the first-line drugs, 61% of the isolates were multi-drug resistant, and 19% were resistant to all first-line drugs. Any resistance to INH, RMP, STM, EMB, PZA was 69%, 61%, 58%, 26% and 55% respectively. Four isolates were resistant to fluoroquinolones (pre-XDR), and belonging to T3_ETH linage. The rest isolates were susceptible to all second-line anti-TB drugs. The linage CAS1_KILI was a risk factor for MDR, resistance to one or more anti-tuberculosis drugs, and drug-resistant to INH, RMP, STM, and PZA. The linage T3_ETH was found to be a risk factor for EMB resistant.

About 119 genetic profiles and 15 clusters were observed. T3_Eth International family constitutes the most predominant followed by the CAS1_KILI and CAS1_DELHI. Other International families found were CAS, H1, H3, H4, LAM9, T1, T2, T3, and T5. Some isolates were designated as an orphan. The predominantly clustered isolates were 3 bands. Clustering among 4 spoligotypes T3_ETH (41.8%), CAS1_KILI (29.4%), CAS_DELHI (10.5%), and T1 (33.3%) were observed with the overall clustering rate of 27.3%. The linage T3_ETH was significantly associated with clustering.

Using spatial analysis, significant high and low rate most likely spatial and space-time clusters were identified. A significant secondary cluster was also obtained. The most likely cluster of

the high rate from both the purely special and space-time analyses was almost from the same area. Population density and per capita income was significantly associated with spatial clustering.

The full roll-out of Xpert MTB/RIF is predicted to be the best option to substantially reduce the TB burden in Addis Ababa and is considered cost effective. Targeted use of Xpert MTB/RIF for HIV positive and high MDR risk groups with same-day LED fluorescence microscopy for all other presumptive TB cases is an alternative choice in case of budget shortage. A substantial number of drug-resistance TB, high rate of molecular clustering and existence of drug resistance hot spot indicated that the presence of recent drug- resistant TB transmission and inefficiency of TB control program in Addis Ababa city. T3_ETH and CAS1_KILI was the predominant linage and related to drug resistance tuberculosis in the city. Thus strengthen TB control program is crucial to interrupt the transmission of TB.

Key words: Tuberculosis, Modeling, drug resistance, molecular typing, spatial analysis, Ethiopia

Chapter 1. General Introduction

Tuberculosis (TB) remains a major global health problem. It causes ill-health among millions of people each year. According to WHO report in 2016, there were almost 10.4 million new cases and 1.4 million TB deaths in 2015(WHO, 2016). High Burden Countries (HBCs) account for approximately 81% of the estimated number of new TB cases (all forms) arising worldwide each year. Globally, 3.9% of new cases and 21% of previously treated cases are estimated to have rifampicin or MDR-TB (MDR/RR) in 2015. MDR/RR-TB caused 250 000 deaths in 2015(WHO, 2016). The problem is becoming challenging by the emergence of extensive drug-resistant (XDR), MDR tuberculosis that is also resistant to the fluoroquinolones and second-line injectable drugs, and totally drug-resistant, having resistance to additional drugs beyond XDR tuberculosis.

The Federal Democratic Republic of Ethiopia is the 2^{nd} among highly populated country in Africa with an estimated population of 103 million (PMC, 2017). Ethiopia is one of the 30 high TB burden countries and one of the four high TB burden countries in Africa (i.e. South Africa, Nigeria, and Democratic Republic of Congo). In Ethiopia the prevalence of all forms of TB was estimated to be $192/10^5$population, TB/HIV co-infection was $16/10^5$population and annual mortality was $4/10^5$population(WHO, 2016). Ethiopia had an estimated 3300 MDR-TB cases in 2015(WHO, 2016). The second drug resistance TB survey result showed 2.7% of new cases and 17.9 % of retreatment cases in Ethiopia were resistance to isoniazid and rifampicin, (MDR-TB)(EPHI, 2014). Ethiopia had a small number of reported cases of extensively drug-resistant TB (XDR-TB)(Agonafir *et al.*, 2010; Bedru Hussein, 2013).

TB is also a major health problem in the capital city Addis Ababa with a total of 9352 all forms of TB reported cases (5% of all Ethiopian cases)(AAHB, 2016). The HIV/TB co-infection rate in the city was 25%. Addis Ababa had an estimated 220 MDR-TB cases and 78 MDR/RR-TB notified cases with the treatment success rate of 72% in 2016(AAHB, 2016). Two XDR-TB cases were reported in the city(Agonafir *et al.*, 2010). The prevalence of latent TB in the city is about 47% by Tuberculin Skin Test (>=10mm) and 44% by Quantiferon^{TM} interferon release assay(Dagnew *et al.*, 2012).

Despite all the efforts and commitments exerted and opportunities availed, our office Addis Ababa City Administration Health Bureau is still facing many challenges which possibly compromise the quality of TB prevention and control activities: some of the challenges are low case detection rate and the emergence of multi-drug resistant TB (MDR-TB).

The major hurdle encountered during TB case detection is the lack of accurate diagnostic tools. To date, TB screening strategies used a combination of symptom screening, radiological and microscopy-center based laboratory diagnostics (smear microscopy). This is a major shortcoming as it is now well established that these standard diagnostic tools perform sub-optimally especially in high HIV prevalent settings. Additionally, data demonstrated that the diagnostic delay of smear microscopy resulted in a significant diagnostic default of active TB patients after initial screening, thus further decreasing the efficacy of this screening strategy. In addition, culture is often very expensive, not routinely available and takes several weeks, which compromises patient follow-up and traceability. Thus, the need for alternative laboratory diagnostic tool to case detection is urgent. With the recent development of a number of novel TB diagnostic tools with superior performance compared to conventional modalities, it is imperative that case-detection strategies be reviewed. The best choice of diagnostic strategy is likely to vary

between settings with different epidemiology (such as levels of TB incidence, HIV co-infection, and drug-resistant TB) and structural and resource constraints (such as existing diagnostic pathways, human resources, and laboratory capacity).

The major hurdle encountered during MDR-TB management is investigations of presumed outbreaks of MDR-TB which require detailed information on drug resistance pattern and the overall burden of the disease in order to build an effective treatment regimen and guide its diagnosis, infection control, and public health management. Investigation of presumed outbreaks of MDR-TB also requires strain typing data to determine the level of recent transmission (effectiveness of TB control program) and identify outbreak-related strains. It requires identifying geographic clustering of cases for targeted interventions such as intensified active case findings and tactical allocation of resources and requires pinpointing factors associated with recent infection for the control of the epidemic and strengthen the disease surveillance.

Thus, the aims of this study were as follows

1.1 General Objective

The overall objective of this thesis was to determine the impact of alternative laboratory diagnostic algorithms on the patient, health system, and identify cost-effective algorithms using virtual implementation approach; to determine drug resistance, molecular and spatial pattern of tuberculosis transmission, and to identify factors associated with the transmission in Addis Ababa, Ethiopia.

1.2 Specific Objectives

Specific Objective 1*:* To project the effects of alternative laboratory diagnostic algorithms on the patient and health system, and identify cost effective algorithms that increase TB case detection in Addis Ababa, Ethiopia.

Hypothesis: Smear microscopy-based screening for TB combined with a package of new TB diagnostics technologies significantly enhances patient, and health system level TB outcomes, and cost effectiveness.

Specific Objective 2: To determine drug resistance pattern of tuberculosis transmission among high MDR risk group in Addis Ababa

Hypothesis: Drug resistance is becoming common for first-line and second-line anti-TB drugs

Specific Objective 3: To identify recent transmission of tuberculosis and responsible strains in Addis Ababa

Hypothesis: Some strains are recently transmitted and genotypically clustered consistently with an aerosol transmission of MTB strains.

Specific Objective 4: To identify and locate outbreaks of Drug Resistance TB

Hypothesis: Drug resistance patients are geographically clustered

Specific Objective 5: To identify factors associated with the molecular and geographic pattern of drug-resistance tuberculosis transmission in Addis Ababa.

Hypothesis: Specific demographic, epidemiologic and microbiologic risk factors are associated with recent DR-TB outbreaks in Addis Ababa.

This Ph.D. thesis is organized as follows:

Chapter one included introduction, hypothesis, significance, and objectives of the study

Chapter two presents the literature review that covers an overview of the tuberculosis disease, *M. tuberculosis* organism, epidemiology of tuberculosis in global and Ethiopia, and methods to detect tuberculosis, drug resistance. Finally, review about the molecular basis of drug resistance.

Chapter three presents result from the assessment of the patient, health system, and population effects of alternative diagnostics algorithms for tuberculosis detection in Addis Ababa city.

Chapter four present first-line and second-line anti-TB drug resistance patterns and its associated risk factors in Addis Ababa city.

Chapter five present results of molecular pattern of tuberculosis transmission and its associated risk factors in Addis Ababa

Chapter six present results of geospatial and space-time clustering of rifampicin resistant tuberculosis and its associated risk factors in Addis Ababa

Chapter seven gives a comprehensive discussion on the findings of the studies.

Chapter eight presents conclusions, recommendations, and perspectives of the whole study.

The reference section provides relevant references on the subject.

Chapter 2. Literature review

2.1 The Burden of Human Tuberculosis

The World Health Organization (WHO) reported that there were an estimated 10.4 million new (incident) TB cases worldwide in 2015, of which 5.9 million (56%) were men, 3.5 million (34%) among women and 1.0 million (10%) among children. People living with HIV accounted for 1.2 million (11%) of all new TB cases. (WHO, 2016)

Worldwide, the rate of decline in TB incidence remained at only 1.5% from 2014 to 2015(WHO, 2016). This needs to accelerate to a 4–5% annual decline by 2020 to reach the first milestones of the End TB Strategy. The strategy calls for a 90% reduction in TB deaths and an 80% reduction in the TB incidence rate by 2030, compared with 2015(WHO, 2015b). Six countries accounted for 60% of the new cases: India (~2.2million), Indonesia (~1million), China (~930,000), Nigeria (~570,000), Pakistan (~500,000) and South Africa (~450,000), and followed by Bangladesh (~360,000), Philippines (~290,000), D.R. Congo (~240,000), Myanmar (~200,000) and Ethiopia (~200,000).(WHO, 2016)

In 2015, there were an estimated 480 000 new cases of multidrug-resistant TB (MDR-TB) and an additional 100 000 people with rifampicin-resistant TB (RR-TB) who were also newly eligible or MDR-TB treatment(WHO, 2016). India, China, and the Russian Federation accounted for 45% of the combined total of 580 000 cases. (WHO, 2016)

There were an estimated 1.4 million TB deaths in 2015, and an additional 0.4 million deaths resulting from TB disease among people living with HIV. Although the number of TB deaths

fell by 22% between 2000 and 2015, TB remained one of the top 10 causes of death worldwide in 2015. (WHO, 2016)

Ethiopia is one of 30 countries with the highest burdens of TB, TB and HIV co-infection (TB/HIV), and multidrug resistance TB (MDR-TB). In 2015, the incidence of all forms of TB in Ethiopia was 192/10^5population (~191,000 cases), TB/HIV co-infection was 16/10^5 population (~16,000), and annual mortality of TB plus TB/HIV cases were 30 /10^5 (~28900 cases)(WHO, 2016). Ethiopia had an estimated 3300 (range 2100–4600) MDR-TB cases in 2015 (WHO, 2016), and has had two cases of extensively drug-resistant TB (XDR-TB)(Agonafir *et al.*, 2010).

2.2 The biology of the causative agent of TB

2.2.1 Organism

The bacterium *Mycobacterium tuberculosis complex (MTBC)* belongs to the class actinomycetes, order Actinomycetales, family Mycobacteriaceae and genus *Mycobacterium* (Popa *et al.*, 2006; Shinnick and Good, 1994; Tortoli, 2006; Tortoli, 2014). It is slowly reproducing an aerobic, non-motile, non-spore forming, rod-shaped organisms that appear positive with acid-fast alcohol staining. They have a lipid-rich, hydrophobic cell wall consisting of peptidoglycan, complexes with mycolic acids unique to mycobacteria and arabinogalactan (Jarlier and Nikaido, 1994; Rastogi *et al.*, 2001). The thickness and composition of the cell wall render mycobacteria impermeable to hydrophilic nutrients and resistant to heavy metals, disinfectants, and antibiotics(Jarlier and Nikaido, 1994; Lambert, 2002; Levy-Frebault and Portaels, 1992; Rastogi *et al.*, 2001).

The MTBC represents a group of species or ecotypes within the genus of Mycobacterium: *M. tuberculosis*(Hoffner SE, 1993), *M. africanum(Castets et al., 1968), M. canettii(van Soolingen et al., 1997)* where the natural host are humans and *M. bovis* (cows)(Grange, 2001; van Soolingen

et al., 1994), *M. caprae*(goats)(Aranaz *et al.*, 1999), *M. microti*(voles)(van Soolingen *et al.*, 1998) and *M. pinnipedii* (seals) (Cousins *et al.*, 1993) which usually have animals as their natural hosts. In addition, rare *M. tuberculosis* complex variants, standing within the *M. tuberculosis* complex are not yet completely described; the *M. suricattae(Parsons et al., 2013)*, *M. orygis* (antelopes)(van Ingen *et al.*, 2012), *M. mungi(Alexander et al., 2010),* chimp bacillus(Coscolla *et al.*, 2013) and the *Dassie* bacillus(Smith, 1960).

2.2.2 Genome

The genome of *M. tuberculosis* (MTB H37RV) contains a circular 4.4 million base pairs that code for approximately 4018 genes (Camus *et al.*, 2002; Cole and Barrell, 1998; Ramakrishnan *et al.*, 2015). The genomic DNA is GC rich (65.6%). The function of 89% of the genes was predicted (Camus *et al.*, 2002; Kelkar *et al.*, 2011; Ramakrishnan *et al.*, 2015). There are a number of repetitive DNA sequences in the *M. tuberculosis* genome including insertion sequences (IS), the direct repeat (DR) region, the major polymorphic tandem repeats (MPTR) and the polymorphic GC-rich repetitive sequence (PGRS), these provide an opportunity for epidemiological investigations. (Poulet and Cole, 1995).

2.2.3 The Global Phylogeny of Mycobacterium

Human-associated *M. tuberculosis* strains are classified into seven phylogenetic lineages. These linages are strongly associated with specific global geographical locations. Lineage 1 is called Indo-Oceanic and prevalent in the Philippines and rim of Indian Ocean. Lineage 2 which is called East Asian (including “Beijing”) is prevalent in East Asia. Lineage 3 which is called Central Asian (CAS) /Delhi is prevalent in India and East Africa. Lineage 4, Euro-American, including the Latin American-Mediterranean (LAM), Haarlem, X-type, and T families prevalent in Europe, America, Middle East, and some part of Africa. Lineage 5 is *M. africanum* 1 located

in West Africa. Lineage 6 is *M. africanum* 2 located again in West Africa and lineage 7 reported in northern part of Ethiopia.(Comas *et al.*, 2013; Nebenzahl-Guimaraes *et al*., 2016)

Many of the phenotypic differences have been observed between main phylogenetic lineages (Krishnan *et al.*, 2011). However, it is also increasingly appreciated that there is diversity within these main lineages. Lineage 7 strains are associated with prolonged patient delay (Yimer *et al.*, 2015). The East African Indian linage is associated with a lower TB transmission (Albanna *et al.*, 2011). The Beijing strains acquire drug resistance at a higher rate than others (Hanekom *et al.*, 2011). *M. africanum* acquires drug resistance at a lower rate than the Euro-American lineage (Yeboah-Manu *et al.*, 2011). The Beijing and Indo-Oceanic strains are associated with co-infection of TB and meningitis than strains from the Euro-American lineage (Comas *et al.*, 2013).

2.3 Drug Resistance

Resistance to all the available anti-tuberculosis drugs can occur. To a large part, resistance is conferred by single nucleotide mutations in the chromosome of MTBC. No resistance plasmids exist in MTBC and horizontal gene transfer does not occur. Due to the clonal nature of MTBC, single SNPs can, therefore, be used as resistance markers. This is discussed in the following table (Table 2.1)

Table 2.1 Common target mutations in drug-resistant *Mycobacterium tuberculosis* isolates

Common mutations		Compensatory mutations
Isoniazid: inhibition of cell wall mycolic acid synthesis		
katG	Thr275Pro, Trp300Gly, Ser315Thr or Ser315Asn, Gln434X, Ala478del	Intergenic region between *oxyR'* and *ahpC*: –9G→A, −15C→T
Rv1910c-furA intergenic region	−12G→A, −10A→C, −7G→A	Unknown
furA-katG intergenic region	Truncation of 134-bp fragment (2156592–2156726)*	Unknown

mabA (fabG)-inhA	−17G → T, −15C → T, −8*t* → A, Ser94Ala	Unknown
Rifampicin: inhibition of transcriptional activity		
rpoB	Asp516Val or Asp516Tyr, His526Arg or His526Asp or His526Tyr, Ser531Leu	*rpoA* mutations: Arg186Cys, Thr187Pro/Ala, Glu319Lys; *rpoC* mutations: Val483Ala, Asp485Asn, Gly594Glu, Asn689Ser, Asn826Lys
Pyrazinamide: interference with membrane energy transduction, inhibition of trans-translation, inhibition of pantothenate and inhibition of CoA biosynthesis		
pncA	Diverse mutations scattered along entire gene	Unknown
rpsA	Thr5Ser, Asp123Ala, Ala438del	Unknown
panD	Met117Ile, Pro134Ser	Unknown
Ethambutol: inhibition of biosynthesis of cell wall arabinogalactan		
embB	Met306Val or Met306Ile, Gly406Ser or Gly406Ala or Gly406Asp or Gly406Cys, Gln497Arg	Unknown
ubiA	Val188Ala, Ala237Val, Arg240Cys, Ala249Gly	Unknown
Fluoroquinolones: inhibition of DNA replication, transcription, and recombination		
gyrA	Ala74Ser, Gly88Ala or Gly88Cys, Ala90Val, Ser91Pro, Asp94Gly or Asp94His or Asp94Ala	*gyrA* mutations: Thr80Ala, Ala90Gly
gyrB	Asp461His or Asp461Asn or Asp461Ala, Gly470Ala, Asp494Ala, Asn499Asp, Glu501Val	Unknown
Streptomycin: inhibition of protein synthesis		
rpsL	Lys43Arg, Lys88Arg	Unknown
rrs	462C → T, 492C → T, 514A → C, 517C → T	Unknown
gidB	n/a†	Unknown
Kanamycin and amikacin: inhibition of protein synthesis		
rrs	1401A → G, 1484G → T	1409C → A, 1491G → T
eis‡	−10G → A, −14C → T, −37G → T	Unknown
whiB7	86del C, 124del C, 128del G, 133del C, 133_134insC, 179del G§	Unknown
Capreomycin: inhibition of protein synthesis		
rrs	1401A → G, 1484G → T	Unknown
tlyA	Asn236Lys	Unknown

Linezolid: inhibition of protein synthesis		
rrl	2061G→T, 2270G→C or 2270G→T, 2576G→T, 2746G→A	Unknown
rplC	Cys154Asn, His155Asp	Unknown
Bedaquiline: inhibition of mycobacterial ATP synthase		
atpE	Asp28Val, Glu61Asp, Ala63Pro	Unknown
Rv0678	Ser68Gly, Arg94Gln, Glu138Gly, Trp92_Phe93insGly, Arg38_Leu39insAla	Unknown
Ethionamide: inhibition of cell wall mycolic acid synthesis		
ethA	Diverse mutations scattered along entire gene	Unknown
ethR	Ala95Thr, Phe110Leu	Unknown
mabA (fabG)-inhA	−17G→T, −15C→T, −8T→A, Ser94Ala	Unknown
Para-aminosalicylic acid: inhibitor of folic acid and thymine nucleotide metabolism		
ribD	−11 G→A	Unknown
thyA	Gln111X, Leu143Pro, Ala182Pro,Thr202Ala,Tyr251X, Val261Gly, X264Arg	unknown
dfrA	Val54Ala, Cys110Arg, Ser66Cys	Unknown
folC	Glu40Ala or Glu40Gly, Iie43Ala or Iie43Thr	Unknown
D-cycloserine: inhibition of cell wall peptidoglycan synthesis		
alr	-26G-T	Unknown
ddl	non identified	Unknown
cycA	Gly122Ser**	Unknown

Nucleotide positions refer to H37Rv genome (NC_000962.3). The table is taken from Keertan Dheda *et al.* (Dheda, 2017).

2.4 Laboratory Diagnostics of Tuberculosis

The bacteriological confirmation of TB and the determination of drug susceptibility are essential to ensure that a patient is correctly diagnosed with TB and started on the most effective treatment regimen. Use of inadequate and ineffective antibiotics may lead to further spread of resistant bacteria and amplification of resistance. Therefore, diagnosis and identification TB, MDR-TB, and XDR-TB is a prerequisite for appropriate treatment. Hence methods with proven clinical utility for the diagnosis of active pulmonary TB and drug susceptibility (microscopy, commercial kits to detect molecular markers, and culture) are discussed below.

2.4.1 Microscopy

Light microscopy

Sputum microscopy was developed in the 1880s. It has remained essentially unchanged since then. It is the most widely and routinely used diagnostic tool for active pulmonary TB in most developing countries. Smear examination by light microscopy after Ziehl Neelsen staining relies on the following principle: Primary stain (carbol fuschion dye) penetrates cell wall, intense decolorization (by alcohol-acid) does not release primary stain from the cell wall of AFB, color of AFB-based on primary stain (red), and counter stain (Methylene blue) provides contrasting background (blue).

It is fast, relatively cheap, and does not require sophisticated equipment. It is specific enough (specificity of ranged from 94%-100%) that no confirmatory testing is needed. Unfortunately, it has the low sensitivity of 29.2% (Getachew *et al.*, 2015). Only as little as 0.2 ml materials are examined. Hence bacteria must be present in high concentrations (over 10,000 acid-fast bacilli per/ml) to be visible. Its intrinsic reliance on sputum production also limits its use in some vulnerable groups such as children and HIV-positive patients who often produce little sputum

with the low bacillary load. In addition, it does not allow a differentiation between viable and non-viable bacteria, or the identification of different mycobacteria species.

Fluorescence microscopy

Auramine O fluorescence microscopy (FM) technique was first described by Hagemann in 1937. The principle includes the primary satin (Auramine O) penetrates the cell wall, Intense decolorization by acid- alcohol does not release primary stain from the mycolic acid of AFB, and quenching was done (Potassium permanganate) to suppress non-specific background fluorescence and provides contrasting background. The color of AFB is based on the primary stain.

The advantage of using Auramine-stained smears overweigh compared to ZN-stained smears. FM has higher sensitivity (10%) and equivalent specificity compared to ZN(WHO, 2011a). The higher sensitivity is due to that for ZN negative results a minimum of 100 microscopic fields must be examined using 100x oil immersion objective which takes about 3-5 min of technician's time. But, in high volume laboratories, smears may not be examined for the recommended time, causing lower sensitivity. FM method uses a lower power objectives lens (25x, while the ZN uses 100x), that takes mean time of 1 min to examine a sputum smear, causing higher sensitivity. FM is very advantageous in high volume labs. In order to improve the diagnostics by smear microscopy, the WHO have recommended the gradual substitution of the ZN microscopy to fluorescent microscopy(WHO, 2011a).

2.4.2 Culture

Isolation of the causative agent provides definite evidence of the disease and is considered the gold standard for diagnosing TB. In addition, it offers the opportunity for obtaining bacterial

isolates that can be used for in-depth studies. Although this technique is highly sensitive and needs only a few viable bacilli to initiate growth, the slow growth rate of MTBC (8 weeks for solid culture and 6 weeks for liquid culture) and the requirement of specific decontamination solution in addition to a costly biosafety level 3 laboratory prevent its usage as a first hand rapid test for the diagnosis of active TB

2.4.3 Molecular testing

Different molecular assays have been proposed for the detection of tuberculosis and mutations associated with resistance to anti-TB drugs besides DNA sequencing which is considered the gold standard for molecular detection of drug resistance. These include real-time PCR and kit based line probe assays. Commercially available common methods for rapid molecular-based detection of MTBC and/or drug resistant MTBC are Xpert MTB/RIF, MTBDR*plus*, and MTBDRsl assays. The basic methodology and usefulness of these tools are described here.

Xpert MTB/RIF

The Xpert MTB/RIF test, designed for the GeneXpert® System (Cepheid, USA), is a single use cartridge-based semi-quantitative real-time Polymerase Chain Reaction (PCR) in-vitro diagnostic test capable of detecting the MTBC while simultaneously detecting rifampicin (RIF) resistance by targeting the rifampicin resistance determinant region of the *rpoB* gene. The GeneXpert machine is a fully automated closed system that performs both sample preparation and real-time PCR, producing results in less than 2 h (Boehme *et al.*, 2011).

Resistance to RIF anti-tuberculosis drug is almost exclusively associated with mutations in the *rpoB* gene that encodes the ß-subunit of RNA polymerase. RIF interferes with transcription by the DNA-dependent RNA polymerase. RNA polymerase is composed of four different subunits

(α, β, β’ and σ) encoded by *rpoA, rpoB, rpoC* and *rpoD* genes respectively. RIF binds to the b-subunit of ribonucleic acid polymerase resulting in inhibition of transcription initiation. Extensive studies on the *rpoB* gene in RIF-resistant isolates of *M. tuberculosis* worldwide identified over 70 distinct mutations and short deletions in the gene. More than 95% of rifampicin-resistant strains are associated with mutations within the 81-base pair region of the *rpoß* gene, which is termed the rifampicin resistance determinant region, corresponding to codons 507 to 533, which code for the beta subunit of the RNA polymerase of *M. tuberculosis*.

Based on a WHO meta-analysis of the sensitivity and specificity of Xpert MTB/RIF, the test has shown very high sensitivity in sputum samples, 98% in smear-positive, culture positive and 79% in people living with HIV. The specificity is notably high, more than 92.5%. Xpert MTB/RIF detects resistance to rifampicin directly from sputum, regardless of the smear status in less than 2 hours. Furthermore, this method requires no additional reagents since all reagents are in-built, minimizing the cost(WHO, 2014d).

GenoType MTBDRplus,

GenoType MTBDRplus is designed to detect *M. tuberculosis* and rifampicin (RIF) and isoniazid (INH) drug resistance. The detection of RIF resistance is enabled by the detection of the mutation of *rpoB* gene and discussed above. Resistance to INH is associated with the mutation in a relatively large number of genes: *katG*, inhA, *KasA, oxyR, and ahpC*. But for detection of INH resistance the method target the most significant resistance associated mutations (katG and *inhA* genes) that coding for catalase-peroxidase and mycolic acid biosynthesis, respectively. *C*atalase-

peroxidase is necessary for activation of INH to act on and inhibits synthesis of mycolic acid production.

The procedure is divided into three steps: DNA extraction from decontaminated sputum sample or cultured materials, a multiplex amplification with biotinylated primers, and a reverse hybridization. It detects resistance to both isoniazid and rifampicin from the pulmonary patient specimen. It is less sensitive for smear-negative TB. The potential for cross-contamination requires extensive training and Infrastructure requirement. (Manufacturer manual)

MTBDRsl

GenoType MTBDR*sl* is qualitative in vitro test for the identification of MTBC and its resistance to fluoroquinolones (e.g. ofloxacin and moxifloxacin), and aminoglycosides/cyclic peptides (e.g. injectable antibiotics such as kanamycin, amikacin, capreomycin, and viomycin) from sputum specimens and cultivated samples. The detection of fluoroqinolone resistance is enabled by the detection of the most significant resistance associated mutation of the *gyrA* and *gyrB* genes which are coding for the A-subunit and B-subunit of the DNA gyrase, respectively. For detection of aminoglycosides/cyclic peptides resistance, the 16s rRNA gene (rrs) is examined. For detection of low-level kanamycin resistance, the promoter regions of the *eis* gene, coding for the acetyltranserase Eis, is examined. (Manufacturer Insert)

2.5 Molecular Typing Techniques for MTBC

Recent developments in molecular biology have resulted in techniques that allow rapid identification and tracking of specific strains of *M. tuberculosis* as they spread through the population. While, previous methods, such as colony morphology, comparative growth rates,

susceptibility to antibiotics, and phage typing were useful but did not provide sufficient information regarding TB epidemiology.

MTBC is a genetically monomorphic bacterium that has little sequence diversity compared to most other bacteria(Supply *et al.*, 2003). Genome sequencing has revealed polymorphic regions at nucleotide and gene level. Hence, various polymorphic markers (example: insertion sequence (IS), direct repeat (DR) and tandem repeats, Figure2.1) have been used successfully to trace on-going chains of TB transmission, differentiate relapse from re-infection cases and detect laboratory cross-contamination. The most widely used tool for epidemiological strain typing of *M. tuberculosis* are Restriction Fragment Length Polymorphism IS6110 RFLP, Spoligotyping, and Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeats (MIRU-VNTR) analysis. Recently, with the cost of high throughput DNA sequencing decreasing, whole-genome sequencing is gradually becoming the ideal tool to infer molecular epidemiology and phylogenetic aspects(Ei, 2016). Each of these methods results in strain-specific genetic profiles (fingerprints). Identical strain fingerprints are called clusters, which are usually associated with recent transmission. While strains with unique fingerprints represent remote transmission or infection acquired in past. The basic methodology and usefulness of these tools are described here.

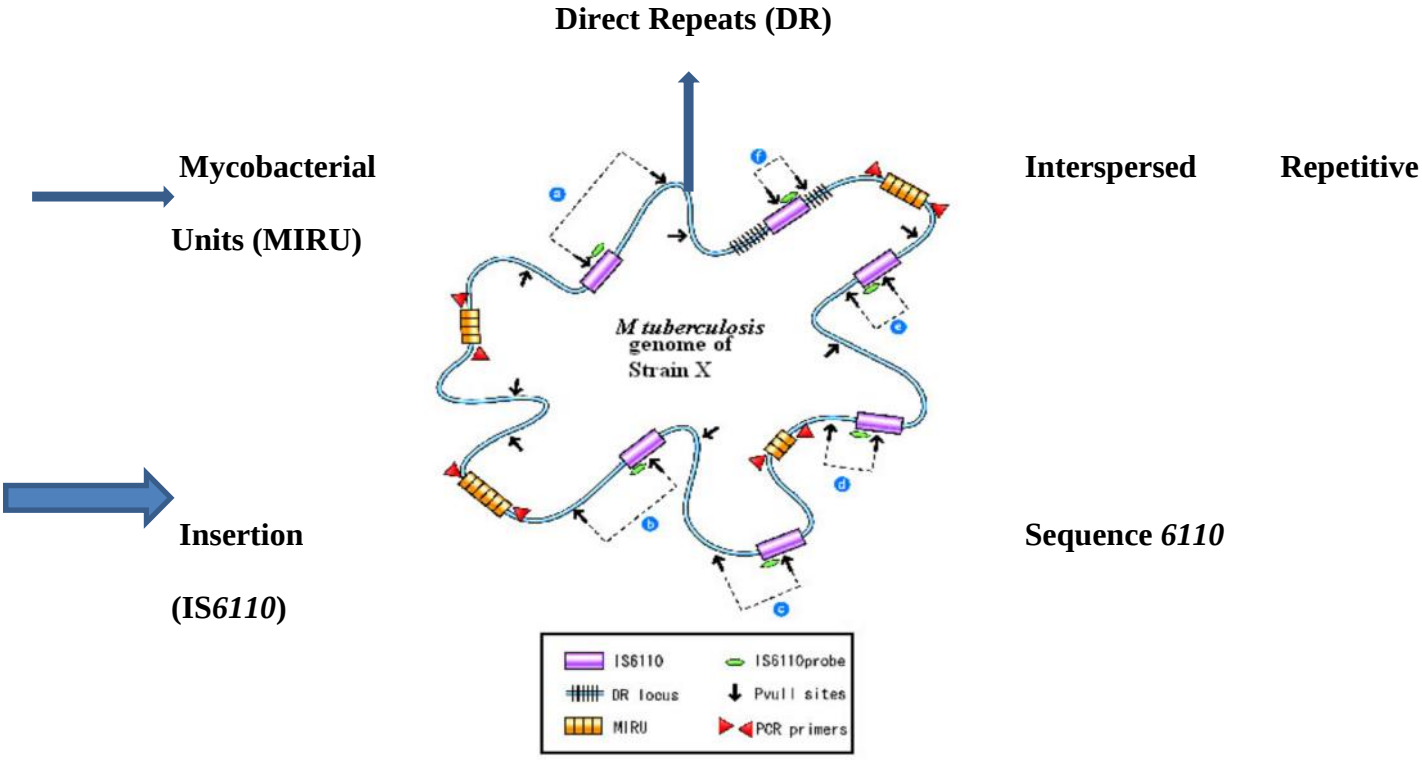


Figure 2:1 Hypothetical Genome of MTB strain and polymorphic repetitive sequences of IS6110, DR and MIRUs (Barnes and Cave, 2003)

2.5.1 Restriction Fragment Length Polymorphism (IS6110 RFLP)

IS*6110*-RLFP was the first standardized molecular typing method developed in the early 1990s by van Embden *et al.*,1993(van Embden *et al.*, 1993). This method is based on the detection of the IS*6110* insertion sequence. IS*6110* is 1355bp in length with imperfect terminal inverted repeats and contain two transposes open reading frames (ORF 1 and 2) which overlap by 1bp. Differences in copy number (between 0 and 25 copies) and locations within the genome make this technique for differentiating MTBC. Isolates with identical IS*6110* DNA fingerprints are interpreted to infer transmission between individuals, while isolates with unique DNA fingerprints are thought to reflect reactivation of a prior infection in the patient.

Molecular fingerprinting using IS*6110* relies on the cultivation of the organism, extraction of genomic DNA, digestion of genomic DNA with *Pvu*II restriction enzyme that cleaves the IS*6110* sequence only once, separation of DNA fragments by agarose gel electrophoresis, Southern transfer of the fingerprinting gel, hybridization, and detection of the IS elements with a labeled probe. Finally, the RFLP patterns are entered into a computerized database and analyzed with an image analysis system.

The major advances that have been achieved through IS6110DNA fingerprinting include identifying the population structure, quantification of the contribution of causal contact to ongoing transmission, discriminating re-infection to relapse, identification of outbreaks, demonstration that drug-resistant strains can be transmitted, understanding human-animal and animal –human transmission of tuberculosis, understanding where transmission occurs, proving nosocomial transmission, proving multiple infections in patients, and demonstrating laboratory cross-contamination.

On the other hand, initially considered as the gold standard, this method has been replaced by MIRU-VNTR and spoligotyping for various reasons: It requires large amount of isolates material when compared to PCR-based tests, the time delay in waiting for cultures to grow, the inability to perform this technique on non-viable culture and not discriminatory enough for strains with 6 or less IS*6110* copy numbers(Warren *et al.*, 2009).

2.5.2 Spoligotyping

Spacer oligonucleotide typing, or spoligotyping, is a rapid, polymerase chain reaction (PCR)-based method for molecular typing of the Mycobacterium tuberculosis complex (MTBC) (Kamerbeek J, 1997)**.** Spoligotyping takes advantage of the Direct Repeat (DR) locus of the

MTBC genome, a region consisting of multiple direct variable repeats (DVR). Each DVR is composed of identical 36 bp-DR and a non-repetitive short sequence (also called spacer) of 35-41 base pairs in length. Multiple copies of 36-bp direct repeats are well conserved but the spacer sequences between those DR sequences are different. The presence or absence of selected 43 spacers allows for discrimination among MTBC strains.

The protocol entails amplification of DR region, hybridization of amplified PCR products to the immobilized spacer-oligos corresponding to each of 43 spacers. The presence of spacers is visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection. The resulting pattern of presence or absence of spacer revealed by chemiluminescence can be compared with the database- SITVITWEB (formerly SpolDB4) for identification.

Spoligotyping method has many strongpoints as compared to RFLP technique. It is simple, cost-effective, its digital data, and high-throughput with accurate and reproducible results within 2 days. It can be performed with considerably less DNA and direct application in clinical samples without the need for prior culture; it also allows genotyping of impure DNA, nonviable specimens, paraffin-embedded material, and material from slides of Ziehl-Neelsen staining. The results are highly reproducible, and the binary (present/absent) data generated can be easily interpreted and computerized and are amenable to inter-laboratory comparisons. These all makes it ideal for molecular epidemiological studies.

However, the method's weaknesses include the inability of spoligotyping to differentiate well within large strain families such as the Beijing family, is less discriminatory than IS*6110* RFLP

analysis & MIRU-VNTR (it targets only a single genetic locus, covering less than 0.1% of the *M. tuberculosis* complex genome), the limited success in improving the assay through expansion, and the difficulty in obtaining the specialized membranes and instrumentation. Furthermore, it is unable to detect contaminated isolates or multi-strain infections (Barlow *et al.*, 2001; Goguet de la Salmoniere *et al.*, 1997).

2.5.3 Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeats

Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeats (MIRU-VNTR) analysis is a PCR-based method that analyses multiple independent loci containing variable numbers of tandem repeats (VNTR) of different families of interspersed genetic elements collectively called mycobacterial interspersed repetitive units (MIRU). VNTRs are microsatellites (1-to 8-bp repeats) and minisatellites (9-to 100-bp repeats) which are located in intergenic regions, in regulatory regions, or within open reading frames and are more or less abundant throughout bacterial genomes. There are a total of 41 MIRU loci and at least 24 of them are considered as polymorphic. Protocols based on PCR amplification of different proposed formats: 12, 15 or 24 loci using primers positioned in flanking DNA sequences have been used to study the transmission dynamics of MTBC.(Supply *et al.*, 2006)

The number of tandem repeats per locus varies among strains and this difference serves as a tool for differentiating MTBC. The number of repeats (allele) per locus is calculated by PCR amplification and comparison of the product sizes of those repeated sequences with a molecular size marker on an agarose gel. The numerical results (number of copies per locus) is matched to an online database (MIRU-VNTRplus) for comparison and identification.(Supply *et al.*, 2006)

The discriminatory power of MIRU-VNTR is related to the number and selection of loci which are used. In general, when only the 12 loci are used, it is less discriminating relative to IS*6110* RFLP genotyping for isolates with high-copy-number IS*6110* insertions but more discriminating than IS*6110* RFLP genotyping for isolates with low-copy-number IS*6110*. When more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power approximates that of IS*6110* RFLP analysis (Ei, 2016; Supply *et al.*, 2006). The advantages of MIRU-VNTR analyses over gold standard IS*6110*-RFLP method are it requires little culture growth for DNA and provides comparable, numerical data by using standard gel electrophoresis or by automated analysis using fluorescence tagged PCR primers and sequencer. MIRU typing is also a method of choice for MTB strains with 0-5 copies of the IS*6110* element (Ei, 2016; Supply *et al.*, 2006).

2.5.4 Whole genome sequencing as a typing method

Whole genome sequencing (WGS) is a molecular method that determines the complete DNA sequence of an organism's genome at a single time. The genotyping methodologies described so far are a snap-shot of the *M. tuberculosis* genome and act as a surrogate marker of their evolution.

WGS, generates complete information of a strain, including the evolutionary background, drug resistance mutations, virulence-associated polymorphisms, and assessment of TB transmission, distinguish between relapse and reinfection and delineates outbreaks (Kato-Maeda *et al.*, 2013; Pettersson *et al.*, 2009; Roetzer *et al.*, 2013). In general, WGS can provide several answers at a single time, making it the ideal tool for studying the pathogen. WGS has become increasingly available, and prices have exponentially decreased in the last years. It can be foreseen that WGS will be implemented in daily research and routine clinical work and replace previous genotyping

methods as well as genotypic drug resistance testing. However, large-scale applications, especially in endemic areas are not yet possible as cost and the analyses remain important hurdles. Targeted sequencing of selected isolates, on the other hand, could be feasible in many settings.

2.6 Treatment of TB in Ethiopia

The following information on treatment of TB in Ethiopia was taken from Tuberculosis, Leprosy and TB/HIV Prevention and Control Programme Manual of Ethiopian Ministry of Health (EFMOH, 2016). Rifampicin, Ethambutol, Isoniazid, Pyrazinamide and Streptomycin drugs are used as first-line treatment of TB in Ethiopia. Rifampicin, Isoniazid, Pyrazinamide and Ethambutol (RHZE 150/75/400/275 mg); Rifampicin and Isoniazid (RH 150/75 mg); Ethambutol and Isoniazid (EH 400/150 mg) are available in fixed-dose combination. Ethambutol 400 mg; Isoniazid 150 mg and 300 mg; Streptomycin sulfate vials, 1 g, are available as a single dose. Streptomycin is administered by injection while the other drugs are to be taken orally.

The treatment of TB has two phases. The intensive phase consists of three or more drugs for the first 8 weeks for new cases and 12 weeks for re-treatment cases. The continuation phase immediately follows the intensive phase and requires at least two drugs to be taken for 4-6 months. Treatment regimens are based on WHO treatment category. Category I New sputum smear-positive, category II Sputum smear-positive Relapse, category III New sputum smear-negative, not seriously ill, and category IV Chronic and MDR-TB cases (still sputum positive after supervised retreatment). Patients belonging to category I & III will be treated with Ethambutol, Rifampicin, Isoniazid and Pyrazinamide during the intensive phase and Ethambutol and Isoniazid during the continuation phase. The treatment regimen for category II is

Streptomycin, Ethambutol, Rifampicin, Isoniazid and Pyrazinamide during the intensive phase and Rifampicin and Isoniazid during continuation phase

In the case of drug resistance, further drugs are available categorized in five groups; 1) Isoniazid, pyrazinamide, ethambutol and rifampicin (as first-line oral agents); 2) streptomycin, kanamycin, amikacin, capreomycin, and Viomycin; 3) Ciprofloxacin, levofloxacin, moxifloxacin, Gatifloxacin and ofloxacin (fluoroquinolones); 4) para-aminosalicylic acid, cycloserine, terizidone, thioacetazone, ethionamide and prototionamide (oral bacteriostatic second-line agents) 5) clofazimine, linezolid, amoxicillin/clavulanate, and carythromycin (with unclear role).

Drug resistance to these second-line drugs can also occur. The definition of extensively drug-resistant strains (XDR) is defined as MDR plus the resistance to any fluoroquinolone and to at least one of the injectable second-line drugs. XDR strains represent a major concern for global TB control, but other drug resistances and combinations can occur as well. New drugs bed aquiline and delamanid and the repurposed drug linezolid are available to treat MDR tuberculosis.

Chapter 3. Modeling the Patient and Health System Impacts of Alternative Xpert® MTB/RIF Algorithms for the Diagnosis of Pulmonary Tuberculosis in Addis Ababa, Ethiopia

3.1 Abstract

Background: To reduce global tuberculosis (TB) burden, the active disease must be diagnosed quickly and accurately and patients should be treated and cured. In Ethiopia, TB diagnosis mainly relies on spot-morning-spot (SMS) sputum sample smear analysis using Ziehl-Neelsen staining techniques (ZN). Since 2014 targeted use of xpert has been implemented. New diagnostic techniques have higher sensitivity and are likely to detect more cases if routinely implemented. The objective of our study was to project the effects of alternative diagnostic algorithms on the patient, health system, and costs, and identify cost-effective algorithms that increase TB case detection in Addis Ababa, Ethiopia.

Methods**:** An observational quantitative modeling framework was applied using the Virtual Implementation approach. The model was designed to represent the operational and epidemiological context of Addis Ababa, the capital city of Ethiopia. We compared eight diagnostic algorithm with ZN microscopy, light emitting diode (LED) fluorescence microscopy and Xpert MTB/RIF. Interventions with an annualized cost per averted *disability adjusted life year* (DALY) of *less than* the *Gross Domestic Product (GDP*) per capita are considered cost-*effective* interventions.

Results: With a cost lower than the average per-capita GDP (US$690 for Ethiopia) for each averted *disability adjusted life year* (DALY), three of the modeled algorithms are cost-effective. Implementing them would have important patient, health system, and population-level effects in the context of Addis Ababa

- The full roll-out of Xpert MTB/RIF as the primary test for all presumptive TB cases would avert 91170 DALYs (95% credible interval [CrI] 54888 – 127448) with an additional health system cost of US$ 11.6 million over the next 10 years. The incremental cost-effectiveness ratio (ICER) is $370 per DALY averted.
- Same day LED fluorescence microscopy for all presumptive TB cases combined with Xpert MTB/RIF targeted to HIV-positive and High multidrug resistant (MDR) risk groups would avert 73600 DALYs(95% CrI 48373 - 99214) with an additional cost of US$5.1 million over the next 10 years. The ICER is $169per DALY averted.
- Same-day LED fluorescence microscopy for all presumptive TB cases (and no Xpert MTB/RIF) would avert 43580 DALYs with a reduction cost of US$ 0.2 million over the next 10years. The ICER is $13 per DALY averted.

Conclusions: The full roll-out of Xpert MTB/RIF is predicted to be the best option to substantially reduce the TB burden in Addis Ababa and is considered cost effective. However, the investment cost to implement this is far beyond the budget of the national TB control program. Targeted use of Xpert MTB/RIF for HIV positive and high MDR risk groups with same-day LED fluorescence microscopy for all other presumptive TB cases is an affordable alternative.

3.2 Introduction

According to the WHO global tuberculosis (TB) report (WHO, 2015a), Ethiopia is one of the 30 high TB burden countries with an estimated prevalence of TB of 200 (161–243) per 100, 000 population. Despite all the efforts and commitments exerted and opportunities availed, the Ethiopian national tuberculosis program (NTP) is still facing many challenges which possibly compromise the coverage and quality of TB prevention and control activities: one of the challenges is low TB case detection rate. A major hurdle contributing to the low TB case detection rate is the lack of an accurate diagnostic algorithm. In Ethiopia screening strategies for presumptive tuberculosis cases mainly depends on a combination of symptom screening and microscopy using 3 samples (spot-morning-spot) and for some cases chest X-ray (EFMOH, 2013). This is a major shortcoming as it is now well established that these standard diagnostic tools perform sub-optimally. In one study conducted in Addis Ababa's health facilities, the sensitivity of Ziehl-Neelsen(ZN) microscopy was found to be 29.2% (Getachew *et al.*, 2015). Other, studies have demonstrated that the diagnostic delay of smear microscopy resulted in a significant loss-to-follow-up of active TB patients after initial screening (Langley *et al.*, 2014; Theron *et al.*, 2014). Screening strategies based on culture are more accurate, but are often very expensive, not routinely available and take several weeks to come to diagnosis (EFMOH, 2013), which compromises patient follow-up and traceability.

Ethiopia is implementing targeted use of Xpert MTB/RIF since 2014 (EFMOH, June 2014), and the utilization is suboptimal. New diagnostic tools for TB have the potential to deliver substantial reductions in the burden of disease through improved sensitivity (WHO, 2013), lower diagnostic default, less delay to starting appropriate treatment and reductions in TB incidence (Langley *et al.*, 2014). Thus, the need for full implementation of newer diagnostic approaches for case

detection in Ethiopian context is urgent. However, these new tools are more costly than the conventional diagnostic tools. Accordingly, policymakers must decide on the best combination of tools to choose; who should be tested with the new tools; whether the new tools complement or replace the existing diagnostics; and on the general impact of implementing the new tools on patients, health systems and epidemiological burden (Cobelens *et al.*, 2012; Langley *et al.*, 2015; Mann *et al.*, 2010). It would be expensive, time-consuming and disruptive to the health system to try and answer all these questions through experimentation in the live environment. Mathematical modeling has the potential of addressing the above questions to help guide policy makers in the most optimal and cost-effective use of new diagnostic tools (Langley *et al.*, 2012; Lin *et al.*, 2011). Modeling can project the effect of rolling out the new algorithm on the number of patients diagnosed, treated and cured. This enables each new diagnostic option to be evaluated and compared in terms of the number of *disability adjusted life years* (DALY's) averted, incremental cost-effectiveness ratio's (ICER), and sustainability. Different combinations or packages of tests and approaches can be modeled. This study is proposed to assess the impact of alternative diagnostic algorithms on tuberculosis detection using a modeling approach in a district of Addis Ababa, Ethiopia. The analysis adds to other work on this topic (Basu *et al.*, 2009; Dye, 2012; Langley *et al.*, 2012; Langley *et al.*, 2014; Langley *et al.*, 2015).

3.3 Materials and Methods

3.3.1 Study setting and algorithms

The study was conducted for public health facilities which provide the Directly Observed Treatment (DOT) services to TB patients in Addis Ababa, Ethiopia. We compared the impact of eight diagnostic algorithms. These were chosen based on WHO (WHO, 2014d) and Ethiopian ministry of Health (EFMOH, June 2014) recommendations. Routine diagnostic algorithms, that are common in the country, were also considered. Under the **ZN-Spot-Morning-Spot**

scenario, Ziehl-Neelsen (ZN) microscopy using three sputum samples provided within two days was modeled. This scenario represents the common diagnostic algorithms for presumptive TB patients when there is no access to Xpert MTB/RIF (expert opinion). In this model, it was considered as the base case for comparison. **FN-Spot-Morning-Spot** scenario replaces ZN microscopy with light-emitting diode (LED) fluorescence microscopy. **FN-Spot-Morning** and **FN-Spot-Spot** scenarios use LED Fluorescence microscopy using two sputum samples provided within two days (on the SPOT-early MORNING) and on the same day (on the SPOT-SPOT), respectively. **Full-Xpert** scenario tests all patients with presumptive tuberculosis with Xpert. **Targeted-Xpert- MDR-HIV-ZN-Spot-Morning-Spot** scenario uses ZN microscopy (SPOT-MORNING-SPOT) as the primary test for tuberculosis diagnosis and target Xpert for presumptive TB cases that are HIV positive, known contact of MDR-TB patients, and retreatment cases. But **Targeted-Xpert-ZN-Negative-Spot-Morning-Spot** scenario targets the use of Xpert to a patient with presumptive TB who are smear negative in addition to a patient who is HIV positive, known contact of MDR-TB patients, and retreatment case. **Targeted-Xpert-MDR-HIV-FN-Spot-Spot** scenario uses LED fluorescence microscopy on the same day (SPOT-SPOT) as the primary test for tuberculosis diagnosis and target Xpert for presumptive TB cases who are HIV positive, known contact of MDR-TB patients, and retreatment cases. Figure 1 Illustrate the diagnostic algorithms used in the analysis.

3.3.2 Model structure

We adopted a modeling framework pioneered by Langley et al. in Tanzania (Langley *et al.*, 2014) to represent the operational and epidemiological context of Addis Ababa, Ethiopia. The model used the discrete-event simulation (DES) approach and Witness software to represent the patient pathways that individual patients follow and the sputum sample pathways in the laboratory (Fig 3.1).

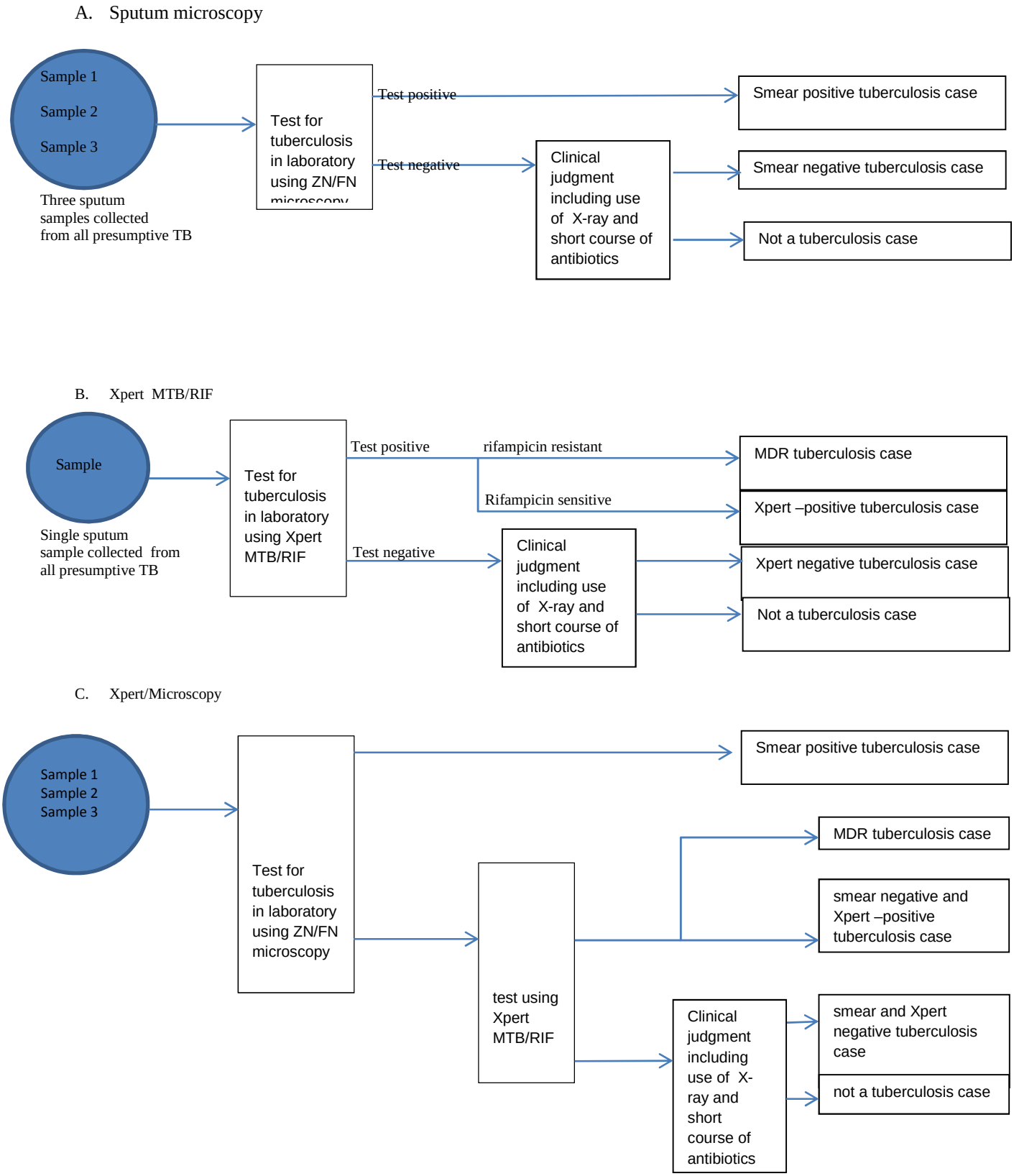


Figure 3.1 Patient pathway for diagnostic algorithms. A. Sputum microscopy. B. Xpert MTB/RIF. C. Xpert MTB/RIF in addition to microscopy

The patient pathways, the patient were categorized as either new cases or retreatment cases, and HIV+ or HIV- with some knowing their status and some not. Multiple visits to the diagnostic center are required by each new patient. The number of visits for each patient is dependent on a number of factors which were modeled. These factors included the diagnostic test; whether the patient became diagnostic lost to follow-up (LTFU); the backlog in the laboratory that could lead to diagnosis not being available; the time the patient was willing to wait in the diagnostic center for results; whether the initial test result was positive; whether X-ray and a short course of antibiotics was required following a negative sputum test; and whether TB treatment would be required. Once TB has been diagnosed a further visit to the diagnostic center is required to receive the first batch of medication and for treatment education. Patients return to the diagnostic center during treatment for sputum smear microscopy testing at the end of each phase of treatment. Patients are finally categorized as cured, completed, failed treatment, or treatment lost to follow-up.

In sputum sample pathways, sputum samples are collected in the diagnostic center from presumptive TB cases and individuals undergoing treatment for TB. Samples follow a pathway through the laboratory depending on the patient characteristics, diagnostic technique, and the diagnostic algorithm being used. There were two different types of diagnostic test that were modeled – microscopy and Xpert. These were modeled separately with control logic defining which test process would be used for which patient sample based on the ‘attributes’ of the patient. This logic enabled a number of different diagnostic algorithms to be evaluated. Samples are prepared for the appropriate test, the test is then conducted and a test result assigned.

For simplicity, we assumed a constant population with no immigration or emigration and constant transmission dynamics. Further details on the model and parameter descriptions are given in the Supplemental material and other references (Langley *et al.*, 2014; Lin *et al.*, 2011). The model was calibrated by using real data from diagnostic centers in Addis Ababa. The outputs from the model were validated against the observed results from diagnostic centers.

Considering the natural history and pathogenesis of tuberculosis that take generations for the effects of intervention to be seen and situational changes due to new diagnostics technologist in pipeline, we choose a timeframe of 10 years for prediction

3.3.3 Diagnostic and cost parameters

A survey of one central referral laboratory and 20 public health facilities that provide TB services provided (randomly selected) estimates for the diagnostic and cost parameters to be used in the model (Supplement Table B). Sensitivity and specificity of diagnostic tools were collated from the literature(Boehme *et al.*, 2011; Swai *et al.*, 2011; WHO, 2011b) (Supplement Table A). Data were collected from August to December 2014. Supplement material part A and B lists the model variables and their sources

Cost variables and source of the data are presented in supplemental material part C. Unit costs were input in US dollars and are based on published data and estimates from Ethiopian National Tuberculosis program, Addis Ababa Health Bureau, and Health facilities. We used public sector salary scale of 2014 to estimate annual employment cost using National Bank of Ethiopia exchange rate (19.7 Birr= 1 USD). Consolidated Health Economic Evaluation Reporting Standards (CHEERS) (Don Husereau, 2013) was used for reporting of economic evaluation results.

3.3.4 Uncertainty Analysis

We conducted uncertainty analysis to estimate the 95% credible interval of projected outcome, accounting for the uncertainty of input parameter values. In the operational component, each simulated year involved running the model for over 2,500 simulated days. Outcomes were recorded for each simulated 90 day period and the mean and standard deviation calculated. From these, 95% confidence limits for key outcome measures were calculated.

3.3.5 Cost-effectiveness analysis

Cost-effectiveness measures were calculated to compare different diagnostic options. The incremental cost of implementing each alternative diagnostic option was derived from the Addis Ababa health system perspective, and included the additional annual running costs (eg, consumables, susceptible and drug resistant -tuberculosis drugs, radiographs, equipment maintenance, and laboratory personnel) and the investment costs (eg, microscopes and the equipment related to Xpert implementation) (supplement material). Other overhead costs were assumed to be unaffected by a change in the diagnostic algorithm. The introduction of new tuberculosis diagnostics is expected to improve the survival of patients co-infected with tuberculosis and HIV, therefore we estimated the incremental costs from additional antiretroviral therapy (ART) on the basis of the projected increase in HIV prevalence resulting from reductions in the number of deaths from tuberculosis with HIV co-infection. The population effect on tuberculosis epidemiology was summarized using *disability- adjusted life years* (DALYs). Costs and DALYs were calculated over 10 years with an annual discount rate of 3%. We calculated the average cost-effectiveness ratio (ACER) by comparing each alternative diagnostic option to the base case scenario. Because the alternative diagnostic options are mutually exclusive interventions that compete for the same resources, we also calculated the incremental cost-effectiveness ratios (ICER) to compare one option with the next less-effective option. The

estimated ICER was compared with the willingness-to-pay threshold for Ethiopia (US$690 based on the gross domestic product [GDP] per capita in 2015) (MOFED, 2015). Interventions with an ICER of *less than* the *Gross Domestic Product* (*GDP*) per capita considered as cost-*effective* interventions(WHO, 2014a) but need to be considered alongside affordability, budget impact, fairness, feasibility and any other criteria considered important in the local .

3.4 Results

3.4.1 Patient-level Impact

The full roll-out of Xpert MTB/RIF is projected to produce the greatest patient-level benefits among the eight alternative diagnostic options (Table 3.1). The modeling result showed that mean patient visits for diagnosis reduces by an average of 1·4 visits, time to start treatment reduces by an average of 7.8 days, and the diagnostic lost to follow-up rate reduces by 9% compared to the base case (**ZN-Spot-Morning-Spot**). The likelihood that a patient with tuberculosis will successfully complete diagnosis and treatment is increased by 8.3%. The next best algorithms from the patient perspective involve targeting of Xpert MTB/RIF to HIV-positive cases and high MDR risk groups alongside same day LED for the other TB presumptive cases (**Targeted-Xpert-MDR-HIV-FN-Spot-Spot**) and Xpert as a secondary test for smear negative new TB suspects (**Targeted-Xpert-ZN-Negative-Spot-Morning-Spot**).

Table 3.1 Patient-level effects of new diagnostic algorithms

Diagnostic algorithms	Diagnosis and treatment initiation			Diagnosis and treatment success	
	Number of visits to the diagnostic center	Time to start Treatment (Days)	Diagnostic LTFU* rate (%)	New Tuberculosis cases diagnosed without a positive sputum test (%)	Likelihood of a true tuberculosis patient completing diagnosis and treatment (%)
ZN-Spot-Morning-Spot	5	16.8	13.3	59.8	76.6
FN-Spot-Morning-Spot	4.9	15	12.6	52.9	78.4
Targeted-Xpert-ZN-Negative-Spot-Morning-Spot	4.4	10.5	11.8	28.6	80.9
Targeted-Xpert- MDR-HIV-ZN-Spot-Morning-Spot	4.7	15.2	12.1	52.5	79.2
Full-Xpert	3.6	9	4.3	28.9	84.9
FN-Spot-Spot	4.4	16	8.6	56.6	80.6
FN-Spot-Morning	4.9	15.5	13.1	52.4	78.2
Targeted-Xpert-MDR-HIV-FN-Spot-Spot	4.1	14.6	6.8	49.4	83.3

*LTFU-Lost to follow up

3.4.2 Health system Impact

Under full scale-up of Xpert, the required number of sputum samples per year is 54,000 compared to 113,000 under the base case. The full roll-out of Xpert requires only 33.3% of the laboratory staff time that is needed for the base case (Table 3.2). There is no significant change in drug sensitive tuberculosis notification among the alternative diagnosis algorithms. After false-positive cases are excluded, full Xpert scale-up is projected to increase the number of true tuberculosis cures by 346 (Table 3.2). The use of Xpert would also identify 207 rifampicin-resistant cases during the diagnostic process compared with only 59 MDR tuberculosis cases under the base case. Xpert reduces missed TB cases (patients with TB but not diagnosed or lost to follow-up) by 341 patients compared with the base case.

3.4.3 Cost-effectiveness Analysis

The modeling analysis indicated that full roll-out of Xpert is expected to avert approximately 9117 DALYs per years at average cost-effectiveness ratio (ACER) of $127 per *disabilityadjusted life year* (DALY) averted compared to the base case (Table 3. 3). The average cost-effectiveness ratio (ACER) of other alternative diagnostic options ranged from $2 to $203 per DALY averted compared with the base case. Xpert as the primary test for HIV-positive cases and high MDR risk group alongside LED same day for all others (**Targeted-Xpert-MDR-HIV-FN-Spot-Spot**) is expected to avert 7360 DALYs per year and Xpert as secondary tests for smear negative new presumptive TB cases (**Targeted-Xpert-ZN-Negative-Spot-Morning-Spo**t) is expected to avert 4724 DALYs. Other diagnostic algorithms would have less effect on DALYs, with the benefits ranging from 1747 (**FN-Spot-Morning**) to 4358 (**FN-Spot-Spot**)

Table 3.2 Health-system-levels effects of new diagnostic algorithms: mean per year in 10years

Diagnostic algorithms	Sputum samples tested for tuberculosis per year			Lab staff utilization (%)	Patients starting tuberculosis treatment per year including false positive			Missed TB* per year	Complete cures per year excluding false positive
	microscopy (000s)	Xpert (000s)	x-ray (000s)		Standard regimen	Treatment complete /cure	Treatment failure /MDR TB		
ZN-Spot-Morning-Spot	113	0	12	30	6200	5919	59	963	3154
FN-Spot-Morning-Spot	112	0	12	22	5874	5608	59	893	3228
Targeted-Xpert-ZN-Negative-Spot-Morning-Spot	93	33	6	30	5257	4927	148	785	3332
Targeted-Xpert- MDR-HIV-ZN-Spot-Morning-Spot	98	7	11	28	6165	5831	118	859	3263
Full-Xpert	17	37	6	10	5588	5189	207	622	3500
FN-Spot-Spot	86	0	12	17	6269	5984	59	800	3322
FN-Spot-Morning	82	0	12	16	6200	5919	59	963	3154
Targeted-Xpert-MDR-HIV-FN-Spot-Spot	76	7	12	1	5874	5608	59	893	3228

*Missed TB- Patient with TB but not diagnosed or lost to follow up

DALYs averted per year. The full rollout of Xpert requires an additional budget of an average US$ 1,161,133 budget per year over 10 years. Incremental cost for the other alternative algorithms varies between –US$ 52,727 and US$950,669 over the next 10 years (Table 3.3, Fig. 3.2)

In the analysis of the incremental cost-effectiveness ratio (ICER) options **FN-Spot-Morning-Spot**, **Targeted-Xpert-ZN-Negative-Spot-Morning-Spot,** and **Targeted-Xpert-MDR-HIV-ZN-Spot-Morning-Spot** are strongly dominated by same-day LED fluorescence microscopy (**FN-Spot-Spot**), LED same day with Xpert (**Targeted-Xpert-MDR-HIV-FN-Spot-Spot**) , and Xpert full rollout; because they produce DALY gains at a higher incremental cost . The ICER of Xpert full rollout is $370.1 per DALY averted , followed by LED Same Day + Xpert for HIV positive and high MDR risk group (**Targeted-Xpert-MDR-HIV-FN-Spot-Spot**) ($176.7), same-day LED fluorescence microscopy ($12.6) and LED fluorescence microscopy with two sample (-$30.2) (Table 3). The ICERs for the above alternative algorithms are lower than the annual per-capita GDP of Ethiopia (US$690) (MOFED, 2015) and are deemed cost-effective.

Table 3.3 Cost-effectiveness analysis of new diagnostic algorithms

Diagnostic algorithms	DALYs averted per year			Additional health system costs per year over 10 years (US$)	Cost-effectiveness ratio (US$ per DALY averted)	
	YLL	YLD	DALY		ACER	ICER
ZN-Spot-Morning-Spot	0	0	0			
FN-Spot-Morning-Spot	1935	25	1965	3456	2	Dominated
Targeted-Xpert-ZN-Negative-Spot-Morning-Spot	4660	64	4724	950669	201	Dominated
Targeted-Xpert- MDR-HIV-ZN-Spot-Morning-Spot	2838	39	2878	582813	203	Dominated
Full-Xpert	8988	128	9117	1161133	127	370.1
FN-Spot-Spot	4299	59	4358	-19748	-5	12.6
FN-Spot-Morning	1728	25	1747	-52727	-30	-30.2
Targeted-Xpert-MDR-HIV-FN-Spot-Spot	7256	104	7360	510831	69	176.7

YLL = years of life lost. YLD=years lost due to disability DALY= disability adjusted life year. ACER= average cost-effectiveness ratio. ICER=incremental cost-effectiveness ratio

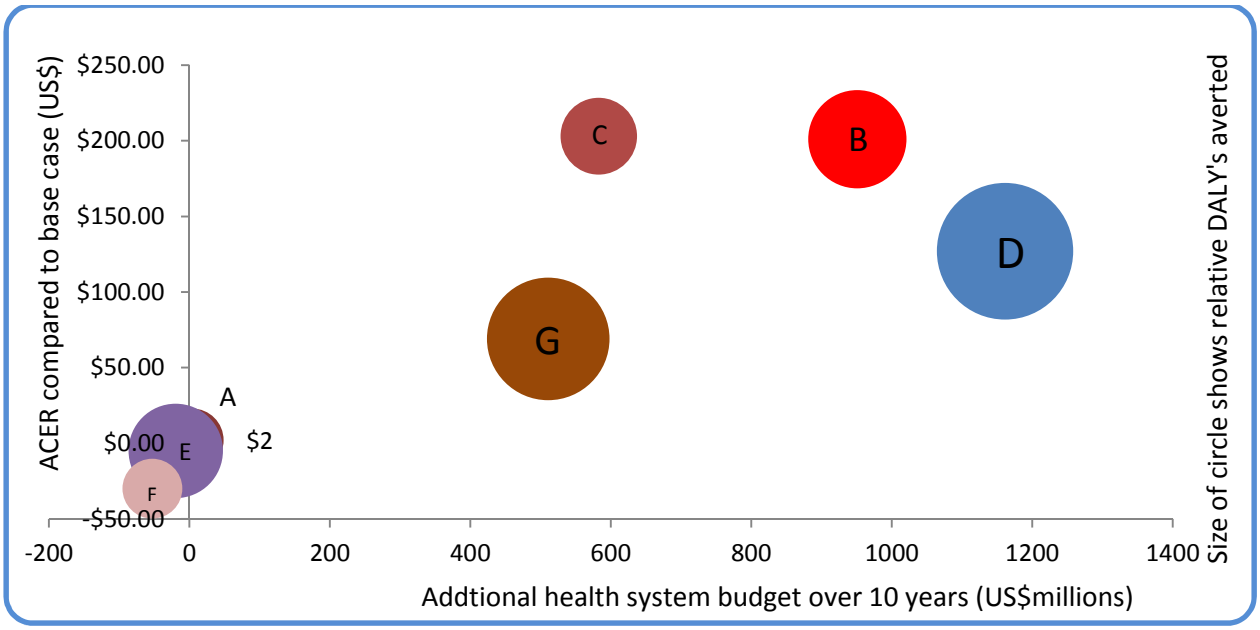


Figure 3.2 Additional health system budget versus average cost-effectiveness ratio (ACER) versus disability-adjusted life-years (DALYs) averted of alternative diagnostic algorithms

A= FN-Spot-Morning-Spot scenario, B= Targeted-Xpert-ZN-Negative-Spot-Morning-Spot scenario, C= Targeted-Xpert- MDR-HIV-ZN-Spot-Morning-Spot scenario, D= Full-Xpert scenario, E= FN-Spot-Spot scenario, F= FN-Spot-Morning scenario, and G= Targeted-Xpert-MDR-HIV-FN-Spot-Spot

3.5 Discussion

In this study, we used a modeling approach to evaluate the potential patient and health system effects and to complete a cost effective analysis of eight diagnostic algorithms. Our results indicated that full roll-out of Xpert in Addis Ababa city would have the greatest patient-level and health system benefits, and would be cost effective with the highest DALYs averted among the alternative diagnostic options. The improved diagnostic sensitivity and the need for only one sputum sample for Xpert reduce mean patient visits for diagnosis, time to start treatment, and diagnostic lost to follow-up rate. This fact result in an increase in the likelihood that a patient with TB will successfully complete diagnosis and treatment. It would result in an additional health system cost, and an incremental cost to the HIV program due to longer life expectancy for

co-infected TB and HIV patients. We note that cost-effectiveness does not necessarily mean that the implementation is affordable to Addis Ababa tuberculosis control program.

A more affordable cost effective alternative is targeted use of Xpert MTB/RIF for HIV positive and high MDR risk groups with same-day LED fluorescence microscopy for all other presumptive tuberculosis cases (**Targeted-Xpert-MDR-HIV-FN-Spot-Spot**). The result is in line with the current effort of national tuberculosis program to scale-up LED fluorescent microscopy and targeted use of Xpert. The third cost effective alternative is same day LED fluorescence microscopy (**FN-Spot-Spot**). The improved diagnostic sensitivity of LED microscopy and the need for sputum with in the same day reduce diagnostic lost-to follow up rate. This fact results in better performance of this option as compared to ZN microscopy with spot-morning, and FN spot-morning. The weak part of this option is do not have a room for drug resistant TB.

The other options are not cost effective, with DALY gains at a higher incremental cost and additional health system costs while they are less effective than the above three for the context of Addis Ababa, Ethiopia. The World Health Organization has endorsed the use of same day LED fluorescent microscopy in 2011 (WHO, 2011b). However, in Ethiopia it has not been implemented. Our result also shows that a diagnosis based on 3 (SMS) samples is not cost effective compared to Spot-spot LED fluorescence microscopy. It requires additional patient visits for diagnosis, sputum, and laboratory staff time. It results in higher diagnostic lost to follow-up rate, higher patient and health system costs, and a decrease in the likelihood that a patient with tuberculosis will successfully complete diagnosis and treatment. The national

technical working group should discuss this issue for giving advice to policy makers on the most optimal and cost-effective diagnostic algorithm.

Our results are in line with the findings of Langley et al in Tanzania (Langley *et al*., 2014) where projections due to full Xpert rollout meant patient visits for diagnosis reduced by 1.2 (95% CrI 1.1 – 1.3)visits, time to start treatment by 6·6 (95% CrI 5·9–7·3) days, and the diagnostic lost to follow-up rate by 7% (95% CrI 6–9), the likelihood that a patient with tuberculosis will successfully complete diagnosis and treatment increased by 18%. They also reported highest DALY averted, and a reduction of the number of sputum samples by 34% and laboratory staff time by 45%. In both studies, the effect of the alternative algorithms on tuberculosis case notification was the same and marginal. The reason behind this is that the increase in case detection is counterbalanced by a reduction in the number of false positives. Our results are also in line with other studies (Sun *et al.*, 2013; Zwerling *et al.*, 2015) where novel diagnostics can substantially reduce TB transmission.

We found a scenario whereby targeted use of Xpert is cost effective (**Targeted-Xpert-MDR-HIV-FN-Spot-Spot**). This is cost effective because besides the targeted use we introduce same day LED fluorescence microscopy to those not benefitting from Xpert. The other algorithms with targeted use of Xpert (**Targeted-Xpert-ZN-Negative-Spot-Morning-Spot** & **Targeted-Xpert-MDR-HIV-ZN-Spot-Morning-Spot**) are not cost effective compared to all other options. The reason for the inferior cost-effectiveness is that the projected costs are substantially higher while the projected gains in DALYs are lower than same day LED fluorescence microscopy in combination with Xpert. Similarly, in the Tanzania study targeting of Xpert either to HIV positive or smear negative HIV-positive cases was not cost-effective (Langley *et al.*, 2014).

A number of limitations were observed in our modeling analysis. All epidemiological modeling results are uncertain (Zwerling *et al.*, 2015). It is affected by uncertainty of variables and assumptions on model structures. Uncertainty analyses were done only for few parameters. Data accuracy can be a constraint with modeling. Full health system costing was not considered. Users of the modeling result should take account that a modeling analysis cannot consider all the practical challenges that might arise from implementation of a new diagnostic methods.

3.6 Conclusions

In conclusion, we suggest that Ethiopian TB program to consider switching to Same-day diagnosis according to WHO recommendation on using the same day LED fluorescent microscopy on two spot samples. The full roll-out of Xpert MTB/RIF was predicted to be the best option to substantially reduce the TB burden in Addis Ababa and was considered cost effective. However, the required resources to implement this are beyond the budget of the TB control program. Targeted use of Xpert MTB/RIF for HIV positive and high MDR risk groups with same-day LED fluorescence microscopy for all other presumptive TB cases is an affordable alternative. In a broader perspective, our results also highlight the importance of a modeling approach to support the selection and implementation of the optimal diagnostic algorithm and outlined the delicate balance between maximizing impact but coming up with an affordable diagnostic algorithm option.

Chapter 4. Drug Resistance Patterns of *Mycobacterium tuberculosis* Isolates and Associated Risk factors in Addis Ababa, Ethiopia

4.1 Abstract

Background: Drug resistance is a growing threat to public health and TB control. The objective of the paper was to assess the level of and risk factors for first and second-line anti-TB drugs resistance among high MDR risk group tuberculosis patients in Addis Ababa, Ethiopia

Methods: A total of 153 isolates collected from pulmonary high Multi-Drug resistance (MDR)-TB risk group in 2013- 2015 were included in this study. Drug susceptibility testing (DST) against first-line anti-TB drugs: isoniazid (INH), rifampicin (RMP), streptomycin (STM), ethambutol (EMB) and pyrazinamide (PZA) was performed using the BACTEC™ MGIT 960™ TB System. DST against second-line ant-TB drugs including fluoroquinolones and aminoglycocides/cyclic peptides, was performed using GenoType MTBDRsl.

Results: About 70% of the isolates were resistance to one or more of the first-line drugs, 61% of the isolates were multi-drug resistant, and 19% were resistant to all first-line drugs. Any resistance to INH, RMP, STM, EMB, PZA was 69%, 61%, 58%, 26% and 55% respectively. Four isolates were resistant to fluoroquinolones (pre-XDR), and belonging to T3_ETH linage. The rest isolates were susceptible to all second-line anti-TB drugs. The linage CAS1_KILI was a risk factor for MDR, resistance to one or more anti-tuberculosis drugs, and drug-resistant to INH, RMP, STM, and PZA. The linage T3_ETH was found to be a risk factor for EMB resistant. The age, sex, HIV and previous treatment history were not associated with drug resistance.

Conclusions: A substantial number of drug-resistance TB is circulating in the community and there is a need for more efforts to limit the spread of TB disease.

4.2 Introduction

Multidrug-resistant tuberculosis (MDR-TB), defined as TB that is resistant to at least isoniazid (INH) and rifampicin (RFP), is difficult to treat and requires prolonged treatment with expensive and often toxic multidrug regimens (WHO, 2014b; WHO, 2014c). The World Health Organization (WHO) estimates that 480,000 people globally had incident multidrug resistant tuberculosis (MDR-TB) and an additional 100 000 people with rifampicin-resistant TB (RR-TB) in 2015. Drug resistance surveillance data showed that 3.9% of new and 21 % of previously treated TB cases was estimated to have had rifampicin or multidrug resistant (MDR/RR) tuberculosis in 2015. MDR/RR - TB caused 250 000 deaths in 2015. About 9.5% of MDR-TB cases have additional drug - resistance, extensively drug - resistant TB (XDR - TB). The global treatment success rate of MDR-TB remains at approximately 52%(WHO, 2016).

Ethiopia is one of the 30 countries with a highest MDR-TB burden. Ethiopia had an estimated 3300 (range 2100–4600) MDR-TB cases in 2015(WHO, 2016), and has had a small number of cases of extensively drug-resistant TB (XDR-TB) (Agonafir *et al.*, 2010). Drug resistance surveillance data in Ethiopia showed 2.7 % among new and 17.9 among previously treated cases were estimated to have had MDR -TB. MDR -TB treatment success rate in Ethiopia is 78%(EPHI, 2014). Addis Ababa had an estimated 220 MDR-TB cases and 78 MDR-TB notified cases with the treatment success rate of 72% in 2016 (AAHB, 2016).

Drug resistance represents a growing threat to public health and TB control, surveillance to monitor the emergence and spread of drug resistance is a crucial component of the national strategy to combat drug resistance. Detailed information on drug resistance patterns of tuberculosis is essential to understand the overall burden of disease; to guide its diagnosis, infection control and public health management; to inform the development of comprehensive,

flexible, multidrug resistant tuberculosis guidance(Stagg *et al.*, 2015); to build an effective treatment regimen and improve treatment outcome (Yuen CM, 2015); however, such data are scarce in Ethiopia. The objective of this chapter, therefore, was to determine first-line and second-line anti-TB drug resistance patterns and its associated risk factors among high MDR risk group in Addis Ababa, Ethiopia.

4.3 Materials and methods

4.3.1 Study subject, area and study period

The Addis Ababa Regional Health Research Laboratory (AAHRL) provides TB drug susceptibility testing using Xpert for 120 public and private TB clinics in a city of 3.5 M persons. A total of 153 isolates were included in this study. These strains were grown from sputa from smear positive pulmonary tuberculosis patients who were referred to the lab for drug susceptibility testing (DST) between April 2013 and July 2015. For the study subjects, socio-demographic data, history of previous tuberculosis treatment, and HIV status were collected. The sputa were stored and transported to Armauer Hansen Research Institute (AHRI) for culture. Drug susceptibility testing and molecular typing were done at Molecular Epidemiology laboratory, Division of Molecular Biology & Human Genetics, Stellenbosch University, South Africa. The study was approved by Addis Ababa City Government Health Bureau ethics committee.

Specimens were kept at -20^{O}C in the AAHRL laboratory, and transported in cold box within 5 days to the mycobacteriology laboratory at AHRI for culture.

4.3.2 Culture methods

N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) method was used to digest and decontaminate the sputum specimens. Specimens were processed and cultured using

Lowenstein-Jensen (LJ) media (WHO, 1998). For details, see chapter 5 Materials & methods part, Isolation, and Identification of *Mycobacterium tuberculosis* section.

4.3.3 Drug susceptibility testing

Drug susceptibility testing (DST) for first-line anti-TB drugs including isoniazid (INH), rifampicin (RMP), streptomycin (STM), ethambutol (EMB) and pyrazinamide (PZA) were performed by BACTEC™ MGIT 960™ TB System (Becton, Dickinson and Company, USA)(Rusch-Gerdes, 2006).

Drug Susceptibility Testing for Streptomycin, Isoniazid, Rifampin, and Ethambutol

Five BACTEC MGIT 960 tubes (7ml of modified 7H9 broth base) were labeled for each test culture. One was labeled as GC (growth control, without drug), one as STM, one as INH, one as RMP, and one as EMB. Aseptically 0.8 ml of BACTEC 960 SIRE Supplement was added to each of the MGIT tubes. Each concentration drug vial (332 µg for STM, 33.2 µg for INH, 332 µg for RIF, 1660 µg for EMB) was reconstituted with 4ml of sterile deionized water, mixed thoroughly and dissolved completely. Aseptically, 100 µL STM drug was added in the STM labeled tube. Similarly using a separate micropipette, other drugs were added in the other labeled tubes.

Aseptically 0.5 ml of the well-mixed two days positive culture suspension was added into each of the drug -containing tubes using a pipette. For the control, 0.1 ml of the test culture suspension was added to 10.0 ml of sterile saline (1:100 dilutions) and mixed well by inverting the tube 5-6 times. About 0.5 ml of this diluted suspension was added into the growth control tube. The caps were tightened and the inoculated broths were well mixed by gently inverting the tube several times. The final critical concentration of drugs in the medium was 1.0 µg/ml of medium for

STM, 0.1 µg/ml of medium for INH, 1.0 µg/ml of medium for RMP, and 5.0 µg/ml of medium for EMB.

The inoculated tubes were placed in the correct sequence in the set carrier (GC, STM, INH, RMP, EMB). The susceptibility set carrier was entered into the BACTEC MGIT 960 instrument using the susceptibility test set entry feature. The instrument interpreted results at the time when the growth unit (GU) in growth control reaches 400 (within 4 -13 days). At this point, the GU values of the drug vial were evaluated. It was assigned as susceptible (S) when the GU of the drug tube is less than 100; resistant (R) when the GU of the drug tube is 100 or more; indeterminate (X) when certain conditions occur which may affect the test result.

The purity of the inoculum was checked by streaking the test culture suspension onto a blood agar plate incubated at 35 ºC for 48 hours. Quality control was done using *M. tuberculosis* H37Rv (ATCC [American Type Culture Collection] number 27294) as a QC strain which is susceptible to all anti-tuberculosis drugs.

Drug Susceptibility Testing for Pyrazinamide

Two MGIT PZA tubes (7ml modified Middlebrook 7H9 broth adjusted to pH 5.9) were labeled, one as GC (growth control) and one as PZA drug. Aseptically 0.8 ml of PZA supplement was added to each of the two tubes using a pipette. The PZA drug vial (20,000 µg of lyophilized PZA) was reconstituted with 2.5 ml of sterile deionized water. Aseptically 100 µL of the reconstituted drug was added into the PZA tube. Aseptically, 0.5 ml of two days positive culture suspension was inoculated into the PZA tube using a sterile pipette. The concentration of PZA

per ml of the medium was 100 µg. For growth control inoculation, the inoculum used for the drug tube was diluted by adding 0.5 ml of the culture suspension to 4.5 ml of sterile saline (1:10 dilution) and mixed well by inverting the tube 5-6 times. Aseptically 0.5ml of the diluted suspension was inoculated into the tube labeled GC. The caps were tightened and the tubes were mixed by gently invert it several times. The GC was placed first and PZA second, in a two AST Set Carrier. The PZA set was entered into the instrument using AST set entry feature. PZA was selected as the drug in 2nd tube AST set carrier. Quality control and instrument interpretation are the same as described for other first-line drugs.

DST for Second Line Anti-TB Drugs

DST for second line drugs including fluoroquinolones (FLQ) (ofloxacin & moxifloxacin) and aminoglycocides (AM)/cyclic peptides (CM) (capreomycin, viomicin/kanamycin and amikacin) were performed using DNA hybridization technology on nitrocellulose strips (GenoType® MTBDR*sl*; Hain Lifescience, Nehren, Germany). Strips were interpreted according to the manufacturer's instructions. For each gene, the test evaluates the presence of wild-type (WT) and/or mutant (MUT) sequences, thus covering all high-confidence resistance mutations. For *gyrA,* these are 90Val, 91Pro, and the codon 94 mutations Ala, Asn, Gly, His, and Tyr. When all the WT probes of a specific gene appear as bands on the strip, there is no detectable mutation within the region examined and the strain is considered sensitive to the corresponding drugs. In the case of a mutation, the amplicon cannot bind to the corresponding WT probe, but it may bind to one of the MUT probes provided this specific mutation is represented on the strip. The absence of a WT band or appearance of a MUT band at least as strong as the amplification control band must be interpreted as resistance to the respective drugs.

For quality control, each test batch included a known pan-susceptible TB strain (H37Rv). Negative controls (water) were included during all steps of the procedure.

4.3.4 Genotyping

The isolates were classified into main phylogenetic lineages by using spoligotyping and linages were assigned according to revised signatures provided in SITVITWEB. For details, see chapter 5 Materials & methods part Spoligotyping section.

4.3.5 Statistical analysis

All data were entered and analyzed using SPSS statistical package software (SPSS Inc., Chicago, IL). Categorical data were compared by the chi-square test or the fisher exact test when expected cell sizes (n) were smaller than 5. In order to determine independent risk factors, odds ratios (OR) and 95% confidence intervals (CI) were calculated for demographic (gender, age), epidemiologic (previous treatment and HIV status), and infection by *Mycobacterium tuberculosis* lineages.

4.4 Results

4.4.1 Demographic characteristics of the study subject

A total of 153 *M. tuberculosis* isolates from MDR suspected groups (Retreatment, history of contact with MDR patient, and HIV patients) pulmonary TB patients were included for fist-line and second-line anti-TB drug susceptibility testing. The mean age ± the standard deviation of the study subjects were 32 ± 11.1. The majority (39%) of the patients were in the 26-35 year age group, followed by 16-25 year age group (32%), 36-45 year age group (15%), ≥ 45 year age group (12%), and 1 patient was 14 years age (1%). Among the study subjects, 29% were HIV co-infected, 77% previously treated, and 52% were male (Table 4.1).

4.4.2 First-line anti-tuberculosis drugs resistance

Among the 137 *M. tuberculosis* isolates, 83 (70%) isolates were resistance to at least one first-line anti-TB drug, 83 (61%) were MDR isolates, and 27 (19%) were resistant to all first-line anti-TB drugs. Large proportions of the MDR-TB isolates (32.5%) were resistant to all five first –line drugs tested. The highest proportion of poly resistance was observed in INH+STM combination (1.5%). Any resistance to INH, RMP, STM, EMB, and PZA was 69%, 61%, 58%, 26%, and 55% respectively. Four isolates (2.9%) were resistant to only a single drug (INH & PZA). Monoresistance to RMP, STM, and EMB was not observed in any isolates (Table 4.1).

Among new cases, resistance to at least one first-line anti-TB drug was observed in 19 (68%) isolates, MDR-TB was detected in 15 (58%) isolates, and 5 (18%) isolates were resistant to all first-line drugs. Monoresistance to first line drug was not observed in any isolates (Table 4.2).

Among previously treated cases, resistance to at least one first-line anti-TB drug was observed in 64 (70%) isolates, MDR-TB was detected in 55 (60%) isolates, and 20 (22%) isolates were resistant to all first-line drugs. Monoresistance to INH in 3 (2%) isolates, and PZA in 1 (1%) isolate was observed. It was not observed RMP, STM, and EMB monoresistance (Table 4.2).

Table 4.1 Characteristics of the study subjects in Addis Ababa, Ethiopia

Characteristics		Number (%)
HIV status	Negative	55 (36)
	Positive	45(29)
	Unknown	53 (35)
Treatment group	New	31(23)
	Retreatment	103 (77)
Sex	Male	73 (52)
	Female	68 (48)
Age groups, years	≤15	1(1)
	16-25	45(32)
	26-35	55(39)
	36-45	22(16)
	≥45	17 (12)

The majority (42%) of MDR isolates was T3_ETH linage and followed by CAS1_KILI (32%), CAS1_DELIHI (9%), T1 (7%), and H3 (1%). T3_ETH linage (59%) dominate isolates among resistance to all first-line drug and followed by CAS1_KILI (23%), T1 (9%), and CAS1_DELHI (5%) (Table 4.3).

Table 4.2 Pattern of resistance to anti-tuberculosis drugs in new and previously treated tuberculosis cases

Drug resistance	New cases (%)	Previously treated cases (%)	Total (%)
Resistance to any drug	19(23)	64(77)	83 (70)
Any resistance :			
INH	16 (17)	78 (83)	94 (69)
RMP	15 (18)	68 (82)	83 (61)
STM	13 (16)	66(84)	79 (58)
EMB	6 (17)	29 (83)	35 (26)
PZA	13 (17)	63 (83)	76 (55)
Monoresistance :			
INH	-	3	3 (2)
RMP, STM, EMB	-	-	-
PZA	-	1	1 (1)
Only two drugs resistance:			
INH+RMP	2	0	2(2)
INH+STM	-	2	2(2)
INH+EMB	-	-	
Only three drugs resistance:			
INH+STM+EMB	-	-	
INH+STM+PZA	-	1	1(1)
INH+EMB+PZA	-	-	
INH+RMP+PZA	1	3	4(3)
Only four drugs resistance:			
INH+RMP+STM+EMB	1	1	2(2)
Five drugs:			
INH+RMP+STM+EMB+PZA	5(19)	22(81)	27 (19)
Pre-XDR		4(100)	4(3)

N= number, INH=Isoniazid, RMP=Rifampicin, STM= Streptomycin, EMB= Ethambutol, PZA= Pyrazinamide

4.4.3 Second-line anti-tuberculosis drugs resistance

Among 137 isolates tested for second-line anti-TB drugs, four were resistance to FLQs (2.9%) and the remaining isolates were susceptible to second-line anti-TB drugs tested. The 4 FLQs resistance isolates were retreatment cases, resistance to INH and RMP (Pre-XDR), and belonging to T3_ETH linage. The MIRU-VNTR analysis indicated that one of the isolates is unique. They were from 23 to 35 year age and 3 were female. Their houses were located in different (Addis Ketema, Akaki Kality, Arada, and Bole) sub city. Information derived from this study was shared with clinicians and public health authorities.

4.4.4 Risk factors associated with drug resistance

In a univariate analysis of the possible association between MDR and demographic, epidemiological and microbiological parameters, only CAS1_KILI linage was associated with MDR (odds ratio 5.4, P=0.003) and resistance to one or more anti-tuberculosis drugs (odds ratio 26, $p < 0.001$)(Table 4.4). A significant risk of resistance to Ethambutol (odds ratio 5.1, $p <0.001$) was observed among patients with T3_ETH linage compared to patients with non T3_ETH linage. CAS1_KILI was identified as a risk factor for Streptomycin resistant (odds ratio 8.5, $p < 0.001$), Isoniazid resistant (odds ratio 8, $p= 0.001$), Rifampicin resistant (odds ratio 5.4, $p= 0.001$), and Pyrazinamide resistant (odds ratio 4.5, $p= 0.006$) (Table 4.5). The age group, sex, HIV co-infection and previously treated cases were not significantly associated with resistance.

Table 4.3 Anti-TB drug resistance stratified for *Mycobacterium tuberculosis* linages

Linages(N)	Anti-TB drugs resistance							
	INH N(%)	RMP N(%)	STM N(%)	EMB N(%)	PZA N(%)	MDR N(%)	RFLD N(%)	Pre-XDR N(%)
CAS (2)	1(50)	0	0	0	0	0	0	0
CAS1_DELHI (19)	10(53)	10(53)	10(53)	1(5)	7(39)	10(53)	1(5)	0
CASI_KILI (28)	26(93)	24(86)	25(89)	5(18)	21(75)	24 (86)	5(18)	0
H1(1)	0	0	0	0	0	0	0	0
H3(4)	1(25)	1(25)	2(50)	0	1(25)	1(25)	0	0
LAM9(1)	0	0	0	0	0	0	0	0
T1(6)	5(83)	5(83)	3(50)	2(33)	5(83)	5(83)	2(33)	0
T2(3)	1(33)	0	0	0	0	0	0	0
T3(1)	0	0	0	0	0	0	0	0
T3_ETH (49)	37(76)	32(65)	29(59)	20(41)	31(63)	32(65)	13(27)	4(8)
T5 (1)	0	0	0	0	0	0	0	0
Orphan (8)	5(63)	3(38)	4(50)	1(13)	3(38)	4(50)	1(13)	0

N= number, INH=isoniazid, RMP=rifampicin, STM= streptomycin, EMB= Ethambutol, PZA= pyrazinamide, MDR= Multidrug resistant, RFLD= Resistant to all first line anti-TB drugs, XDR= extended drug resistance

Table 4.4 Risk factors for *Mycobacterium tuberculosis* resistance to first-line drugs in Addis Ababa, Ethiopia

Characteristic		Susceptible to all first line drugs N (%)	Resistance to one or more drugs N (%)	OR(95CI)	p- value	MDR-TB N (%)	OR(95CI)	p- value
Gender	Male	21(33)	42(67)	0.7(0.3-1.6)	0.583	37(58)	0.8(0.4-1.6)	0.198
	Female	16(27)	43(73)	1		38(63)	1	
Age groups	≤15	0	1(100)	-		1(100)		
	16-25	11(28)	28(72)	1.2(0.5-2.7)	0.890	24(62)	1.1(0.5-2.3)	1.000
	26-35	17(35)	32(65)	0.7(0.3-1.6)	0.510	29(59)	0.9(0.4-1.9)	0.959
	36-45	5(29)	12(71)	1.1 (0.3-3.2)	1.000	10(56)	0.8(0.3-2.2)	0.840
	≥45	4(25)	12(75)	1.4(0.4-4.5)	0.613	11(65)	1.2(0.4-3.6)	0.907
Previous TB	Yes	28(31)	62(69)	1.2(0.5-3.0)	0.921	55(60)	1.1(0.5-2.6)	1.000
treatment	No	9(35)	17(65)	1		15(58)	1	
HIV status	Positive	15(41)	22(60)	0.5(0.2-1.2)	0.176	18(46)	0.4(0.2-1.0)	0.075
	Negative	12(26)	37(76)	1		33(67)	1	

Mycobacterium tuberculosis linage							
CAS	1(50)	1(50)	0.4(0.02-6.7)	1	-		
CAS1_DELHI	8(44)	10(56)	0.5(0.2-1.3)	0.211	10(53)	0.7(0.3-1.9)	0.647
CAS1_KILI	1(4)	26(96)	14.9(1.9-114.7)	<0.001	24(86)	5.4 (1.8-16.8)	0.003
H1	1	0			-		
H3	2(50)	2(50)	0.4(0.1-3.0)	0.376	1(25)	0.2(0.02-2.1)	0.148
H4	1	0			-		
LAM9	1	0			-		
T1	1(20)	4(80)	1.7(0.2-15.6)	0.630	4(80)	2.8(0.3-25.5)	0.330
T2	2(67)	1(33)	0.2(0.02-2.3)	0.175	-		
T3	1	0			-		
T3_ETH	12(25)	37(75)	1.5(0.7-3.3)	0.456	32(65)	1.5(0.7-3)	0.432
T5	1	0			-		

N=number, OR= odds ratio, CI=confidence interval

Table 4:5 *Mycobacterium tuberculosis* T3_ETH and CAS1_KILI linages and its association with anti-tuberculosis drug resistance

Drug resistance		T3_ETH N (%)	OR (95CI)	P-value	CAS1_KILI N (%)	OR (95CI)	P-value
Streptomycin	Resistant	29(40)	25(34)	1.00	25(34)	8.5(2.4-30.1)	<0.001
	Sensitive	20(39)	3(6)		3(6)	1	
Isoniazid	Resistant	37(43)	26(30)	0.270	26(30)	8(1.8-35.8)	0.001
	Sensitive	12(31)	2(5)		2(5)	1	
Rifampicin	Resistant	32(43)	24(32)	0.432	24(32)	5.4 (1.8-16.8)	0.001
	Sensitive	17(34)	4(8)		4(8)	1	
Ethambutol	Resistant	20(69)	5(17)	<0.001	5(17)	0.7(0.2-1.9)	0.437
	Sensitive	29(30)	23(24)		23(24)	1	
Pyrazinamide	Resistant	31(45)	21(30)	0.512	21(30)	4.5(1.6-12.8)	0.006
	Sensitive	21(38)	5(9)		5(9)	1	
Resistant to all FLD	yes	13(59)	5(23)	.089	5(23)	1.1(0.4-3.2)	0.916
	No	39(37)	23(22)		23(22)	1	

N=number, OR=odds ratio, CI= confidence interval, FLD= first-line anti-TB drugs

4.5 Discussion

The global control of tuberculosis remains a challenge from the standpoint of anti-TB drug resistance. Our study aimed at determining the level and risk factors associated with first and second-line anti-TB drug resistance among high MDR risk group in Addis Ababa, Ethiopia. The strains were grown from sputa from smear positive pulmonary tuberculosis patients who were referred to Addis Ababa regional laboratory (AARL) for drug susceptibility testing (DST). HIV positive presumptive new TB cases, presumptive New TB cases who have known contacts of MDR-TB patients, and all retreatment cases were eligible for DST service at AARL. The present study subjects could be classified as a high MDR risk group.

The overall rates of MDR-TB observed in our study were 61%. The anti-tuberculosis drug resistance survey in Ethiopia had shown MDR-TB rate of 2.7 % among new cases and 17.9% of previously treated cases(EPHI, 2014). A report from Addis Ababa had shown a lower rate of MDR-TB, 43 % (Agonafir *et al.*, 2010) and 46.3% (Abate *et al.*, 2012) among TB patients referred to the St Peter TB specialized hospital in Ethiopia for treatment and DST due to suspicion of MDR-TB. A higher level of MDR-TB among new cases in our study as compared to the previous study in a St peter TB specialized hospital may reflect an increasing trend of MDR-TB, and the control measures in place to prevent the development and the transmission of drug –resistant TB are insufficient; the study subject of our cases and the two papers were almost similar (high MDR risk group). Other studies in Addis Ababa also showed lower level of MDR-TB among new cases: 0.6% in 1996/97(Demissie *et al.*, 1997), and 0.6% in 2005 (Asmamaw *et al.*, 2008) among all cases. In other part of the country, studies had shown that MDR-TB prevalence was up to 3.7% in new cases, and 32.8% in previously treated cases(Abdella *et al.*,

2015; Asmamaw *et al.*, 2008; Tessema *et al.*, 2012). The difference in the rate of MDR-TB between our study and other reports in the country might be due to the difference in study subjects. In our study new cases were HIV positive or who are known contact of MDR-TB patients and referred to the laboratory due to suspicion of MDR.

In this study, 83 (70%) of the cases were resistant to at least one or more first-line anti-TB drugs and our results is comparable to the rate reported from St. Peter's TB specialized hospital in Ethiopia 72.9%(Abate *et al.*, 2012). In other studies in Ethiopia, a lower rate of resistance was also reported in ranges from 15% to54%, and the figures vary from place to place (Abate *et al.*, 1998; Asmamaw *et al.*, 2008; Demissie *et al.*, 1997; Meskel *et al.*, 2008; Tessema *et al.*, 2012). The discrepancy of the result between our finding and other studies may reflect variation in the study population. Resistant to all of the five first- line anti-TB drugs (INH, STM, RMP, EMB &PZA) were observed at a rate of 19%, our finding is lower than a rate reported in studies from St. Peter's TB specialized hospital 89% (Agonafir *et al.*, 2010) and 37.2% (Abate *et al.*, 2012).

Our finding indicated that single-drug resistance to INH was the highest (69%) and resistance to EMB was the lowest (26%) among first-line drug. In other studies in Ethiopia resistance to INH was 11.9% in 1984 (Lemma *et al.*, 1984), 14.8% in 1986(Wolde *et al.*, 1986), 8.4% in 1994(Demissie *et al.*, 1997) ,56% in 2004-2008 (Abate *et al.*, 2012) reports, and lower than resistance to streptomycin (Abate *et al.*, 2012; Demissie *et al.*, 1997). An increase in the proportion of INH resistance was also reported in a study in Addis Ababa(Abate *et al.*, 2012). An increase in the proportion of INH resistance could be seriously considered since this drug is the core component of the standard and short-course chemotherapy regimens. In 1994 primary

resistance to EMB was not observed in the city (Demissie *et al.*, 1997). Other study conducted in Addis Ababa (2011) reported that EMB resistance was 43.5% (Abate *et al.*, 2012). The prevalence of PZA resistance among our study subjects (high MDR risk group) were 55%. Pooled PZA resistance prevalence estimate was 16.2% among all TB cases, 41.3% among patients at high MDR-TB risk, and 60.5% among MDR-TB cases(Michael G. Whitfield, 2015).

The highest rate of monoresistance observed in our study was associated with INH (2%) among previously treated cases. INH monoresistance was not observed among new cases. The highest rate of monoresistance associated with INH was reported among new cases in a range of 2.3%-2.8%(Agonafir *et al.*, 2010; Tessema *et al.*, 2012). In contrast to our finding, monoresistance to streptomycin was found to be in the highest proportion (14.8%) followed by INH monoresistance, 2.9%(Abate *et al.*, 2012) in a study conducted in Addis Ababa. Monoresistance to PZA was only observed in 1(1%) isolates from previously treated cases, and monoresistance to RMP, STM, and EMB was not observed in our study. Other studies in Ethiopia also reported an absence of monoresistance to RMP(Abate *et al.*, 2012) and EMB (Abate *et al.*, 2012; Agonafir *et al.*, 2010), or low levels of monoresistance to RMP and EMB (Abate *et al.*, 2012).

Among second-line anti-TB DST, only resistance to the fluoroquinolone was detected in four (2.9%) isolates, lower than a rate reported in Addis Ababa 17.4%(Agonafir *et al.*, 2010). The patients were not exposed to the second-line anti-TB treatment. The explanation could be due to the use of fluoroquinolone for the treatment of other bacterial infection which may select for resistance isolates of *M. tuberculosis*. In the present study, the isolates were susceptible to the remaining second-line anti-TB drug tested, indicative of the low use of the drugs in Addis

Ababa. Studies of second line DST in another part of the country indicated a varied proportion of resistant(Bedru Hussein, 2013). In our study, pre-XDR TB was observed in four isolates (2.9%) and XDR-TB was not detected, Two of the pre-XDR isolates were resistant to STM, ETM, and susceptible to PZA. The other two of the pre -XDR isolates were susceptible to EMB, resistant to PZA, and varies in resistant to STM. In another study, XDR-TB was reported in two isolates (4.4%) in Addis Ababa (Agonafir *et al.*, 2010) and reported in one isolate (1%) in other part of the country (Bedru Hussein, 2013).

This study showed a significant association between infection with isolates of the CAS1_KILI lineage and multi-drug resistance, resistance to at least one or more first-line anti-TB drugs, and resistance to each INH, RMP, STM, and PZA. A significant association to EMB was observed among patient with T3_ETH linage. A previous study from the northwest part of Ethiopia showed that Haarlem linage has a significant association with multi-drug resistance, resistance to all first-line anti-TB drugs and resistance to each first line anti-TBs including INH, RMP, STM, EMB and PZA(Tessema *et al.*, 2012). Another study from the southwest part of the central Ethiopia reported that there was no association between linage and drug resistance(Bedewi *et al.*, 2017).These indicated that the dominant strain among drug resistance is not the same across the country.

In the present study, age, sex, HIV status, history of the previous treatment of the patients was not significantly associated with resistance to anti-TB drugs. Similar data were reported in Addis Ababa: HIV, age, and sex were not associated with anti-TB drug resistance excluding history of previous retreatment (Abate *et al.*, 2012; Abebe *et al.*, 2012; Maru *et al.*, 2015; Mitike *et al.*,

1997). In contrary to our result, HIV (Yimer *et al.*, 2012), age group(Adane *et al.*, 2015) and being male (Abate *et al.*, 2012; Biadglegne *et al.*, 2013)were reported as a risk factor for MDR-TB in the country. In Ethiopia, studies reported that previous treatment history was highly associated with resistance to anti-TB drug resistance (Abate *et al.*, 2012; Agonafir *et al.*, 2010; Ahmed Esmael, 2014; Berhan *et al.*, 2013; Hirpa *et al.*, 2013; Tessema *et al.*, 2012). The difference between our result and other studies might be explained by the difference in study subject among new cases. In our cases, new cases were among high MDR-TB risk groups. The new cases in this study had history of contact with MDR-TB patient or were HIV positive who frequently visit health facility, and our finding (similar result among new and retreatment cases) might suggest that primary infection with a drug-resistant strain rather than acquired during a previous treatment episode is now driving the spread of resistance among the previously treated and new cases. In Ethiopia, drug side effects during treatment, treatment not directly observed by a health worker, interruption of treatment of at least a day, duration of treatment between 2 and 7 months, and treatment with a category II regimen were reported as a risk factors for MDR-TB (Hirpa *et al.*, 2013).

The study has its own limitation. There is a potential bias in estimation of drug resistance in new and previously treated cases. It may be subjected to selection bias.

4.6 Conclusions

The remarkable percentage of drug resistance against first-line anti-TB drugs indicated inefficiency in TB control program which has led to accumulation and transmission of resistant isolates. This led to the use of less effective, more toxic and more costly second-line anti-tuberculosis drugs. This suggests that strengthen TB control program and strengthens diagnostic facilities of first- and second-line DST for early case detection and treatment of MDR-TB in Addis Ababa, Ethiopia is significant. Improved infection control measures need to be implemented.

Chapter 5. Molecular Epidemiology of *Mycobacterium tuberculosis* Isolates from Pulmonary Tuberculosis Patients in Addis Ababa, Ethiopia

5.1 Abstract

Background: Genotyping can augment classic epidemiological methods for detecting TB outbreaks. The objective of this study was to identify recent transmission of tuberculosis and responsible strains, and factors associated with the transmission in Addis Ababa, Ethiopia.

Methods: IS6110 Restriction Fragment Length Polymorphism (RFLPs), spoligotyping, and mycobacterial interspersed repetitive unit - variable number tandem repeat 24-loci typing (MIRU-VNTR)were used to analyze DNA fingerprinting and perform cluster analysis of 148 *Mycobacterium tuberculosis* isolates collected from pulmonary tuberculosis patient lives in Addis Ababa, Ethiopia.

Results: About 119 genetic profiles and 15 clusters were observed. T3_Eth International family constitutes the most predominant followed by the CAS1_KILI and CAS1_DELHI. Other International families found were CAS, H1, H3, H4, LAM9, T1, T2, T3, and T5. Some isolates were designated as an orphan. The predominantly clustered isolates were 3 bands. Clustering among 4 spoligotypes T3_ETH (41.8%), CAS1_KILI (29.4%), CAS_DELHI (10.5%), and T1(33.3%) were observed with the overall clustering rate of 27.3%. The linage T3_ETH, <5 IS6110 RFLP bands, MDR, resistance to one or more anti-tuberculosis drugs, and drug-resistant to INH, RMP, STM, and PZA was significantly associated with clustering.

Conclusions: High clustering rate and recent transmission index indicated the presence of recent drug- resistant TB transmission in Addis Ababa, Ethiopia. T3_ETH and CAS1_KILI was the predominant linage.

5.2 Introduction

Tuberculosis (TB) is the second leading cause of death from infections worldwide, causing illness, interference with education and employment, and premature death. The Federal Democratic Republic of Ethiopia is the tenth largest country in Africa with an estimated population of 103 million(PMC, 2017). Incidence of all forms of TB in Ethiopia was $192/10^5$population (~191,000 cases) and annual mortality is 4 $/10^5$ (~3900 cases)(WHO, 2016). A total of 9352 TB cases were also reported in Addis Ababa in the year 2016 (AAHB, 2016; PMC, 2017) . The prevalence of latent TB adults (averaged of 21 years) in Addis Ababa is about 47% by TST (≥10 mm) and 44% by Quantiferon^{TM} interferon release assay with high agreement between the methods (Dagnew *et al.*, 2012). Thus, TB latency, reactivation and transmission are very common in Ethiopia as in the remainder of sub- Saharan Africa and other developing countries. In 2016, about 8% of all new TB cases are HIV co- infected(WHO, 2016). The HIV/TB co- infection rate in Addis Ababa city is 25% (AAHB, 2016).

Multidrug-resistant tuberculosis (MDR-TB) is a threat to global TB control and is of major public health concern worldwide. Ethiopia is one of the 30 countries with a highest MDR-TB burden. Ethiopia had an estimated 3300 (range 2100–4600) MDR-TB cases in 2015 (WHO, 2016), and has had a small number of cases of extensively drug-resistant TB (XDR-TB)(Agonafir *et al.*, 2010). The programmatic response to MDR-TB in Ethiopia began in 2009, when the Green Light Committee (GLC) of the World Health Organization (WHO) approved a pilot program to treat 45 MDR-TB patients at St Peter's Specialized TB Hospital in Addis Ababa.

Aerosol transmission of TB and other respiratory infections leads to clustering of cases (outbreaks) (Weinstock, 1981). Identifying clusters and associated *M. tuberculosis* strain

lineages can be useful in investigating virulence, transmissibility and drug resistance (Kulldorff *et al.,* 2005). Genotyping can augment classic epidemiological methods for detecting TB outbreaks. Integration of genotyping with demographic, epidemiologic, and microbiologic data can reveal risk groups. DNA typing of *M. tuberculosis* is an efficient tool to reveal transmission links among TB cases (van Embden *et al.*, 1993). Thus, in developing countries such as Ethiopia with high levels of endemic TB, effective investigation of outbreaks should be substantially facilitated by strain typing. Only a few molecular epidemiological studies have been done in Addis Ababa city. More data are, therefore, needed to better understand tuberculosis transmission dynamics in the city.

For molecular typing of *M. tuberculosis*, the gold standard is IS*6110* restriction fragment length polymorphism (RFLP) (van Embden *et al.*, 1993). However, IS*6110* RFLP is relatively slow and discriminates poorly among isolates containing similar fragments sizes. Some TB strains possess low IS*6110* copy numbers and therefore cannot be adequately discriminated by this method(Ei, 2016; Kamerbeek J, 1997). However, similar discriminatory power and greater convenience have been demonstrated with other methods such as spacer oligonucleotide typing (spoligotyping), which detects 43 spacer sequences interspersed with direct repeats (DRs) in the genomic region uniquely present in members of *M. tuberculosis* complex (Kamerbeek J, 1997). Mycobacterial interspersed repetitive unit - variable number tandem repeat (MIRU-VNTR)) typing is an alternative to IS*6110* RFLP typing. Among more than 40 MIRU-VNTR loci scattered on the *M. tuberculosis* chromosome, 15 and 24 MIRU-VNTR loci have been proposed as the international standard(Iwamoto *et al.*, 2007).

In this study, we used a combination of MIRU-VNTR 24-loci typing, spoligotyping, and IS*6110*RFLP for higher resolution of *DNA* fingerprinting to investigate *Mycobacterium*

tuberculosis strains isolated from patients living in Addis Ababa. The objectives of this study were to identify recent transmission of tuberculosis and responsible strains, and factors associated with the transmission (predominant strains, age, HIV status and history of previous treatment) in Addis Ababa, Ethiopia.

5.3 Materials and methods

5.3.1 Study subject, area and study period

The Addis Ababa Health Research Laboratory (AARL) provides TB drug susceptibility testing using Xpert for 120 public and private TB clinics in a city of 3.5 M persons. A total of 212 isolates were included in this study. These strains were grown from sputa from smear positive pulmonary tuberculosis patients who were referred to the lab for drug susceptibility testing (DST) between April 2013 and July 2015. For the study subjects, socio-demographic data, history of previous tuberculosis treatment, and HIV status was collected. The sputa were stored and transported to Armauer Hansen Research Institute (AHRI) for culture. Molecular typing was done at Molecular Epidemiology laboratory, Division of Molecular Biology & Human Genetics, Stellenbosch University, South Africa. The study was approved by Addis Ababa City Government Health Bureau ethics committee.

Specimens were kept at -20^{o}c in the AAHRL laboratory, and transported in cold box within 5 days to the mycobacteriology laboratory at AHRI for culture.

5.3.2 Isolation and Identification of Mycobacterium tuberculosis

Culture methods

Specimens were processed and cultured using Lowenstein-Jensen (LJ) media (WHO, 1998).

Preparation of LJ media

Ingredients were consists of mineral salt solution, malachite green solution and homogenized eggs. The mineral salt is composed of Potassium dihydrogen phosphate anhydrous (KH_2PO_4) 2.4g, Magnesium sulfate ($MgSO_4$. $7H_2O$) 0.24g, Magnesium citrate 0.6g, Asparagine 3.6g, Glycerol (reagent grade) 12ml and Distilled water 600ml. The ingredients were dissolved in the distilled water by heating and autoclaved at 121°C for 30 minutes. This solution was cooled to room temperature and stored in suitable amounts in the refrigerator for use. Malachite green solution, 2% were made from Malachite green dye 2.0g and sterile distilled water 100ml. Using aseptic techniques the dye was dissolved in sterile distilled water by placing the solution in the incubator for 1-2 hours. The fresh solution was used.

The homogenized whole eggs were prepared as follows. Fresh hens' eggs, not more than seven days old, were cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Eggs were soaked for 30 minutes in the soap solution. Eggs were then be rinsed thoroughly in running water and soaked them in 70% ethanol for 15 minutes. Finally, the eggs were cracked with a sterile knife into a sterile flask and mixed with a sterile blender.

Mineral salt solution 600ml, Malachite green solution 20ml, Homogenized eggs (20-25 eggs, depending on size) 1000ml were aseptically pooled in a large, sterile flask and mixed well to prepare the complete medium. The complete egg medium was distributed in 7ml volumes in

sterile 14ml culture bottles. The medium was inspissated within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Before loading, the inspissator was heated to 80°C to quicken the build-up of the temperature. The culture bottles were placed in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C. After inspissation, the whole media batch was incubated at 35°-37°C for 24 hours as a check of sterility. The LJ medium was dated and stored in the refrigerator. For optimal isolation from specimens, LJ medium was not older than 4 weeks.

5.3.3 Specimen processing, inoculation, and incubation

N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC-NaOH) METHOD (WHO, 1998) was used to digest and decontaminate the sputum specimens. A fresh 4% NaOH solution was prepared by adding 40% NaOH to sterile distilled water in the proportion 1:10. Then equal proportion of the decontaminant along with 5g of NALC per 100 ml of NAOH solution was then added into equal ml of fresh sputum in a sterile tube which was tightly closed and shaken to digest inner contents which was allowed to stand for 15min at room temperature with occasional shaking. Afterward, the sample was centrifuged at 3000 x g for 15min which separate the supernatant from sediment. Forty milliliters of phosphate buffer (pH =6.8) was added to neutralize the Na OH and this was centrifuged for 15 minutes at 3000 rpm. After decanting the supernatant, a few drops of phosphate buffer (pH =6.8) was added to reconstitute the sediment. Finally, an aliquot of 50 µl of the sample was then be inoculated into two bottles of Löwenstein- Jensen (LJ) medium. The inoculated media were then be incubated at 37 ^{o}C in a slanted position, with the screw caps slightly loosened to allow for the evaporation of the inoculum. After 24-48 hours, screw caps were tightened and tubes were further incubated. The growth of the bacteria was read every week

starting from the third week to the eight weeks. Isolates were identified by Xpert® MTB assay following the manufacturer's instructions.

5.3.4 Storage of strain

For storage of Mycobacterium strains cryovial tubes, with a screw cap and a content of about 2ml was used. Three- gram Tryptone Soya Broth (Oxoid, Basingstoke, UK), 20ml glycerol and 80 mL of distilled water were mixed and used as storage medium. It was autoclaved at 120^{O}C for 15 min and stored at 4^{O}C until use. Two loops full of strain was transferred into two cryovials and stored at -70^{O}C in Armauer Hansen Research Institute (AHRI) laboratory until further use(Kristin Kremer, 2001).

5.3.5 Extraction of Genomic DNA

DNA was extracted as described by Ausubel et al. (Ausubel, 1988). DNA was extracted from all isolates by heating *Mycobacterium tuberculosis* cultures in a petri-dish at 80°C for 2h which obtained from 7H10 media. The scraped of bacteria were suspended in 3ml extraction buffer (monosodium glutamate, Tris-HCl & EDTA) in a sterile 50-mL polypropylene tube which contained 20 x 5 mm glass beads (enough to fill the bottom part of the tube). The bacterial clumps were disrupted by vigorous vortexing for approximately 2 min. Five hundred microliter of lysozyme (100µg/mL) and 2.5 µL of RNAse A (10mg/mL) were added to the tube. The contents of the tube were mixed by gentle inversion and then incubated at 37 °C for 2 hours. After incubation, 700 µL of 10×Proteinase K buffer and 300 µL of Proteinase K (10g/mL) were added. The sample was gently mixed by inverting the tube a few times and then incubated overnight at 45 °C.

Proteins were removed by adding phenol/chloroform/ isoamyl alcohol (25:24:1) and repeatedly gently mixed by inversion every 15min for 2h at room temperature. Phase separation was done

by centrifuge each sample at 3000rpm for 20min at room temperature. The top phase was aspirated and transferred into new polypropylene tube containing 5mL of chloroform/ isoamyl alcohol (24:1), and centrifuge at 3000rpm for 20min at room temperature. The top phase was carefully aspirated and transferred to a new polypropylene tube containing 700 µl 3M sodium acetate pH 5.2. DNA was then precipitated with the addition of 7 mL of cold (-20 °C) isopropanol. The precipitated DNA was collected using a thin glass rod and washed with 1 mL of 70% ethanol for approximately 10 minutes in a 1.5mL tube. DNA was then air-dried overnight, rehydrated by adding 500 µL TE buffer (pH 8.0) and released from the glass rod by gentle mixing. The DNA was redissolved by incubating at 4°C overnight. DNA was stored at -20^{o}c until used.

5.3.6 IS6110 Restriction Fragment Polymorphism Analysis

IS6110 Restriction Fragment Length Polymorphism (RFLP) typing was conducted for 120 tuberculosis isolates as described by Warren and colleagues (Warren *et al.*, 2009). The DNA concentration was determined using spectrophotometry

PvuII Restriction Endonuclease Digestion

The extracted genomic DNA was restricted with *Pvu* II in a reaction mix of final volume 100µL consisting of 6 µg of genomic DNA, and 30 units of *Pvu* II in 10µL of 10x restriction buffer. The restriction mix was incubated for 16 hours in an oven at 37 °C. At the end of digestion, the reaction was incubated at 65 °C for 10 minutes to inactivate any remaining enzyme activity.

Gel Electrophoresis of Restricted DNA (Test Gel)

Completeness of restriction digestion and estimation of the amount of DNA present in each digestion reaction were checked. A 10 µL of an aliquot of the digestion reaction were transferred

to a 0.5ml tube. Four µL of 6x loading buffer were added and mixed by repeated pipetting. A 1% agarose gel was prepared by dissolving 3g of agarose in 300mL of 1x TBE (pH 8.3). The mix was gently heated in a microwave oven until all of the agaroses was dissolved and then caste into a 20cm x 25cm glass gel electrophoresis tray containing a gel comb. The comb was removed when the gel cool to room temperature.

The PvuII-digested DNA was separated by electrophoresis using 1% agarose gel in 1x TBE at 40 voltage for 4h. The gel was stained in 500mL of 1x TBE containing 50uL of a 10mg/mL ethidium bromide stock solution with shaking for 30 min at room temperature. The DNA was visualized at 245nm on a UV light box and the image was captured by photography. Digested DNA showed a smear with an evident banding pattern. The intensity of the digested DNA was used to estimate the amount of DNA present in each digestion reaction.

Ethanol precipitation before final gel

The remaining PvuII-digested DNA (90 µL) was precipitated by adding 10 µL 3M sodium acetate, pH 5.2 and 300 µL ice cold 100% ethanol. Mixed and incubated at -20°C for 16h. The PvuII-digested DNA was pelleted by centrifugation at 10 000 x g for 30 min at 4°C. After centrifugation, the supernatant was aspirated to 50ul without disturbing the DNA pellet. DNA pellet was washed with 500 µL ice cold 70% ethanol and centrifuge at 10 000 x g for 30 min at 4°C to remove salts. The supernatant was aspirated to 50ul without disturbing the DNA pellet. The DNA pellet was dried at room temperature for 16h.

Gel Electrophoresis of Restricted DNA (Final Gel)

DNA was re-dissolved in 1X loading buffer/Internal Molecular Weight Marker (IMWM) mix at 4^{o}C for 16h. The volume of 1X loading buffer/IMWM mix added was different according to the intensity of the DNA detected in Gel Electrophoresis of Restricted DNA (Test Gel). A 0.8% agarose gel was prepared by dissolving 2.4g of agarose in 300 ml 1x TBE (pH 8.3). DNA fragments were separated by adding 10 µL of *Pvu*II-digested DNA along with an external marker MTB14323 in the 0.8% agarose gel in 1x TBE (pH8.3) through electrophoresis at a constant voltage of 40 for 16h. The gel was stained in 500mL of 1x TBE containing 50uL of a 10mg/mL ethidium bromide stock solution with shaking for 30 min at room temperature. The bands were visualized at 245nm on a UV light box and the image was captured by photography.

Southern Blot Transfer of the Fingerprinting Gel

The inverted gel was placed into a plastic container. The gel was incubated in 500 mL denaturing buffer at 25^{o}C with gentle shaking for 30 min in order to denature the DNA. The denaturing solution was removed and the gel was neutralized by incubating the gel with 500 mL neutralizing buffer with gentle shaking at 25^{o}C for 30 min.

The nylon membrane (Hybond N+) was labeled with a black ballpoint pen to allow future recognition and spotted with 0.2 µL aliquots of orientation marker at six different positions on the upper and lower edges of the membrane to allow for future alignment of resultant autoradiographs. The membrane was hydrated briefly in H_20 and transferred and equilibrated in 20 X SSPE solutions.

The inverted agarose gel was placed onto the Whatman 3MM paper pre-soaked in 20X SSPE. The air bubbles were removed from under the gel by gently rolling a 10 mL pipette over the gel. Strips of parafilm were placed around the gel to ensure that the fluid flows through the gel during Southern transfer. The membrane was placed onto the agarose gel with orientation markers facing the agarose gel and air bubbles were removed by gently rolling with a 10 mL pipette over the membrane. Two layers of Whatman 3MM blotting paper (see Note 24) pre-wetted in 20X SSPE (pH 7.4) were placed onto the nylon membrane and again air bubbles were removed. To ensure fluid flow through the agarose gel, folded paper towels were stacked onto the Whatman 3MM papers and contact was ensured with the addition of a glass plate and 1 kg weight. The blotting tray was filled with 20X SSPE (pH7.4) and southern transfer proceeded for 16h.

After transfer, the nylon membrane was removed and washed in 2X SSPE for 10 min. The nylon membrane was baked at 80^{o}C for 2 h between 2 sheets of Whatman 3MM blotting paper to fix the DNA to the membrane. The membrane was sealed in a plastic sleeve and stored at 4^{o}C until further use.

Preparing the *IS6110* Probe by PCR Amplification

A PCR amplification mixture was prepared with 0.2 µg DNA template (*M.tuberculosis* DNA) , 5 µL Q-Solution, 2.5 µL 10X PCR Buffer , 2 µL 25 m*M* MgCl2 , 4 µL 10 m*M* dNTP's, 1 µL of each *IS6110* primer (50 pmol/ µL , 5 CGT GAG GGC ATC GAG GTG GC 3 and 5/ GCG TAG GCG TCG GTG ACA AA 3/), 0.125 µL HotStartTaq DNA polymerase and made up to 25 µL with sterile nuclease-free H2O. The amplification was initiated by incubating for 15 min at 95^{O}C. This was followed by 35-45 cycles of 94^{O}C for 1 min, 62^{O}C for 1 min, 72^{O}C for 1 min.

After completion of the last cycle, the sample was incubated at 72^{O}C for 10 min. To check for the efficiency of amplification, a 5 µL aliquot was electrophoretically fractionated in a 2% agarose gel (1 x TBE pH 8.3) and stained with ethidium bromide. The Wizard SV Gel and PCR Clean-up System was used to purify the *IS6110* amplification product as instructed by the manufacturer. The DNA was eluted in nuclease-free H_2O. The *IS6110* probe concentration was determined using spectrophotometrically and stored at -20^{O}C.

ECL Labeling of *IS6110* and Internal Marker Probes

Probe DNA (200ng, IS6110 DNA prepared by PCR amplification or Marker X) was added to a 0.5ml tube and made up to 15 µL with nuclease-free H_2O. It was incubated at a 100^{O}C for 5 min to denature the probe DNA. The denatured probe was snap cooled on ice (to 4^{O}C) for 5 min. 15 µL of the labeling mix (horseradish peroxidase, ECL^{tm} direct nucleic acid labeling and detection kit, Amersham, UK) was added to the probe and mixed well. Then 15 µL of glutaraldehyde solution was added and mixed well. The labeled probe was incubated for 10 min at 37^{O}C and used immediately.

Hybridization

The nylon membrane was rehydrated by incubating in 500 mL distilled water for 10 minutes and sealed it in a plastic bag (25 cm x 35 cm). ECLTM Gold Hybridization Buffer (48 ml) was added to the membrane in the plastic bag. All air bubbles were removed by rolling with a 10 mL pipette. The bag was then sealed with a heat sealer. The ECLTM Gold Hybridization Buffer was spread by rolling with 10 mL pipette thoroughly. It was pre-hybridized in a flat plastic container by incubating at 42 °C in a shaking water bath for 60 minutes at 90 rpm. To gently massage the

membrane while incubating in the shaking water bath, a plastic bag containing 300 mL of water was placed on top of the bag containing the membrane. To initiate hybridization, the plastic bag containing the membrane was removed from the water bath and the labeled IS6110 probe was added directly to the ECLTM Gold Hybridization Buffer. All air bubbles were removed using a 10 mL pipette and the bag was resealed. It was hybridized at 42 °C for 16 hours with shaking at 90 rpm. Again, the sealed bag of water was placed on top of the bag containing the membrane

Following hybridization, the nylon membrane was removed from the hybridization bag, placed in a clean, flat plastic container and washed by incubating the membrane in 400 mL the pre-warmed Primary Wash at 42 °C for 20 minutes at 90 rpm. The wash step was repeated with a second 400 mL of pre-warmed Primary Wash. The membrane was washed twice in 400 mL 2X SSC for 5 minutes at room temperature on the shaker.

Detection of Hybridization

The membrane was placed in a new plastic bag and all excess liquid was removed by rolling with a 10 mL pipette. Four mL of each Amershem ECLTM Detection Reagents were mixed in a McCartney bottle and the mixture was added to the membrane in the bag and the bag was sealed. The Detection Reagent was spread over the membrane continuously for 90 seconds by rolling with a 10 mL pipette. All Detection Reagent was removed by rolling out with a 10 mL pipette and the plastic bag was sealed. All air bubbles were removed. The plastic bag containing the membrane was placed into an X-ray cassette box, with the writing facing upwards. In a dark room, the membrane was exposed to an X-ray film for 15 min. The X-ray film was developed according to the standardized method.

Stripping the membrane

The membrane was placed in a flat plastic container and the boiling 400 mL 0.1% SDS was added over. It was incubated with shaking until the solution reached room temperature. The nylon membrane was placed into a plastic bag and excess fluid was removed by using a 10 mL pipette. The steps was repeated from labeling with the Marker X in order to obtain the hybridization pattern of the marker

IS6110 DNA Pattern Analysis

The IS*6110* DNA patterns were analyzed with BioNumerics software (version 3.1b; Applied Maths BVBA, Kortrijk, Belgium) following scanning of the radiographs. On the basis of the molecular sizes of the hybridization fragments and the number of IS*6110* copies for each isolate, the fingerprinting patterns were compared by the unweighted pair group method of arithmetic averaging by using the Dice coefficient. Dendrograms were constructed to show the relatedness among the strains, and similarity matrices were generated to visualize the relatedness between the banding patterns of all isolates. An RFLP cluster was defined by completely identical patterns after analysis.

5.3.7 Spoligotyping Analysis

DNA dilution and Amplification

Spoligotyping was done based as described by kamerbeek (Kamerbeek *et al.*, 1997). The extracted DNA was diluted at 1:20 into TBE buffer in a new tube. A diluted solution was stored at -20 °C until further use. A total PCR mix of 25 µL (H2O 6.5 µL, Kapa Taq ready mix 12.5 µL, DRa (supplied) 2 µL, DRb (supplied) 2 µL, diluted DNA 2 µL) per 40 specimens including

control was amplified using the following PCR cycles: 95˚C for 3 min, (94˚C for 1 min , 55˚C for 1 min x30, 72˚C for 30 sec) x30 cycles and 72˚C for 10 min .

Hybridization

PCR product (20µL) was added to 150 µL 2xSSPE/0.1%SDS. The diluted PCR products were heat-denatured for 10min at 99^{O}C and cooled on ice immediately. The membrane was washed at 60^{O}C in 2xSSPE/0.1%SDS for 5 min. The membrane was placed on a support cushion in mini blotter (slots perpendicular to the line pattern) and residual fluid was removed from the slots by aspiration. The slots were filled with diluted denatured PCR product without having air bubbles and hybridized for 60min at 60^{O}C on a horizontal surface. The samples were removed from mini blotter by aspiration and the membrane was washed twice in 2xSSPE/0.5%SDS for 5-10min at 60^{O}C. 40ml 2xSSPE/0.5%SDS (42^{O}C) with 10 µL Strepavidine-peroxidase conjugate (500U/ml) were added and incubated at 42^{O}C for 45-60min. The membrane was washed twice with 2xSSPE/0.5%SDS at 42^{O}C for 5-10min and rinsed with 2xSSPE for 5min at room temperature. Incubate membrane was incubated in 20ml (10ml solution1 + 10ml solution2) ECL mix for 1½min and exposed to X-ray film for 20min.

Stripping

The hybridized PCR product was dissociated from the membrane in order to regenerate the membrane for the next hybridization. The membrane was washed twice in 1%SDS at 80^{O}C for 1hour and stored at 4^{O}C in 20mM EDTA pH8.

Analysis of spoligo

Spoligotypes results in the binary format were entered in the SITVIT2 database of the Pasteur Institute of Guadeloupe (available at http://www.pasteur-guadeloupe.fr:8081/SITVITDemo). In this database, spoligotype international type (SIT) designates a spoligotyping pattern shared by two or more patient isolates and "orphan" designates patterns reported for a single isolate. Major spoligotyping-based phylogenetic clades were assigned according to revised signatures provided in SITVITWEB (Levy-Frebault and Portaels, 1992).

A cluster was defined as two or more strains with similar genetic patterns. Strains that did not yield identical genetic patterns were referred to as "non clustered."

5.3.8 MIRU-VNTR Analysis

PCR amplification

MIRU-VNTR analysis was conducted based on methods as described by Supply (Supply *et al.*, 2006). In a DNA-free area, the PCR reaction premixes were prepared with the concentration of nuclease- free H_2O 33.5 µL, Buffer 10 X 10 µL, Q- Solution 20µL, MgCl2 8 µL, dNTPs 16 µL, reverse primer 2 µL, forward primer 2 µL and Taq polymerase 0.5 µL per 7 specimens. Each PCR reaction tube was labeled and dispensed with 11.5 µL of the PCR reaction premixes. A negative control (sterile water) and a positive control (H37Rv) were included, to validate the analysis.

In another PCR product-free area, 1 µL of extracted DNA previously diluted 1:20 was dispensed into each tube. The PCR tube was tightly sealed to avoid evaporation during amplification. Separate amplification of 24 loci was performed using primers specific for the flanking regions of each VNTR region (Table) with the following PCR cycles conditions 95^{O}C for 15 min, (94^{O}C for 1min, 62 ^{O} C for 1min, 72 ^{O} C for 1 min, with 45 cycles), 72 ^{O} C for 10 min, and down to 4^{O}C.

Table 5.1 Primer sequence of 24 MIRU-VNTR loci with unit size

No	Locus	Alias	Primer sequence	Unit (bp)
1	0154	MIRU 02	TGGACTTGCAGCAATGGACCAACT/ TACTCGGACGCCGGCTCAAAAT	53
2	0580	MIRU 04	GCGCGAGAGCCCGAACTGC/ GCGCAGCAGAAACGTCAGC	77
3	0960	MIRU 10	GTTCTTGACCAACTGCAGTCGTCC/ GCCACCTTGGTGATCAGCTACCT	53
4	1644	MIRU 16	TCGGTGATCGGGTCCAGTCCAAGTA/ CCCGTCGTGCAGCCCTGGTAC	53
5	2059	MIRU 20	TCGGAGAGATGCCCTTCGAGTTAG/ GGAGACCGCGACCAGGTACTTGTA	77
6	2531	MIRU 23	CTGTCGATGGCCGCAACAAAACG/ AGCTCAACGGGTTCGCCCTTTTGTC	53
7	2687	MIRU 24	CGACCAAGATGTGCAGGAATACAT/ GGGCGAGTTGAGCTCACAGAA	54
8	2996	MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC/ CATAGGCGACCAGGCGAATAG	51
9	3007	MIRU 27	TCGAAAGCCTCTGCGTGCCAGTAA/ GCGATGTGAGCGTGCCACTCAA	53
10	3192	MIRU 31	ACTGATTGGCTTCATACGGCTTTA/ GTGCCGACGTGGTCTTGAT	53
11	4348	MIRU 39	CGCATCGACAAACTGGAGCCAAAC/ CGGAAACGTCTACGCCCCACACAT	53
12	0802	MIRU 40	GGGTTGCTGGATGACAACGTGT/ GGGTGATCTCGGCGAAATCAGATA	54
13	0424	Mtub 04	CTTGGCCGGCATCAAGCGCATTATT/ GGCAGCAGAGCCCGGGATTCTTC	51
14	0577	ETR-C	GTGAGTCGCTGCAGAACCTGCAG/ GGCGTCTTGACCTCCACGAGTG	58
15	1955	Mtub 21	AGACGTCAGATCCCAGTT/ ACCCGACAACAAGCCCA	57
16	2163 b	QUB - 11b	CCGATGTAGCCCGTGAAGA/ AGGGTCTGATTGGCTACTCA	69
17	2165	ETR-A	AAATCGGTCCCATCACCTTCTTAT/ CGAAGCCTGGGGTGCCCGCGATTT	75
18	2347	Mtub 29	GCCAGCCGCCGTGCATAAACCT/ AGCCACCCGGTGTGCCTTGTATGAC	57
19	2401	Mtub 30	CGTCGTCGCCGAGCTGGATT/ CACCGGGGCTGGCAGCTAAG	58
20	2461	ETR-B	GCGAACACCAGGACAGCATCATG/ GGCATGCCGGTGATCGAGTGG	57
21	3171	Mtub 34	GGTGCGCACCTGCTCCAGATAA/ GGCTCTCATTGCTGGAGGGTTGTAC	54
22	3690	Mtub 39	CGGTGGAGGCGATGAACGTCTTC/ TAGAGCGGCACGGGGGAAAGCTTAG	58
23	4052	QUB 26	GTGCCGGCCAGGTCCTTCC/ CACCGCGTGTTTGACCCGAAC	111
24	4156	QUB 4156	ACCGCAAGGCTGATGATCC/ GTGCATCTCGTCGACTTCC	59

PCR product analysis using agarose gel electrophoresis

Gel preparation and electrophoresis

Two percent (2%) gel was prepared by dissolving 6g agarose with 300ml 1x SB solution and melted in a microwave oven. After solidification, the gel was placed into an electrophoresis tank containing 1 XSB solution. 10 µL of a 100-bp ladder size standard marker was placed in both external wells and in the central well of the gel. For each reaction, a mixture of 2 µL of PCR product with 2 µL of loading buffer was loaded per well. DNA fragments were separated by electrophoresis at 150 V for 4 hours. The gel was stained with 50 µL ethidium bromide for 30 min. The gel was exposed to UV light and a photo was captured.

Sizing and allele assignation

The size of the PCR product was determined by comparison with the position of the size standard marker (100bp). The corresponding repeat number was determined using the provided table (Table 4.2). The allele assignation of the H37Rv control was verified and correct.

The MIRU- VNTR 24-loci profiles were used to classify the strains into main phylogenetic lineages by using the reference strain collection and identification tools available online at http://www.miru-ntrplus.orgwebcite).

5.3.9 Comparison of discriminatory power

The MIRU-VNTR allelic diversity (*h*) at each of the 24 MIRU-VNTR loci was calculated by the formula: $h=1-\sum x_i^2$, where x_i is the frequency of the *i*th allele at the locus (Sun *et al.*, 2004). The discriminatory power of each typing method and combined typing schemes was calculated by the Hunter-Gaston discriminatory index (HGDI) (Hunter and Gaston, 1988).

.
$$\mathrm{HGDI} = 1 - \left[\frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1) \right]$$

It is the probability that two unrelated strain sampled from the test population was placed into different typing groups. HGDI is given by the following equation: Where N is the total number of strains in the sample population, n is the total number of types described, and nj is the number of strains belonging to the jth type.

5.3.10 Statistical analysis

All data was entered and analyzed using SPSS statistical package software (SPSS Inc., Chicago, IL). Categorical data was compared by the chi-square test or the fisher exact test when expected cell sizes (n) were smaller than 5. In order to determine independent risk factors, odds ratios (OR) and 95% confidence intervals (CI) was calculated for demographic (gender, age), epidemiologic (previous treatment and HIV status), and microbiological variables (drug resistance, and infection by *M. tuberculosis* lineages). The recent transmission index (RTI) was calculated as (Number of clustered patients – number of clusters)/total number of patients.

5.4 Results

5.4.1 Demographic characteristics

A total of 212 *M. tuberculosis* isolates were transported to Stellenbosch University South Africa for molecular typing and drug susceptible testing. Out of these, 35 isolates did not survive transportation and 21 isolates were contaminated. The mean age of the study subjects was 31.6 ± 11.1 (range, 66 years). Permanent residence of the patient was Addis Ababa, and 51.8 patients were male. Among the patients, 66% were previously treated cases, 29.5% were HIV co-infected, and 35.2 % were there HIV status was unknown.

5.4.2 Spoligotyping patterns

Spoligotying results are summarized in Table 5.2. Spoligotyping of 143 isolates detected 26 different genetic profiles. A total of 128 clinical isolates were grouped into 10 clusters (clustering rate of 89.5%) and 16 isolates displayed unique patterns (1 isolate only). One hundred thirty-three (93.0%)of *M. tuberculosis* isolates belonging to 18 shared types (SITs), five patterns containing 7 isolates were designated as an orphan strain according to SITVIT2 database and newly created. Three orphan patterns containing 3 isolates matched preexisting patterns; 1 matched with an orphan from Ethiopia, another with an orphan from Kenya, and 1 isolates (bovin_1) matched with an orphan from Sweden. The most frequently detected family was T3_Eth with 55 strains (38.5%), followed by 34 isolates (23.8%) belonging to the CAS1_KILI and 19 isolates (13.3%) to CAS1_DELHI. Other International families found were CAS (2/143), H1 (1/143), H3 (4/143), H4 (1/143), LAM9 (1/143), T1 (12/143), T2 (3/143), T3 (2/143), T5 (1/143) and bovin_1 clad (1/143). The majority (54.9%) of the strains belonging to lineage 4 (Euro-American), and was followed by Lineage 3 (Central Asian, CAS, 38.2%), and while others represent 7.6%. Among the 79 Euro-American strains, sub-lineages T (51%), and H (4.2%) were most prevalent.

Table 5.2 Description of isolates compared with a preexisting shared type in the SITVIT2 database of the Pasteur Institute of Guadeloupe

Lineage	International family	SIT	Spoligotype pattern*	N	%
CAS	CAS1_DELHI	25		16	11.2
CAS	CAS1_DELHI	26		2	1.4
CAS	CAS1_DELHI	247		1	0.7
CAS	Cas	142		1	0.7
CAS	Cas	1551		1	0.7
CAS	CAS1_KILI	21		34	23.8
H	H1	47		1	0.7
H	H3	50		2	1.4
H	H3	134		1	0.7
H	H3	764		1	0.7
H	H4	817		1	0.7
LAM	LAM9	42		1	0.7
T	T1	53		9	6.3
T	T1	205		1	0.7
T	T1	635		1	0.7
T	T1	single		1	0.7
T	T2	52		3	2.1
T	T3	37		2	1.4
T	T3_ETH	149		55	38.5
T	t5	single		1	0.7
Orphan	bovin_1 clad	single		1	0.7
	Orphan 1			2	1.4
	Orphan 2			1	0.7
	Orphan 3			1	0.7
	Orphan 4			1	0.7
	Orphan 5			2	1.4

***** The black and white boxes indicate the presence and absence, respectively, of hybridization

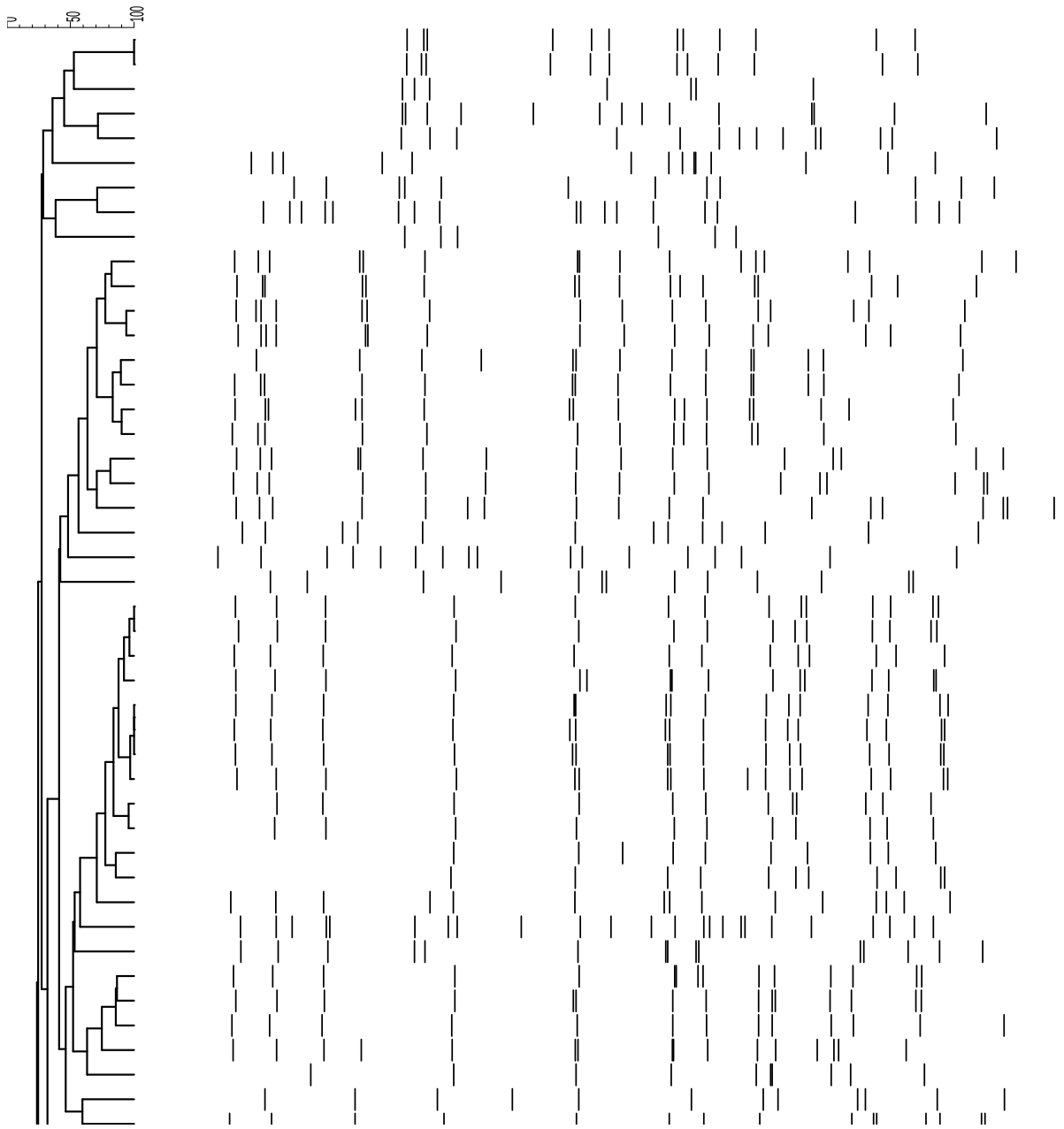
5.4.3 IS6110 RFLP patterns

Among the 118 *M. tuberculosis* isolates, RFLP analysis revealed 80 distinct patterns (0 to 24 IS*6110* copies number), 66 of which were unique (Fig. 4.1). The remaining 52 isolates (44.1%) belonging to 14 clusters. The overall proportion of clustered to not- clustered isolates was 78.8% (95% confidence interval [CI], 65 to 93%). In six isolates (5%) IS6110 element was not found (0 copy number). Further, 43.2% (51/118) isolates were found to be low-copy-number strains having less than six copies of the IS6110 element, and the remaining 56.8% (67/118) were multiple-copy-number isolates with six or more copies of the element.

Among the clusters two of the cluster contained 8 isolates with 3 copy numbers, two clusters of 4 isolates (3 copy numbers), one cluster of 3 isolates (15 copy numbers), two clusters of 3 isolates (3 copy numbers), one cluster of 3 isolates (16 copy numbers), two clusters of 2 isolates (16 copy numbers), one cluster of 2 isolates (14 copy numbers), one cluster of 2 isolates (12 copy numbers) and one cluster of 2 isolates (1 copy number).

Thirty-one isolates (26.1%) were with three bands, 13 isolates with sixteen bands, 12 isolates with fifteen bands, 8 isolates with twelve bands, 7 isolates with fourteen bands, 7 isolates with 4 bands, 6 isolates with seventeen, 6 isolates with zero bands, 5 isolates with eleven bands, 5 isolates with thirteen bands, and the remaining isolates were with different band numbers (Fig 5.1).

IS6110 RFLP analysis of 37 SITs 149 isolates (the largest Spoligo cluster) revealed that 32 (78.4%) isolates were with 3 bands (with 6 clusters) followed by 4 isolates (10.8%) with 4 bands (all were unique), 3 isolates (8.1%) with 0 copy number and 1 isolates (2.7%) with 7 bands (Table 5.3).


0
50
100

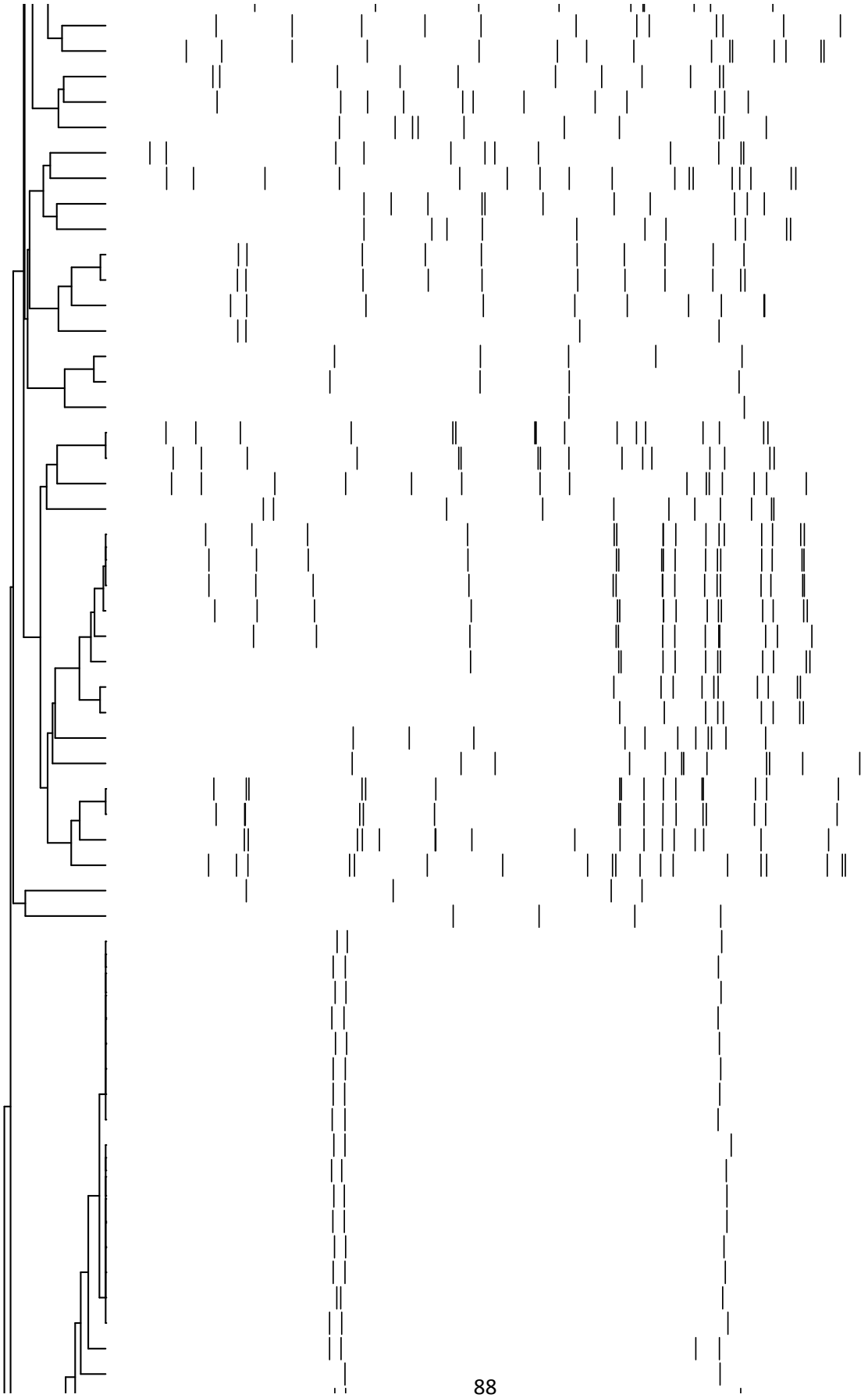


Figure 5.1 *M. tuberculosis* isolates IS6110 RFLP banding patterns and similarity matrices *r*

Table 5.3 IS6110 RFLP patterns of SITs 149 isolates and 2 related isolates (Orphan)

Isolate number	SITs	Spoligo pattern	Copy number	IS6110 RFLP pattern
32	149		3	
1	149		4	
1	149		4	
1	149		4	
1	149		4	
1	149		7	
3	149		0	0 copy
2	Orphan		3	

The filled rectangles depict positive hybridization signals, and empty rectangles represent a lack of hybridization.

5.4.4 MIRU-VNTR Patterns

MIRU-VNTR analysis (24 loci) of 38 isolates, belonging to the biggest spoligo cluster (SITs 149 T3_ETH family), detected 12 different genetic profiles. Twenty -nine (29/38) isolates grouped into 3 clusters that contained one cluster of 21 isolates, one cluster of 6 isolates and one cluster of 2 isolates. Nine isolates (9/38) displayed unique patterns (Figure 5.2). About 76% were identified as TUR linages while 24% were not classified. Figure 5.3 indicate the relative position of our isolates compared to reference isolates in MIRU-VNTR Plus website.

Allelic diversity was observed on eleven loci (11/24 loci). The highest allelic diversity was detected on 2401 (Mtub 30) loci (h=0.41) followed by 4052 (QUB 28960) loci (*h* value= 0.15), 960 (MIRU 10) loci (*h* value = 0.15), and 4156 (QUB 4156) loci (*h* value = 0.10). The allelic diversity of 2059, 3007, 802, 577, 2165, 2461, and 3690 loci were 1.05 *h* value (Table 5.4).

Aellelic divesity was not observed on the remaining 13 International MIRU-VNTR loci. Hunter –Gaston discriminatory index (HGDI) for SITs 149 was 0.679.

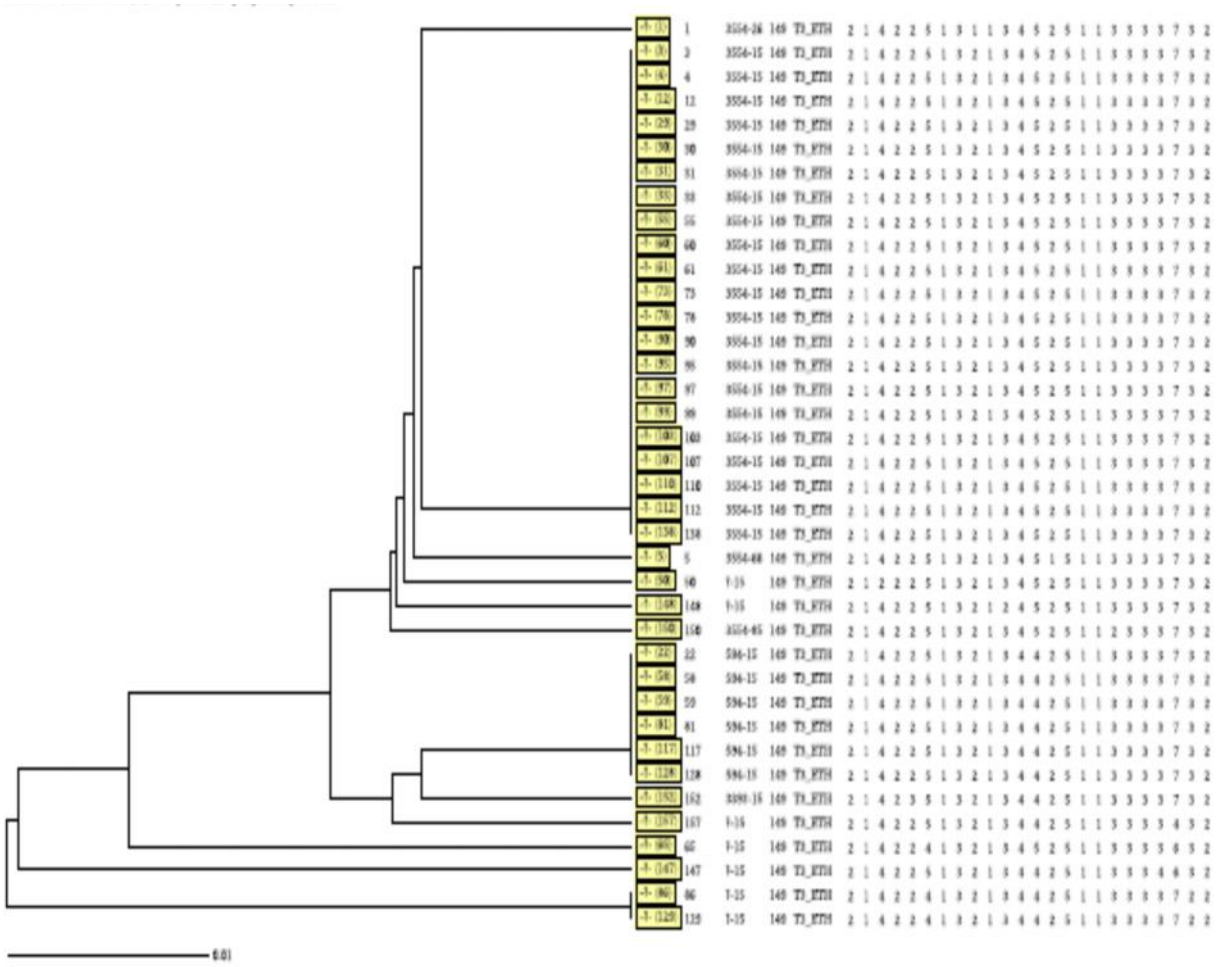


Figure 5.2 Dendrogram deduced from clustering analysis of 38 spoligo T3_ETH isolates using MIRU-VNTR Plus website

Figure 5.3 The relative position of spoligo T3_ETHisolates compared to reference material in MIRU-VNTR Plus website

Table 5.4 Allelic diversity and h value of 24 Mycobacterium tuberculosis MIRU-VNTR loci determined by SITs 149 T3_ETH family isolates

	Allele							*h* value
VNTR locus (alias)	1	2	3	4	5	6	7	SITs 149
154 (MIRU 02)	-	38	-	-	-	-	-	0
580 (MIRU 04)	-	38	-	-	-	-	-	0
1644 (MIRU 16)	38	-	-	-	-	-	-	0
960 (MIRU 10)	-	-	-	3	35	-	-	0.15
2059 (MIRU 20)	1	37	-	-	-	-	-	0.05
2531 (MIRU 23)	-	-	-	-	38	-	-	0
2687 (MIRU 24)	38	-	-	-	-	-	-	0
2996 (MIRU 26)	38	-	-	-	-	-	-	0
3192 (MIRU 31)	-	-	38	-	-	-	-	0
4348 (MIRU 39)	-	38	-	-	-	-	-	0
3007 (MIRU 27)	-	1	37	-	-	-	-	0.05
802 (MIRU 40)	-	37	1	-	-	-	-	0.05
577 (ETR C)	-	1	-	37	-	-	-	0.05
2401 (Mtub 30)	-	-	-	11	27	-	-	0.41
424 (Mtub 04)	38	-	-	-	-	-	-	0
2347 (Mtub 29)	-	-	-	38	-	-	-	0
4052 (QUB 26)	-	-	-	1	-	2	35	0.15
2165 (ETR-A)	-	1	37	-	-	-	-	0.05
1955 (Mtub 21)	-	-	38	-	-	-	-	0
2163b (QUB 11b)	38	-	-	-	-	-	-	0
3690 (Mtub 39)	-	-	37	1	-	-	-	0.05
4156 (QUB 4156)	-	2	36	-	-	-	-	0.10
3171 (Mtub 34)	-	-	38	-	-	-	-	0
2461 (ETR-B)	1	37	-	-	-	-	-	0.05

5.4.5 Clustering and recent transmission based on the three molecular typing methods

Cluster analysis based on a composite data of IS6110 RFLP, spoligotyping, and 24 International standard MIRU-VNTR analysis detected 119 genetic profiles of 143 isolates. The isolates were grouped into 15 clusters ranging in size from 2 to 7 isolates, and 104 isolates displayed unique pattern (Table 5.5). Among the clustered isolates 59% were SITs 149 T3_ETH family (clustered into 8 groups), and followed by SITs 21 CAS1_KILI with 25.6% (clustered into4 groups), SITs 53 T1 with 10.5% (clustered into 2 groups) and SITs 25 CAS1_DELHI with 5.1% (1 cluster group) with clustering rate of 41.8% (SITs 149), 29.4% (SITs 21), 33.3% (SITs 53), and 10.5% (SITs 25). The overall clustering rate was 27.3%. The overall recent transmission index (RTI) was 20.5%. The RTI for T3_ETH was the highest (37.8%) followed by CAS1_KILI (20.7%), T1 family (18.2) and CAS1_DELHI (5.3%) (Table 5.6). The largest cluster was T3_ETH family with 3 bands, and 5 copy number at loci 2401 (Mtub 30) and 960 (MIRU 10). One cluster with 2 isolates was 4 copy number at loci 2401(Mtub 30), and 1 cluster with 2 isolates was 4 copy number at loci 960 (MIRU 10).

5.4.6 Discriminatory power of IS6110 RFLP, Spoligotyping, and MIRU-VNTR

A highest and equal HGI value (HGI=0.996) was observed between only IS6110 RFLP, and in combination with spoligotyping in groups with greater than or equal to five IS6110 RFLP bands (Table 5.6). They differentiated 60 types out of 68 isolates. On the other hand, spoligotyping can only differentiate 21 types (HGI= 0.779). There was no added HGI value from doing spoligotyping. In groups with less than five IS6110 RFLP bands (49 isolates), spoligotyping differentiated 8 types (HGI=0.427), IS6110 RFLP differentiated 19 types (HGI= 0.924), and in combination spoligotyping & IS6110 RFLP, differentiated 23 types (HGI= 0.941). Among 38 T3_ETH family isolates (clustered group by spoligotyping), 24 loci MIRU-VNTR differentiated 11 types (HGI=0.649), IS6110RFLP differentiated 13 types (HGI=0.907), and in combination (MIRU-VNTR & IS6110RFLP) it was able to differentiate 23 types (HGI=0.954).

Table 5.5 Clusters of isolates based on IS6110 RFLP, Spoligotyping, and MIRU-VNTR analysis

Clusters	No of isolates	Typing Method	SITs number (families)	Characteristics of molecular typing
Cluster 1	7	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	3 bands of IS6110 RFLP, 5 copy at loci 2401 (Mtub 30) and 960 (MIRU 10)
Cluster 2	3	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	3 bands, 5 copy at loci 2401 & 960
Cluster 3	3	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	3 bands, 5 copy at loci 2401 & 960
Cluster 4	2	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	3 bands, 5 copy at loci 2401 & 960
Cluster 5	2	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	3 bands, 5 copy at loci 2401 & 960
Cluster 6	2	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	3 bands, 4 copy at loci 2401 & 5 copy at loci 960
Cluster 7	2	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	3 bands, 4 copy at loci 960 & 5 copy at loci 2401
Cluster 8	2	Spoligo IS6110 RFLP MIRU-VNTR	149 (T3_ETH)	0 bands, 5 copy loci 2401 & 960
Cluster 9	2	Spoligo IS6110 RFLP	53 (T1)	12 bands,
Cluster 10	2	Spoligo IS6110 RFLP	21(CAS1_KILI)	14 bands,
Cluster 11	3	Spoligo IS6110 RFLP	21(CAS1_KILI)	16 bands,
Cluster 12	2	Spoligo IS6110 RFLP	21(CAS1_KILI)	16 bands,
Cluster 13	3	Spoligo IS6110 RFLP	21(CAS1_KILI)	15 bands,
Cluster 14	2	Spoligo IS6110 RFLP	21(CAS1_DELHI)	16 bands,
Cluster 15	2	Spoligo IS6110 RFLP	53 (T1)	0 bands

Table 5:6 Comparison of discriminatory power according to DNA typing method alone or in combination

Isolates	Typing method	No. of isolates	Total type pattern	No.of unique isolates	Number of clusters	No. of clustered isolates	Maximum no. of isolates in a cluster	RTI %	HGI
All	Spoligo typing	143	26	15	11	128	55	81.8	0.785
	IS6110 RFLP	118	81	66	14	52	8	32.2	0.985
	Spoligo + IS6110 RFLP	117	84	69	15	48	8	28.2	0.988
	Spoligo + IS6110 RFLP + VNTR*	117	93	78	15	39	3	16.8	0.993
≥ 5 bands	Spoligo typing	68	21	15	6	53	28	69.1	0.779
	IS6110 RFLP	68	60	54	6	14	3	11.8	0.996
	Spoligo + IS6110 RFLP	68	60	54	6	14	3	11.8	0.996
< 5 bands	Spoligo typing	49	8	4	4	45	37	83.7	0.427
	IS6110 RFLP	49	19	11	8	38	8	61.2	0.924
	Spoligo + IS6110 RFLP	49	23	14	9	35	8	53.1	0.941

T3_ETH family	Spoligo	55	1	0	1	55	55	98.2	0
	24 VNTR	38	11	8	3	30	22	71.7	0.649
	IS6110 RFLP	37	13	6	7	31	8	64.9	0.907
	24 VNTR+ IS6110 RFLP	37	23	16	7	21	7	37.8	0.954
CAS1_KILI family	Spoligo typing	34	1	0	1	34	34	97.1	0
	IS6110 RFLP	29	23	19	4	10	3	20.7	0.980
	Spoligo + IS6110 RFLP	29	23	19	4	10	3	20.7	0.980
CAS1_DELHI family	Spoligo typing	19	3	1	2	18	16	89.5	0.292
	IS6110 RFLP	19	18	17	1	2	2	5.3	0.994
	Spoligo + IS6110 RFLP	19	18	17	1	2	2	5.3	0.994
T1 family	Spoligo typing	12	4	3	1	9	9	66.7	0.455
	IS6110 RFLP	11	9	7	2	4	2	18.2	0.636
	Spoligo + IS6110 RFLP	11	9	7	2	4	2	18.2	0.636

VNTR*= MIRU-VNTR only for T3_ETH isolates

5.4.7 Factor associated with strain clustering

A univariate analysis of the possible association between clustering with demographic, epidemiological, and microbiological variables was done. Positivity for T3_ETH ($P < 0.001$) was significantly associated with clustering. Fifty-five percent of T3_ETH isolates belonging to clusters (Table 5.7).

The odds of clustering was nearly 4 fold higher among isolates with < 5 IS6110 RFLP copy number as compared to ≥ 5 copy numbers (P=0.002), 28-fold higher among Isoniazid resistant isolates (P <0.001), 12-fold higher among Rifampicin (RMP) resistant isolates as compared to RMP-susceptible isolates (P< 0.001), 11-fold higher among Pyrazinamide (PZA) resistant isolates as compared to PZA susceptible isolates ($P < 0.001$), 4-fold higher among Streptomycin (STM) resistant isolates as compared to STM susceptible isolates (P=0.04) .

Multidrug resistance (MDR) isolates had 11-fold higher odds of clustering (P<0.001) compared to non-MDR isolates and isolates that were resistant to one or more first-line anti-TB drugs had 26-fold higher odds of clustering (P< 0.001) as compared to fully susceptible isolates. The isolates that were resistant to all first-line anti-TB drugs was not significantly clustered as compared to isolates that were susceptible to at least one first-line anti-TB drugs.

Table 5.7 Demographic characteristics of the study subject, drug resistance pattern phylogenetic linage, and their association with strain clustering

Characteristic		Genotyping pattern		OR(95CI)	p- value
		Clustered (%)	Unique(%)		
Gender	Male	18 (29)	44 (71)	0.8 (0.4-1.7)	0.659
	Female	19 (41)	27 (59)	1	
Age groups	<=45	35(34)	69(66)	1.7(0.4-6.5)	0.430
	>45	3(23)	10(77)	1	
Previous TB treatment	Yes	33(34)	65(66)	1.2 (0.4-3.1)	0.959
	No	7 (30)	16 (70)	1	
HIV status	Positive	9(27)	25(73)	0.6(0.2-1.5)	0.345
	Negative	18	28	1	
IS6110 RFLP band no.	<5	25(51)	24(49)	3.7(1.7-8.2)	0.002
	>=5	15(22)	53(78)	1	
Streptomycin	Resistant	27(44)	34(56)	4.3(1.7-11.2)	0.04
	Sensitive	7(16)	38(84)	1	
Isoniazid	Resistant	33(46)	39(54)	27.9(3.6-215.3)	<0.001
	Sensitive	1(2.9)	33(97)	1	
Rifampicin	Resistant	31	34	11.6(3.2-42.2)	<0.001
	Sensitive	3	38	1	
Ethambutol	Resistant	13	14	2.6(1.0-6.3)	0.067
	Sensitive	21	58	1	
Pyrazinamide	Resistant	30	28	11.0 (3.5-34.6)	<0.001
	Sensitive	4	41	1	
Resistance to one or more FLD	Yes	33 (45)	40(55)	26.4(3.4-203.7)	<0.001
	No	1(3)	32(97)	1	

MDR-TB	Yes	31(47)	35(53)	10.9(3.0-39.0)	<0.0001
	No	3(7.5)	72(92.5)	1	
Resistance to all FLD	Yes	9(41)	13(59)	1.6(0.6-4.3)	0.459
	No	25(29.8-70.2)	59	1	
FLQ	Resistant	3	1	6.9(0.7-68.9)	0.072
	Sensitive	31	71	1	
KAN/AMK/CAP	Resistant	-	-	-	-
	Sensitive	34	72	-	-
KAN/CAP/VIO	Resistant	-	-	-	-
	Sensitive	34	72	-	-
KAN/AMK/ CAO/VIO	Resistant	-	-	-	-
	Sensitive	34	72	-	-
Low level KAN	Resistant	-	-	-	-
	Sensitive	34	72	-	-
M. tuberculosis linage					
CAS		0	2	-	-
CAS1_DELHI		2	17	0.2(0.1-0.9)	0.013
CAS1_KILI		11(39)	18(61)	1.4(0.6-3.4)	0.593
H1		0	1	-	-
H3		0	4	-	-
H4		0	1	-	-
LAM9		0	1	-	-
T1		6 (55)	5 (45)	2.7(0.8-9.3)	0.219
T2		0	3	-	-
T3		0	2	-	-
T3_ETH		21(55)	17(45)	4.1(1.8-9.3)	0.001
T5		0	2	-	-
Orphan		0	8	-	-

5.5 Discussion

Molecular technologies have provided powerful tools for analyzing *Mycobacterium tuberculosis* genotype and transmission patterns, and generate valuable evidence for developing effective TB control. In this study, we present the population structure, transmission patterns and factor associated with transmission of *M. tuberculosis* isolate in Addis Ababa based on a combination of IS6110RFLP, MIRU-VNTR 24-loci typing, and spoligotyping. Our finding showed that highly diverse genetic profiles, though dominated by three types, namely T3_ETH, CAS1_KILI and CAS1_DELHI (>75.6%).

Clustering is a marker for recent transmission to a good extent. Clustered isolates have a high probability to be involved in the same chain of recent TB transmission. Our result indicated that 27.3 % of *M. tuberculosis* isolates were belonging to clusters and the rate of recent transmission was 20.5%, of which most of (70%) the isolates were drug resistant. A higher rate of clustering was reported in Addis Ababa and in another part of the country which ranged from 41.2% to 48.2% (Agonafir *et al.*, 2010; Bruchfeld *et al.*, 2002; Hermans *et al.*, 1995; Tessema *et al.*, 2012). A high discrepancy between our findings and other studies could be explained by differences in discriminatory power of the molecular typing method used. Most of the studies utilized only spoligotyping method that is the level of discrimination is generally low(Warren *et al.*, 2009). Other studies utilized only IS6110 RFLP or Spoligotyping with MIRU-VNNTR. In our study, we used a combination of IS6110 RFLP, spoligotyping and MIRU-VNTR typing that is with higher discriminatory power compared with the typing technique separately.

A relatively high rate of clustering, recent transmission, prevalence and drug resistant underline the importance of T3_ETH and CAS1_KILI spoligotypes in TB control program in Addis Ababa, Ethiopia. T3_ETH was found to be the most prevalent (38.5%) with highest clustering

rate (41.8%) and rate of recent transmission (RTI=37.8%) using a 0.954 discriminatory power (HGI) molecular typing techniques followed by CAS1_KILI with 23.8% prevalence , 29.4% clustering rate and 20.7% rate of recent transmission (HGI=0.980). T3_ETH and CAS1_KILI had been reported to be prevalent in Addis Ababa (Agonafir *et al.*, 2010; Bruchfeld *et al.*, 2002; Mihret A., 2012; Mitike *et al.*, 1997). CAS1_KILI has also been reported to be prevalent in East Africa(Kibiki *et al.*, 2007). T3_ETH has been described in the international database as a genotype predominant in Ethiopia. It has also been reported as a 3-bands type on IS6110 RFLP analysis (Bruchfeld *et al.*, 2002; Hermans *et al.*, 1995). In our study, most of the T3_ETH types were 3 bands but some of the isolates were with 4 bands, 7 bands and no band of the IS6110 element. In addition, two of the orphan isolates (orphan 2) might be recently mutated from T3_ETH. They lacked spacer 22 & 23 compared with T3_ETH spoligotype. They were genetically similar with T3_ETH based on IS6110 RFLP and 24 MIRU-VNTR analyses

The dominant isolates in Addis Ababa were with 3-bands IS6110 RFLP element, of which the level of discrimination by IS6110 RFLP is generally low. IS6110 RFLP is time -consuming and technically demanding molecular typing method (Warren *et al.*, 2009). In our study, an addition of MIRU-VNTR typing increased the discrimination power for the T3_ETH family (most of them were 3-bands) isolates but below the discrimination power of IS6110 RFLP, indicating that IS6110 RFLP typing is very useful for sub-classifying MIRU-VNTR clusters of T3_ETH spoligotypes. Allelic diversity was observed on eleven MIRU-VNTR loci (11/24) on differentiating the T3_ETH family genotype. Mtub 30 (2401) loci provided the highest differentiation power and followed by QUB 28960 (4052) and MIRU 10(960). However, it must be pointed out that higher differentiation power does not necessarily imply usefulness of such loci in epidemiological studies. At present, limited studies on the discriminatory power of

MIRU-VNTR loci on T3_ETH family could be found in the literature. Since this family is predominant and highly transmitted in Addis Ababa, more studies on the potential useful loci in differentiating closely related members of the T3_ETH family isolates are urgently needed.

In our study, one (0.7%) *M. bovis* was observed. In agreement with our study, 0.4% frequency of *M. bovis* was reported in Ethiopia (Firdessa R, 2013). The *M. bovis* isolates from humans showed typical bovine spoligotype profiles lacking spacers 3, 9, 16, and 39–43. In addition, they lacked spacers 3–7, which are features that define strains of the African 2 clonal complex of *M. bovis* reported from TB-infected cattle in Ethiopia (Stefan Berg, 2011). The Beijing genotype was not detected in our study but the presence of this isolate in Addis Ababa was reported by another study (Mihret A., 2012). Lineage 7 was not found in our study, reported in northwest part of the country (Tessema *et al.*, 2012). None of our isolates exhibited the specific spoligotyping patterns of *M. africanum* or *M. canetti*. None of them showed the simultaneous absence of spacers 8, 9, and 39, which is characteristic of *M. africanum* isolates (Viana-Niero *et al.*, 2001); and they all lacked the typical *M. canetti* spoligotyping patterns (presence of spacers 30 and 36 and a lack of all others spacers).

In our study, lineage 4 cluster was dominant and the result is in agreement with previous reports (Agonafir *et al.*, 2010; Mihret A., 2012). In Amhara region of Ethiopia, lineage 3 cluster was dominant (Tessema *et al.*, 2012). This indicated that the population structures of *M. tuberculosis* are not the same across the country and it is advisable to continuously monitor the molecular epidemiology and mapping of the genotypes in the country.

This study had shown a significant association between infection with isolates of the T3_ETH lineage, multi-drug resistance, resistance to at least one or more first-line anti-TB drugs, resistance to each INH, RMP, STM, PZA, and clustering. This might suggest that drug-resistant

isolates are more transmissible than drug-sensitive isolates, it is in agreement with another report (Toit *et al.*, 2014); many others have shown the opposite. The age group, gender, HIV status, the previous history of treatment of the patients was not significantly associated with clustering. This data is in agreement with a report in Addis Ababa(Bruchfeld *et al.*, 2002). Although in our study male TB patients were in the majority, a trend toward increased clustering was observed in isolates from the female patient and is in agreement with the results of previous studies in Addis Ababa (Bruchfeld *et al.*, 2002). A trend toward increased clustering was also observed with isolates from previously treated patients compared with isolates from new patients. The previous study in Ethiopia and other part of the world reported that HIV-infected patient was associated with clustering (Bruchfeld *et al.*, 2002).

This study has its own limitation. Epidemiological links between patients infected with isolates of identical genotypes confirm that the clustered cases are involved in the same recent transmission chain. In our study, a detailed epidemiological contact investigation was not performed; it, therefore, remains unclear how person-to-person transmission occurred within each cluster.

5.6 Conclusions

Our finding of a relatively high clustering rate among isolates from T3_ETH and CAS1_KILI suggests that these isolates may be an important cause of rising incidence of drug- resistant TB in Addis Ababa. The high rate of recent transmission underlines active transmission of *M. tuberculosis* including drug resistant isolates. This underlines the importance of strengthening case finding and treatment of TB patients to interrupt the chain of transmission within the community. Using of high-resolution molecular typing tools like Whole genome sequencing is crucial for differentiating closely related isolates.

Chapter 6. Geospatial Patterns of Drug Resistant Tuberculosis and Associated Risk Factors in Addis Ababa, Ethiopia

6.1 Abstract

Background: According to the World Health Organization report, Ethiopia is one of the 30 highest MDR-TB burden countries with an estimated 3300 DR-TB cases in 2015 and reported cases of extensively drug-resistant TB. The distribution and clustering of DR- TB in terms of time and geographic area in Addis Ababa has never been investigated while this could help to strengthen disease surveillance. The objective of this study was to characterize the purely spatial and space-time clustering of drug resistance TB at wereda level and associated risk factors in Addis Ababa, Ethiopia.

Methods: The information about drug-resistant tuberculosis cases that occurred between April 2013 and March 2017 in Addis Ababa was used. A purely spatial and space-time analysis were conducted using SaTScan and mapping was done using ArcMap. In total, 250 (78.9%) diagnosed drug resistance pulmonary TB cases were utilized for spatial analysis.

Results: Significant high and low rate most likely spatial and space-time clusters were identified. A significant secondary cluster was also obtained. The most likely cluster of the high rate from both the purely special and space-time analyses was almost from the same area. Population density and per capita income was significantly associated with spatial clustering.

Conclusions: There is evidence of clustering of drug resistance pulmonary TB in Addis Ababa. Clusters are mainly found in a community of low socioeconomic conditions. Contact tracing of tuberculosis patients and development work should be strengthened in the hot-spot area to interrupt the clustering.

Keywords: tuberculosis, spatial analysis, clustering analysis, drug resistance, Ethiopia

6.2 Introduction

Ethiopia is one of the 30 highest MDR-TB burden countries globally. The prevalence of all forms of TB was estimated to be 192/ 100,000 population and annual mortality was 4/100,000 population (WHO, 2015a). TB is also a major public health problem in the capital city Addis Ababa with a total of 9,352 TB reported cases (all forms, 5% of all Ethiopian cases), and 78 MDR-TB cases were notified in 2016 (AAHB, 2015). Ethiopia had a small number of reported cases of extensively drug-resistant TB (XDR-TB) (Agonafir *et al.*, 2010).

Aerosol transmission of TB and other respiratory infections leads to spatial and temporal clustering of cases (Kulldorff and Nagarwalla, 1995). Different countries have used Spatio-temporal analysis to study TB disease distribution and have shown a distinct geographical clustering of TB cases over time (Chamie *et al.*, 2015; Lim *et al.*, 2015; Liu *et al.*, 2012; Pinto *et al.*, 2015; Watase and Nakanishi, 2006; Wubuli *et al.*, 2015; Ying and Chen, 2012; Zhao *et al.*, 2013). In studies from southern, and northern part of Ethiopia, significant variations in Spatio-temporal clusters of smear-the positive pulmonary tuberculosis were reported (Dangisso *et al.*, 2015; Tadesse *et al.*). A cluster analysis helps identify areas with unusually high disease rates, which are less likely to occur by chance.

In Ethiopia, where the distribution of risk factors such as HIV and social inequities show great heterogeneity, a better understanding of the spatial –temporal epidemiology of DR-TB may help to strengthen disease surveillance, to identify risk factors for the spread of the disease, and to better plan targeted interventions such as intensified active case findings and tactical allocation of resources such as Xpert MTB/RIF^{R} equipment location and mobile TB care clinic service. It also provides information on the stability of the epidemic over time and could serve as a baseline to evaluate future interventions. Moreover, a graphical demonstration of the mapping to a wider

audience may assist in advocacy and can have a role to play in fund raising for the purpose of addressing the problem. However, information about the spatial distribution of the disease is limited and this is the first study that attempts to characterize the spatial distribution, the purely spatial and space-time clustering of DR-TB at wereda level (smallest administrative unit) in Addis Ababa, where most DR-TB cases are reported.

6.3 Materials and methods

6.3.1 Study area and setting

The study was conducted in Addis Ababa, the capital city of Ethiopia, situated between 9° 0' 19.4436" N and *38*° 45' *48.9996*" E. As a chartered city, Addis Ababa has the status of both a city and a state. It is the largest city in Ethiopia with a population of 3,352,009 people according to Central Statistical Agency estimation for the year 2015 (CSA, 2013). The city is divided into 10 sub-cities and covers a geographic area of 527 square kilometers (Fig 6.1). There are 116 weredas, which are the smallest administrative units within a sub-city.

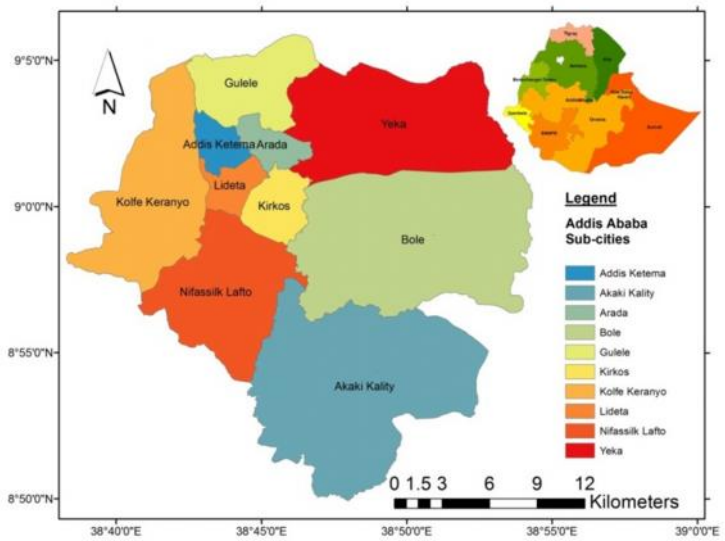


Figure 6.1 Map of Ethiopia and Addis Ababa City

The city has a total of 139 health facilities (92 public and 42 private centers) that provide the Directly Observed Treatment Short courses (DOTs) to TB patients (AAHB, 2015). The programmatic response to DR-TB in Ethiopia began in 2009 in Addis Ababa and currently, two DR-TB treatment initiating Centers (TICs) are operating in the city: St Peter's TB Specialized Hospital and Alert Hospital. The role of TICs includes the investigation of presumptive DR-TB cases, initiation of treatment, provision of mentoring and programmatic support to treatment follow-up center (TFCs) and quarterly reporting on progress to the national TB program (NTP). Each TFC is required to manage all referred patients by providing daily medications to patients, referring patients with severe complications and drug side effects back to the TIC, and monthly standardized recording and reporting to the TIC.

There are laboratories that provide TB drug susceptibility testing (DST) service in the city. The Ethiopian Public Health Institute (EPHI), St Peter's Specialized TB Hospital and International Clinical Laboratory (ICL) have TB culture and DST facilities. Addis Ababa Health Research Laboratory (AAHRL), Yekatit 12 Hospital, Zewditu Memorial Hospital, Menelik II Hospital, Tirunesh Beijing Hospital, Ras Desta Hospital, Alert Hospital (AH), Federal Police Hospital, Federal Prison Hospital, Black Lion Hospital, Armed Force Hospital, Kality Health Center, Koteb Health Center, Addis Ketema Health Center, Kolfe Health Center, Nifas Silk Lafto wereda 9 Health Center, and Tekelahymanot Health Center provide DST service using Xpert MTB/RIF^{R}.

Description of the population

Study population: All multidrug resistance (MDR-TB) or rifampicin resistance (RR-TB) patients with permanent address in Addis Ababa

Study subject: Patients who were being treated as MDR-TB patient at treatment initiating centers and/or confirmed as MDR-TB or RR-TB at one of the above DST centers, and whose permanent address is in Addis Ababa or were living in Addis Ababa before diagnosis/during treatment.

Inclusion criteria

All MDR-TB or RR-TB patients registered in Addis Ababa Health facilities were eligible for enrollment.

Exclusion criteria

Those drug resistance TB patients who were identified as living outside of Addis Ababa city.

Data collection and management

Three hundred seventeen TB patients with a history of drug resistant TB treatment, who were registered from April, 2013 to March, 2017 were selected from TICs and DST centers. For all those eligible as per the criteria listed above data were collected from the DR-TB patient medical record found at the TICs, and from TB laboratory registers at the DST centers. The main purpose of TB laboratory register was to extract information that was missing in the medical records of the TICs as well as to obtain patient information for those diagnosed at the DST centers but who did not start treatment at the TICs. Data from TICs and DST centers was matched using names and address to avoid double registration. Some essential missed data was collected from the initial patient medical record available at DOTs health facility.

Socio-demographic data, history of previous tuberculosis treatment, HIV status, sex, age and permanent residence address (wereda name) were collected. The size of the population of each

wereda was collected from the City Government of Addis Ababa. The incidence of DR-TB per wereda was calculated using the population of each wereda, 'population at year-end', covering the year 2013-2016.

The digital map of the city was obtained and the wereda physical address of the patient was geo-referenced. Data was collected using a structured, pre-tested questionnaire by trained data collectors. Data was double entered into SPSS software and cleaned and cross-checked for consistency.

6.3.2 Geospatial mapping and clustering analysis

Using ArcGIS software (http://www.esri.com/software/arcgisto), the Addis Ababa city digital map and geocoded address of the patients, the distribution of DR-TB in Addis Ababa city was mapped.

A purely spatial and space-time clustering analysis was carried out at wereda level of the patients using Kulldroff's scan statistics (SaTtscan9.4 software) to test for the presence of statistically significant clusters of DR-TB (Kulldorff *et al.*, 2005; Kulldorff and Nagarwalla, 1995). The scan statistics carried out during cluster analysis detected cluster size and locations, computed the relative risk (RR) and provided a p-value using Monte Carlo Simulation. We used the number of cases per wereda, population at the wereda level, and coordinates as input files, as well as the discrete Poisson model with the assumption that the number of cases at each location was Poisson distributed with a known population at risk. Scan circles of various sizes were used to identify the most likely spatial clusters of DR-TB. For maximum spatial cluster size, the upper limit which is 50% of the population at risk was used. The test of significance of the identified clusters was based on comparing the likelihood ratio test statistics against a null distribution obtained from Monte Carlo Simulation (Kulldorff *et al.*, 2005; Kulldorff and Nagarwalla, 1995). The number of permutation was set at 999 and the significance level was set at 0.05. The Spatio-

temporal clustering analysis was done for the period from 2013 to 2016 on yearly base to investigate a presence of recent space-time cluster of disease.

6.3.3 Statistical Analysis

All data were entered and analyzed using SPSS statistical package software (SPSS Inc., Chicago, IL). Categorical data were compared by the chi-square test or the Fisher exact test when expected cell sizes (n) were smaller than 5. In order to determine independent risk factors, odds ratios (OR) and 95% confidence intervals (CI) were calculated for demographic (gender, age), epidemiologic (previous treatment and HIV status), and infection by *Mycobacterium tuberculosis* lineages. The study was reviewed and approved by the Ethical Clearance Committee of the Addis Ababa City Administration Health Bureau.

6.4 Results

6.4.1 Demographic characteristics of the study subjects

During the study period (1/04/2013 to 1/04/2017), 317 incidences of rifampicin-resistant pulmonary TB patients who were permanent residents were reported in Addis Ababa city. Of the 317 cases 67(21.1%) did not have a wereda address and were excluded from the spatial analysis. The mean age of the study subject was 28.86± 12.13 the standard deviation. The majority (35.8%) of the patients were in 26-35year age group, followed by 16-25 year age group (33.4%) and 36-45(16.7%). Among the study subjects, 54% were male, 148 (56%) were previously treated cases excluding unknown cases and 48 (43%) HIV co-infected excluding cases were HIV status unknown (Table 6.1).

Table 6.1 Characteristics of the study subjects in Addis Ababa, Ethiopia

Characteristics		Number (%)
Age groups, years	≤15	18 (5.8)
	16-25	106(34.3)
	26-35	111(35.9)
	36-45	53 (17.2)
	45-65	16 (5.2)
	≥65	5(1.6)
Sex	Male	172 (54.23)
	Female	145 (45.74)
HIV status	Reactive	48 (15.1)
	Non-reactive	65 (20.5)
	Unknown	204 (64.3)
History of anti-TB treatment	New	118 (37.2)
	Retreatment	148(46.7)
	Unknown	51 (16.1)
Residence area (Sub-city)	Addis Ketema	52 (18.9)
	Akaki Kality	6 (2.2)
	Arada	16 (5.8)
	Bole	27 (9.8)
	Gulele	20 (7.3)
	Kirkose	12 (4.4)
	Kolfe Keranyo	50 (18.2)
	Lideta	18 (6.6)
	Nifas Silk Lafto	46 (16.7)
	Yeka	28 (10.2)

Figure 6.2 presents the distribution of excess risk across the weredas. Excess risk is defined as a ratio of the observed number of cases over the expected number of cases. Weredas in red color had higher observed cases than the expected cases. Weredas in blue color had lower observed cases than the expected cases.

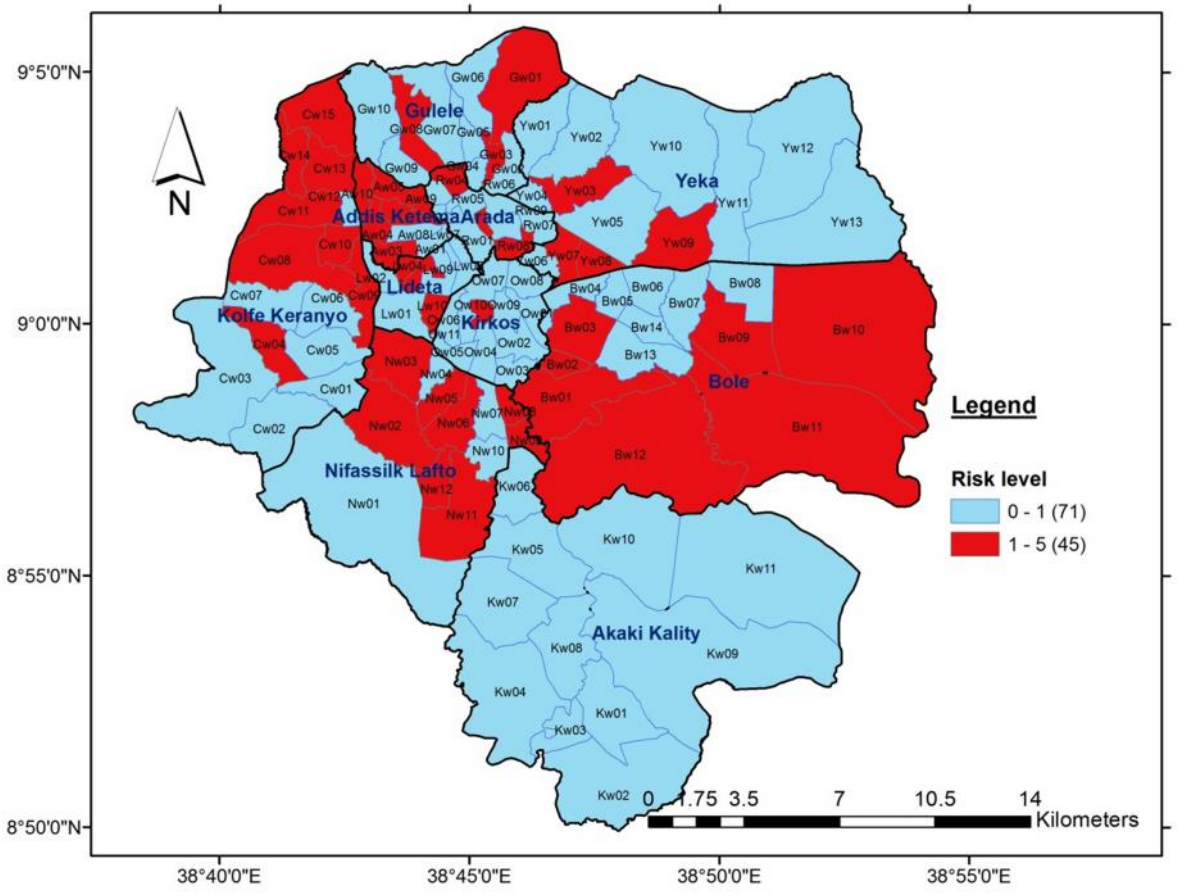


Figure 6.2 Excess risk map of drug resistance TB in Addis Ababa, Ethiopia

The number of rifampicin-resistant notification per wereda in the study period (1/04/2013-1/042017) varied from 0-9 patients. The highest number of rifampicin–resistant cases were reported from Kolfe Keranyo sub city wereda-8 (9 cases) followed by Addis Ketema sub city wereda-2 and wereda-7 (8 cases each) (Figure 6.3). The mean rifampicin-resistant case notification across the city over four years was 2.3 per 100,000 people, ranging from 0 to 10.5 per 100,000 people in the weredas (Figure 6.4). The incidences were increased for the last four years.

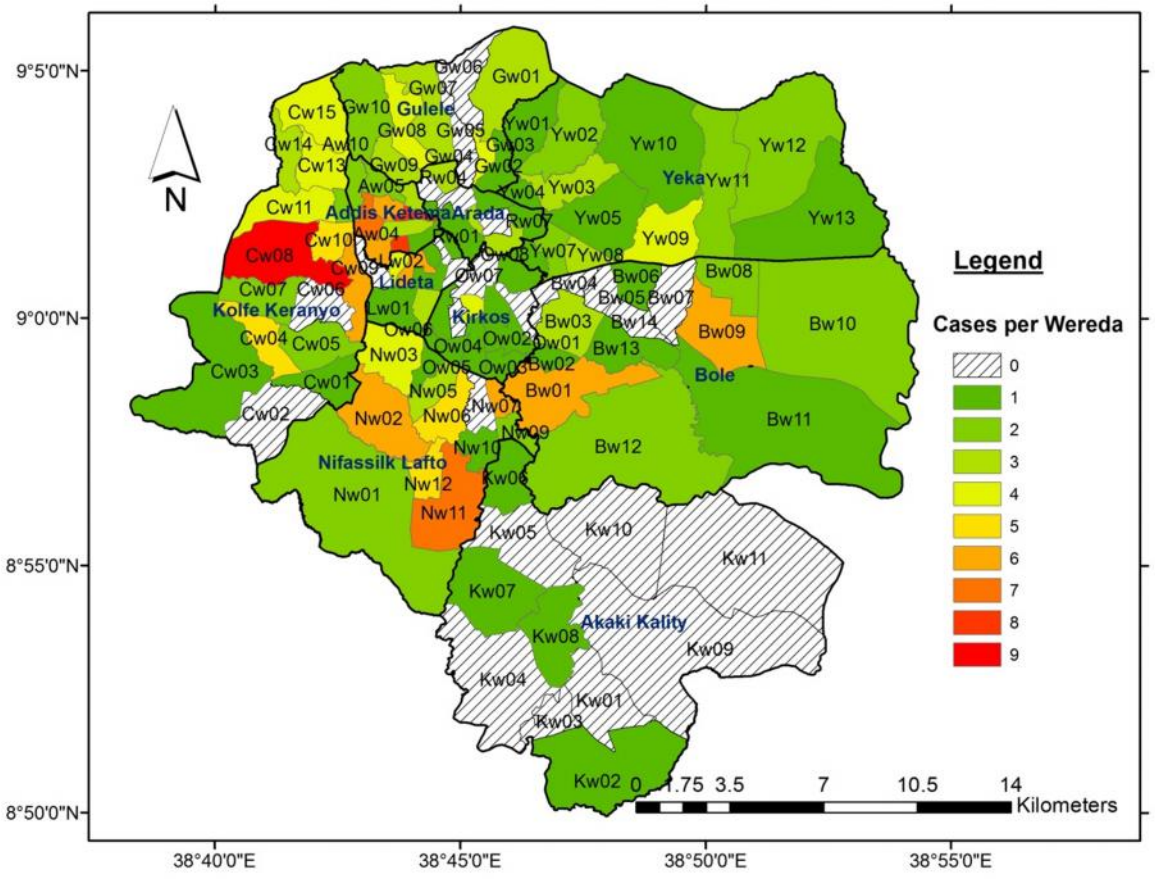


Figure 6.3 Number of rifampicin-resistant TB incidences in Addis Ababa, Ethiopia

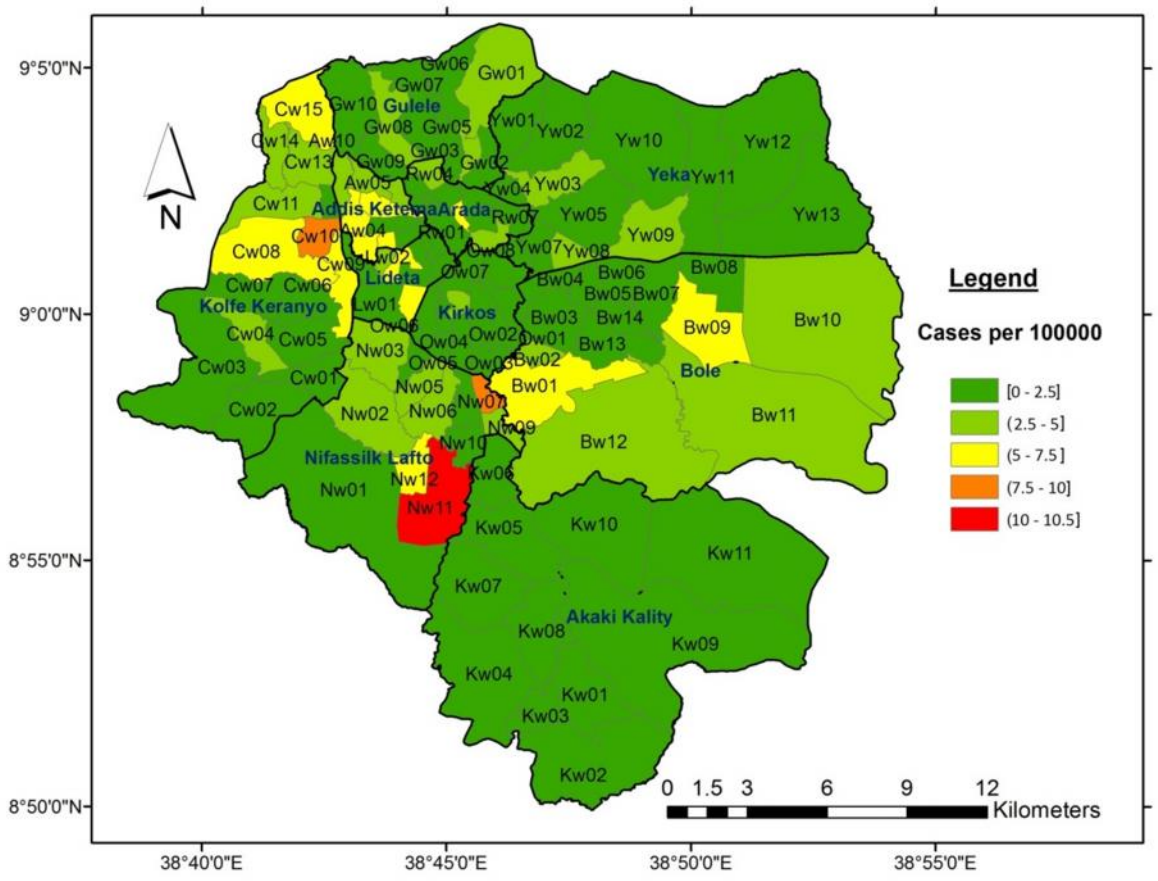


Figure 6.4 Rifampicin-resistant case notifications per 100,000 per wereda in Addis Ababa, Ethiopia

6.4.2 Spatial Pattern of Rifampicin-resistant TB

Using the maximum spatial cluster size of ≤ 50% of the population, the purely spatial analysis of high rates identified most likely significant cluster (hot spot) of rifampicin-resistance TB (RR= 2.70, $p < 0.001$) in 25 adjacent weredas belonging to Kolfe Keranyo sub-city (11 weredas), Addis Ketema sub-city (9 weredas), Lideta sub-city (3 weredas), and Gulele sub-city (2 weredas) (Fig 6.5). Furthermore, a secondary cluster of rifampicin-resistant pulmonary TB (RR= 2.82, $p < 0.001$) was detected in 11 adjacent weredas belonging to Nifas Silk Lafto sub-city (9 weredas),

Arada sub-city and Kirkose sub-city (1 wereda per each sub-city). In the most likely cluster, the case notification was 3.9 per 100,000 people, the cases in this cluster accounted for 41.6% of all cases reported in the city. Using a purely spatial analysis with a maximum spatial cluster size of ≤ 50% of the population, statistically significant low rate of clustering (cold spot) was observed in 43 weredas (RR=0.48, $p < 0.001$). The number of the observed count, expected count, relatively risk, log likelihood, and p-value for statistically significant clustering are shown in Table 6.2.

To investigate the possibility of a smaller cluster, an analysis was performed with a maximum spatial cluster size of ≤ 25% of the total population. This analysis identified the most likely significant cluster in the same geographic locations.

Table 6.2 Statistically significant purely spatial clusters of rifampicin- resistant TB in Addis Ababa, Ethiopia

Cluster ID	No of weredas	No of cases	Expected cases	Relative risk	Log likelihood ratio	p-value
Most likely High-rate cluster	25	104	61.34	2.19	17.49	< 0.001
Secondary cluster	11	42	18.29	2.82	13.55	< 0.001
Low rate cluster	43	54	91.28	0.48	13.00	< 0.001

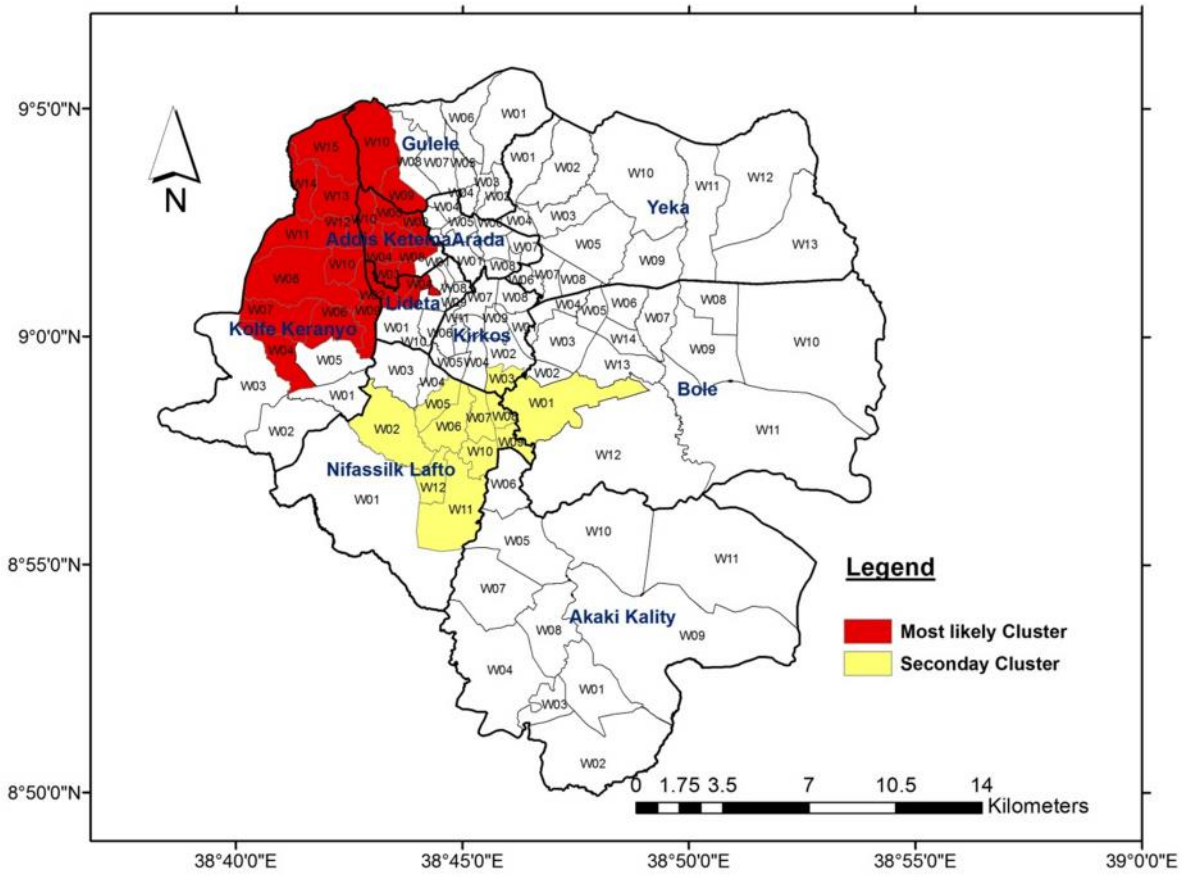


Figure 6.5 Spatial clusters (hotspots) of the rifampicin resistance TB in Addis Ababa, Ethiopia

6.4.3 Space-time cluster

Using a spatial window that included 50% of the population at risk, the results of the space-time analysis are similar to the purely spatial analysis in that the areas of with excess incidence of TB from the purely spatial analysis were also statistically significantly high in the space-time analysis. Two statistically significant space-time clusters were detected for the high occurrence of TB. The first cluster in 18 adjacent weredas of Addis Ketema sub-city (9 weredas), Kolfe Keranyo sub-city (6 weredas), Lideta sub-city (2 weredas), and Gulele sub-city (1 wereda) for the year 20015 and 2016 (RR = 2.75, $p < 0.001$), with 55 observed cases and 23.23 expected

cases. The second cluster was observed in Nifas Silk Lafto sub-city and Yeka sub-city in adjacent weredas (RR=9.6, p<0.001) for the year 2015, with 8 observed cases and 0.86 expected cases (Table 6.3). This shows that the most likely cluster from the purely spatial remained statistically significant only for the year 2015 and 2016.

Table 6.3 Statistically significant space-time clusters of rifampicin- resistant TB in Addis Ababa, Ethiopia

Cluster ID	No of weredas	No of cases	Expected cases	Relative risk	Log likelihood ratio	p-value
First cluster	18	55	23.23	2.75	17.97	< 0.001
Second cluster	2	8	0.86	9.60	10.82	< 0.001

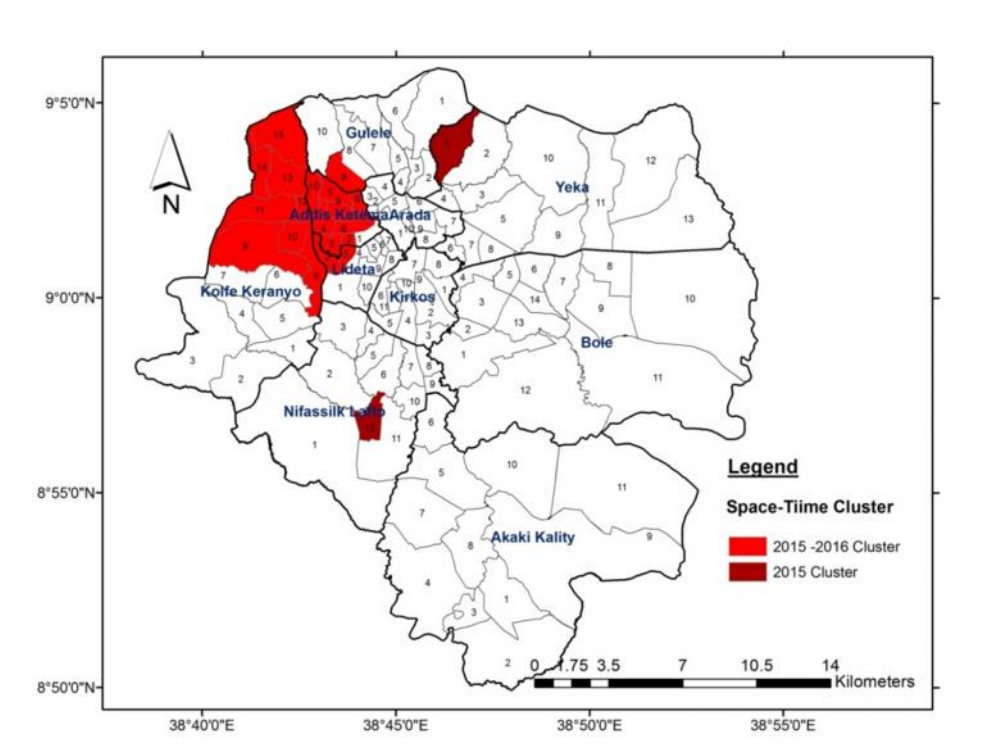


Figure 6.6 Space-time cluster of the rifampicin resistance TB in Addis Ababa, Ethiopia

6.4.4 Factors associated with geospatial clustering

A univariate analysis of the possible association between purely spatial clustering with individual-level (demographic, epidemiological, genotypic) and census- level variables was done. Census-level variables population density (people/hectare) of weredas and per capita income (consumption) were significantly associated with spatial clustering. HIV co-infection was significantly dissociated with spatial clustering. Gender, Age, previous TB treatment history, genotypic clustering, T3_ETH and CAS1_KILI strain types were not statistically associated with spatial clustering.

Table 6.4 Risk factors for most likely spatial clustering of rifampicin-resistant *Mycobacterium tuberculosis isolates* at wereda level in Addis Ababa, Ethiopia

Characteristic		Spatial pattern		OR(95CI)	p- value
		Clustered (%)	Unique (%)		
Individual-level variables					
Gender	Male	64(46)	76(54)	1.5(0.9-2.4)	0.162
	Female	46(37)	80(64)	1	
Age groups	≤15	3(43)	4(57)	1.0(0.2-4.8)	0.955
	16-64	103(41)	149(59)	0.4(0.1-1.4)	0.137
	≥65	4	0		
Previous TB	No	34(34)	66(66)	0.6(0.4-1.1)	0.078
treatment	Yes	63(45)	76(55)	1	
HIV status	Positive	14(33)	28(67)	0.3(0.2-0.7)	0.012
	Negative	38(60)	25(40)	1	
Genotypic	Clustered	6(25)	18(75)	0.5(0.2-1.5)	0.306
	Unique	16(41)	23(59)	1	
CAS_KILI	Yes	11(50)	11(50)	2.7(0.9-8.1)	0.118
	No	11(27)	30(73)	1	
T3_ETH	Yes	6(27)	16(73)	0.6(0.2-1.8)	0.512
	No	16(39)	25(61)	1	
Census-level variables					
Population	≤200	7(9)	72(91)	0.1(0.04-0.3)	<0.001
density	≥201	18(49)	19(51)	9.7(3.6-26.7)	<0.001
(people/hectare)					
Per capita	≤8000	9(90)	1(10)	50.6(6.0-427.4)	< 0.001
income *	8000-10000	3(15)	17(85)	0.6(0.2-2.2)	0.418
	10000-12000	13(35)	24(65)	3(1.2-7.5)	0.018
	>12000	0	49(100)		

- Per capita income for consumption 2015 data aggregated at sub-city level

6.5 Discussion

Geospatial, spatio-temporal and temporal analyses are important in epidemiology in order to detect aggregation of disease cases, to test the occurrence of clustering with its associated risk factors. To the best of our knowledge, this study is the first report to assess and detect statistically significant clusters of drug resistance TB in Addis Ababa, Ethiopia. The present study indicates that the spatial distribution of drug resistance TB in the city was nonrandom. Significantly clustering was detected with hotspots in some areas of the city. The reason for the high occurrence of drug resistance TB in the identified hotspots area may be attributable to either there is a true high rate of disease in a clustered area, or there are falsely low measured rates in other areas. The first possibility may arise from the poor socio-economic conditions of the people, and poor nutritional status. Several studies have reported that the socioeconomic situation in an area influences the incidence of TB in that area (Deribew *et al.*, 2009; Liu *et al.*, 2005; Mishra *et al.*, 2005; Ray *et al.*, 2005; van den Hof *et al.*, 2016). Population density, poverty and overcrowding are the major factors for disease transmission(Acevedo-Garcia, 2001; Haase *et al.*, 2007). We found similar risks in our population. The weredas in the most likely cluster are characterized by low socioeconomic status, high unemployment rates, homelessness, and poor quality housing conditions (AACA, 2014a; AACA, 2014b; AACA, 2014c; AACA, 2014d; Tegegne Gebre-Egziabher, 2015). About, 90% of Addis Ketema sub-city was included in the most likely hot-spot zone. This sub-city has consistently shown a very high poverty across years (Tegegne Gebre-Egziabher, 2015). It is a destination area for the migrants from all over the country. It is also a workplace for many people in the surrounding. *Mercato*, considered to be the largest open-air market in Africa, is located in this hot-spot area. It could be a source point for the dissemination of tuberculosis across the country, because every trader in the country spend some time in Mercato. The second possibility may arise where there is a problem with health

seeking behavior. Hotspots areas for tuberculosis were reported in many parts of the world using GIS tools (Onozuka and Hagihara, 2007; Tiwari *et al.*, 2006; Touray *et al.*, 2010).

Knowledge about the presence of hotspots of drug resistance TB can help in intensifies the response measures in the identified areas including improving infection prevention measures in health facilities. Administrative health authorities should focus more seriously in the case finding activities in identified areas; strengthen public awareness at the community level, further promotion of general health and hygiene.

The observed increase in notification of drug resistance TB cases for the last four years was in line with the Federal Ministry of Health and the Health Bureau efforts to expand the number of laboratories provide tuberculosis drug susceptibility test service using Xpert MTB/RIF.

The present study has some potential limitations. The study period is short (4 years 1/04/2013-1/04/2017). Additional studies are needed to evaluate the spatial and temporal changes of drug resistance TB or a longer study period. Data for cluster analysis was aggregated to the wereda level, better to go for individual level. It is an established fact that the incidence of TB increases with cigarette smoking, alcohol abuse, injection drug use and malnutrition (Comstock *et al.*, 1974). They adversely affect the immune system, and so could enhance the TB incidence. The present study focus on the cluster and some associated factors. Future studies could focus on the effect of various socio-economic and environmental risk factors on the high occurrence of drug resistance TB in the hot spot areas. Extrapolation of census data from 2004 to provide up to date denominator population numbers could be affected by uneven population growth and massive housing project across the city. The data on per capita income was at sub-city level, in future it

would be better to conduct poverty assessment study at wereda level or beyond that to investigate the association between hot-spot and poverty.

6.6 Conclusions

This study utilized spatial analysis and GIS tool to analyze the spatial pattern and associated risk factors of drug resistance TB in Addis Ababa city. Both the purely spatial and spatial-temporal analyses of scan statistic (SaTScan) were applied to reveal spatial characteristics of the TB.

Using data from 2013 to 2017, the study has shown the presence of most likely and secondary cluster of TB in Addis Ababa city. It showed heterogeneity of spatial pattern of TB within the city. This finding can help the TB program to strengthen the control measures in the hotspot areas and to issue future strategies for more effective control of the disease.

More detailed individual level investigations are needed in the identified clusters to ascertain the most important determinants of disease distribution and analyses the burden of drug resistance TB in the identified areas. The use of cluster analysis and GIS tools for regular surveillance of TB incidence in the city may help the TB program in disease control activities.

Chapter 7. General discussion

Introducing best algorithms for diagnosis of tuberculosis has the potential to make a huge difference to the lives of millions of people. Choosing among the available several diagnostic tools and possible combination requires thinking beyond looking for just the accuracy of individual diagnostic tool. In this study a mathematical modeling was applied using the virtual implementation approach to select the best algorithms among eight possible alternatives. The full roll-out of Xpert MTB/RIF is selected as the best option and followed by Targeted use of Xpert MTB/RIF for HIV positive and high MDR risk groups with Same-day LED fluorescence microscopy for all other presumptive TB cases in case of budget shortage. As of 2017 the city government of Addis Ababa Health Bureau has started implementing Xpert MTB/RIF for all presumptive TB cases and we expect huge difference on reducing the TB burden in the city in the future.

From TB-control point of view, it is relevant to understand whether specific genotypes are associated with drug-resistant cases and, in particular, if these resistant strains are successfully transmitted within the community. In our study, CAS1_KILI was significantly associated with first-line anti-TB drugs resistant cases except the drug Ethambutol. However, CAS1_KILI family was not statistically a risk factor for clustering, a trend toward increased clustering was observed; in addition, it was among isolates that are at a high rate of recent transmission. In contrary, infected with T3_ETH isolates was not statistically a risk factor for first-line anti-TB drugs resistant cases and the reverse was true for the drug Ethambutol; but a trend toward increased association with first-line anti-TB drugs were observed. T3_ETH was significantly associated with clustering and it was at a highest rate of recent transmission. All of the FLQ drug resistant isolates observed in this study was belonging to this family. This scenario might

indicate that compensatory mutations in CAS1_family favors the fitness of drug-resistant CAS1_KILI family and continuing being transmitted in the community; or infection by CAS1_KILI could be mild as compared to infection by T3_ETH family. Searching the responsible gene for fitness is urgently required. High proportion (42.1%) of multi-drug resistant groups was observed among T3_ETH. They were the most abundant (35.5%) isolates in this study.

Naturally, larger sample sizes and longer recruitment periods increases the percentage of clusters among recovered *Mycobacterium tuberculosis* isolates. Considering the comparatively small size in this study, our results likely underestimate the proportion of isolates that were recently transmitted and also the proportion of resistant isolates transmitted among TB patients in Addis Ababa city

Chapter 8. Conclusions and recommendations

In conclusion, we recommend Addis Ababa Health Bureau TB program to consider implementing the full roll-out of Xpert MTB/RIF. It is predicted to be the best option to substantially reduce the TB burden in Addis Ababa and was considered cost effective.

The high rate of drug resistance against first-line anti-TB drugs, existence of pre-XDR TB, high rate of recent transmission, high rate of molecular clustering, and geospatial clustering indicated that ineffectiveness in the TB control program. A substantial number of drug-resistance TB is circulating in the community. CAS1_KILI and T3_ETH spoligotypes are important strain of rising incidence of drug-resistant TB in Addis Ababa city. Rifampicin –resistant TB are clustered around Addis Ketema sub city and in the surrounding weredas. High population density and poverty are among the risk factors for spatial clustering.

The findings suggest that strengthen TB control program and strengthen diagnostics facilities for first- and second line drug susceptibility testing is crucial. Establishing molecular typing (IS1610 RFLP, Spoligotyping, MIRU-VNTR, Whole genome sequencing) laboratory is also important for the city. The use of molecular typing and GIS tools for regular surveillance of TB incidence in the city help the TB program in disease control activities. Improved infection control measures need to be implemented. Drug resistance TB is not randomly distributed in the city. Targeted intervention in hot-spot areas is urgently needed. Intensify case finding effort using health extension workers, mass screening, allocate more resources, establish Xpert MTB/RIF test in health centers, improving the income of people particularly in hot-spot area, and improving the

housing conditions are among the required interventions. In general there is a need for more efforts to limit the spread of TB disease.

We also recommend further studies on molecular epidemiology using high resolution whole genome sequencing, spatial clustering at individual home address level having longer period of time, associated risk factors for drug resistance TB transmission, and further detailed study on the virulence of CAS1_KILI and T3_ETH strains.

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Supplement material

Supplement material 1: Key input variables to the integrated model

A. Sensitivity and specificity data

Sensitivity of test for TB	HIV+	44.6%	50.6%	80.0%	**(Boehme *et al.*, 2011)**
	HIV-	72.3%	78.3%	89.0%	
Specificity of test for TB	HIV+	100%	100%	97%	
	HIV-	99.4%	99.4%	99%	
Sensitivity of clinical diagnosis for test negative suspects	HIV+	51.9%	51.9%	80%	**(Swai *et al.*, 2011)**
	HIV-	51.9%	51.9%	80%	
Sensitivity improvement of LED fluorescence microscopy over ZN microscopy		+6·0%			**(WHO, 2011a)**
Specificity improvement of LED fluorescence microscopy over ZN microscopy		0·0%			**(WHO, 2011a)**
Sensitivity improvement of the 3rd sputum over two sputum		0.2%			

B. Base case data

Annual new smear-positive and tuberculosis cases per health center	**46**	**(AAHB, 2015), survey**
Annual new smear-negative and tuberculosis cases per health center	**67**	**Through survey**
Annual number of retreatment patients per health center	**7**	**Through survey**
Proportion of new presumptive TB cases that are smear positive at diagnosis	**9.0%**	**Through survey**
Proportion of tuberculosis cases needing re-treatment	**2%**	**Through survey**
Proportion of HIV-positive tuberculosis cases	**25.6%**	**Through survey**
Proportion of smear-negative presumptive tuberculosis cases that had radiograph and antibiotic trial	**50%**	**Through survey**
MDR tuberculosis in new tuberculosis cases	**2.7%**	**(Program, 2015)**
MDR tuberculosis in retreated tuberculosis cases	**17%**	**(Program, 2015)**
Diagnostic lost to follow-up rate	**13.5%**	**Through survey**

C. Costs variables

LED fluorescence microscope	$1250·0	**(Diagnostics, 2013)**
Xpert cartridge cost per test	$9.98	**(Diagnostics, 2013)**
Xpert MTB/RIF machine 4 cell	**$17500**	**(Diagnostics, 2013)**
Xpert annual maintenance 4 cell	**$1800**	**(Diagnostics, 2013)**
Drug sensitivity cost per test	**$19**	**Ethiopian NTP**
Microscopy cost per test	**$1.5**	**Ethiopian NTP**
Radiograph	**$6.9**	**Ethiopian NTP**
Monthly drug cost for standard regimen	**$3**	**Ethiopian NTP**
Monthly drug cost for retreatment regimen	**$18.8**	**Ethiopian NTP**
Monthly drug cost for MDR tuberculosis regimen	**$119.4**	**Ethiopian NTP**
Annual employment costs for a laboratory technician	**$3200**	**Addis Ababa Health Bureau**
Annual employment costs for a laboratory assistant	**$2400**	**Addis Ababa Health Bureau**

LED-light emitting diode. ZN- Ziehl Neelsen. WHO- World Health organization. MDR- Multi drug resistant NTP- National tuberculosis program

According to Witness DES model, '*entities*' , '*attributes*' , '*activities*', '*queues*' and '*resources*' were defined (supplemental material 2). In addition, the DES Witness model developed in this study contained '***variables***' (where input parameters read in from Excel spreadsheets and outputs were recorded), '***histograms***' (to graphical report the distribution of particular outputs) and '***time series***' (to graphical record outputs over time).

Supplement material 2: Element types

Element type	**Used to represent**
Entities:	Patients (Presumptive TB cases and TB cases) Sputum samples
Attributes:	HIV status TB status New or retreatment presumptive TB case Test result Time diagnosis began
Activities:	Patients - Reception - Sputum Collection - Direct Observed Treatment Short Course (DOTS) training - Receiving diagnosis - Treatment stages Sputum samples - Prepare sample for microscopy - Stain - Complete microscopy examination - Prepare for Xpert - Load Xpert machine - Run Xpert machine - Complete Xpert test
Queues:	Patients - Waiting at reception - Waiting for sputum collection, diagnosis, or DOTS training - Waiting before returning from home to provide sample or receive diagnosis Sputum sample - Waiting for preparation - Waiting for staining

	- Waiting for examination - Results waiting to communication to patient
Resources	Laboratory technicians Laboratory technologist

Supplement material 3: Additional parameters

Input Parameter	Description	Value	
Diagnostic Centre Working days Minutes in day Max wait time	No. of workings days in 1 year No. of working minutes in day Maximum time a patient will wait for diagnosis.	251 days 480 minutes 90 minutes	
Processing Times Diagnostic Centre process times	Duration of processes at the diagnostic centre - Sample Collection - Clinician time - 3 - DOTS training – 10	10 Minutes 3 Minutes 10 Minutes	
Laboratory process times Microscopy	Batch Stain – ZN - LED - Overall drying time - Examination time - Smear+ ZN - Smear+ LED - Smear- ZN - Smear - LED	14 minutes 24 minutes 45 minutes 5 minutes 2.5 minutes 10 minutes 2.5 minutes	
Laboratory process times Xpert MTB/RIF	Preparation time - Test time - Examine time	15 minutes 115 minutes 0.5 minutes	
X-ray	Time allowed to get X-ray	1 day	
Key Probabilities Treatment follow-up test is Smear Positive	Standard Regimen after - Intensive Phase - Continuation Phase 1 - Continuation Phase 2 Retreatment Regimen - Intensive Phase - Continuation Phase 1 - Continuation Phase 2	5% 5% 0.2% 8% 0.2% 0.1%	
HIV	HIV Status unknown at point of TB diagnosis	89.6%	
X-Ray	Requested following a negative	70%	

	smear Requested following a negative Xpert	25%	
GeneXpert	Failure Rate	2%	
Treatment Times Standard Regimen Retreatment Regimen MDR-TB Regimen	Intensive Phase Continuation Phase 1 Continuation Phase 2 Intensive Phase Continuation Phase 1 Continuation Phase 2 Overall time	2 Months 3 Months 1 Month 3 Months 2 Months 3 Months 24 Months	
Short course antibiotics	Time to complete a short course of antibiotics for smear negative patients with TB symptoms	2 weeks	
Laboratory Staffing assumptions Availability Numbers Xpert staffing	Lab staff will be available for the full working day Enough staff will be available to avoid bottlenecks of longer than 10 days. If not achieved increased staffing will be used and the model re-run The same level of staffing will be in place if Xpert is implemented as are available currently. Utilization recorded and cost of staffing will be pro-rated by change in utilization.	480 minutes per day & 251 days per year. Variable by diagnostic center. As above	

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