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**Modified Diagnostic approach and Molecular
Epidemiology of Childhood Tuberculosis in Hawassa,
Ethiopia**

**By
Moges Desta Ormago**

*A Dissertation Submitted to the School of Graduate Studies of Addis Ababa University
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in
Microbial, Cellular and Molecular Biology (Applied Microbiology)*

June, 2018

Addis Ababa, Ethiopia

**ADDISABABA UNIVERSITY SCHOOL OF GRADUATE
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Declaration

I, the undersigned, declare that this PhD Dissertation is my own original work and has not been presented for a degree in any other University, and all sources of materials used for the Dissertation have been duly acknowledged.

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This Dissertation has been submitted for examination with our approval as the research supervisors of the candidate.

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Abstract

Childhood TB accounts to 1 million (10%) of the estimated 10.4 million new cases of TB globally. It is known to be the major contributor of childhood morbidity and mortality. The prevalence of childhood TB/HIV co-infection and multidrug resistance (MDR) presumed to have grown up with the advent of HIV. Unfortunately it has been given less attention as a challenge at international level; there are no sufficient epidemiological data even in high burden countries and the effects are not included with the national policies and strategies i.e. is neglected. Due to this scarcity of epidemiological data the attempts that have been made to develop tools for childhood TB diagnosis, treatment, control and to understand how the immunological and pathological responses differ from that of the adult is very little. It is in recognition of these gaps of knowledge and attention that the present work was set with the following objectives: to use modified approach for diagnosis and determine the prevalence of childhood active TB cases, identify the strains of *Mycobacterium tuberculosis*, determine their drug susceptibility pattern and assess the treatment outcome.

A prospective cross sectional study was done on 700 children clinically suspected for TB and 350 of them were used to determine the prevalence, molecular characterization and drug susceptibility pattern of TB isolates and the remaining 350 were used to evaluate modified childhood TB diagnostic approach. A five year retrospective cross sectional study was also done to evaluate the treatment outcome of children with TB in the study area.

The results showed an overall prevalence of smear positive (7.4%) and Culture confirmed TB cases (9.7%). Drug susceptibility testing showed one (2.9%) MDR case from the isolates and Spoligotyping of 34 isolates revealed 15 different patterns, 11 of which corresponded to Shared International Types (SITs) and 4 to orphan strains. The dominantly identified strains

were SIT 53, SIT 149, and SIT 442. The isolates belonged to Euro-American lineage (94.1%) and East-African-Indian lineage (5.9%). A 13.2% incremental yield was obtained due to testing of pooled samples compared to either of the first or second sample using GeneXpert MTB/RIF assay in diagnostic evaluation study. The overall treatment success rate among all children with TB at the study facilities was 39.7%. The lower prevalence of pediatric pulmonary TB could be due to the less sensitive nature of culture and microscopy diagnostic tools thus the modified diagnostic approach may show the real magnitude by increasing case detection. Clustering of strains in spoligotyping suggested an on-going and active transmission of *M. tuberculosis* in the study area. The identified MDR case showed DST is warranted to optimize the management of childhood TB. The Overall treatment success rate among all children with TB was unsatisfactory compared to WHO target of 85% and 54.7% of children with TB in the study facilities were transferred out. There should be a system to track transfer out cases are on treatment.

Keywords: Children, *M. tuberculosis*, Prevalence, Diagnosis, Drug Susceptibility Testing, Treatment outcome

Acknowledgement

First of all, I would like to express my heartfelt gratitude to my supervisor, Dr Adey Feleke for her genuine approach, her excellent guidance, full support, I am thankful to her for timely comments and corrections and critical revision of the monograph. My sincere appreciation goes to my advisor Dr Mekuria Lakew for his support and critical comments on developing the content of the proposal before the start of the research and his input in the preparation of the manuscript, especially his generous advice and continuous follow-up throughout my study period.

Moreover, I gratefully acknowledge Dr. Daniel Gemechu, Dr. Kefyalew Taye, Professor Luis E.Cuevas and Dr Silvia Blanco conceived the study and gave attention to detail, and in depth understanding that they extended to me during the entire work starting from designing of the research and their timely comment in manuscript preparation.

Aklilu Lemma Institute of Pathobiology (ALIPB) specially Mr Aboma Zewde and Mr Temesgen Mohammed for their technical support in spolypotyping and RD9 deletion typing. My special thanks also go to all laboratory team of South Nations Nationalities and People Public Health Laboratory (SNNPRPHL), especially Ms Zeyneba Temam, Mr Wolde Abreham and Mr Merihun Dawit for their technical assistance during TB culture, SD test and Genotypic DST . My appreciation goes to staff of Hawassa University Comprehensive Specialized Hospital and Adare General Hospital for their participation and help in enrolling study subjects and providing important information about the study subjects without whom this study couldn't have been completed. I gratefully acknowledge Addis Ababa University, Hawassa University and Grand challenges Canada through TB REACH PROJECT ETHIOPIA (grant number S40252-01) for their financial support.

Last not least, I would like to thank my wife Mrs Netsanet Nigussie for her assistance and supporting me all the way in all aspects of this thesis. I am grateful for all individuals who offered me invaluable suggestions and comments on various issues. Above all, let all praises and thanks be to GOD.

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Abbreviations /Acronyms

APC	Antigen presenting Cells
BCG	Bacillus Calmette - Guerin
CAMP	Antimicrobial Peptide Cathelicidine
CBN	Conformal Bayesian network
CDC	Center of Disease Control
CFP-10	Culture Filtrate Protein 10
CI	Confidence intervals
DCs	Dendritic Cells
DC-SIGN	The dendritic cell specific intercellular adhesion molecule3 grabbing non integrin
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Treatment Strategy
DR	Direct Repeat
DST	Drug Susceptibility Testing
EA	Euro-American
EAI	East-African -indian
EPTB	Extra Pulmonary Tuberculosis
ESAT-6	Early Secretory Antigenic target 6 KDa
FMOH	Federal Ministry Of Health
HIV	Human immunodeficiency virus
IFN	Interferon
INH	Isoniazid

IO	Indo-occenic
IRAK	Interleukin-1 receptor associates kinase-4
IS	Insertion Sequence
ITAM	Immunoreceptor tyrosine containing activation motif
KBBN	Knowledge based Bayesian network
LAM	Lipoarabinomanan
LJ	Löwenstein–Jensenmedium.
LPA	Line probe assay
LSPs	Large Sequence Polymorphism
MDR-TB	Multi Drug Resistance Tuberculosis
MGIT	Mycobacteria growth indicator tube
MIRU-VNTR	Mycobacterial interspersed repetitive unit-variable number tandem repeat
MR	Mannose Receptors
MTB	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> complex
MUT	Mutation
NTM	Non-tuberculous mycobacteria
NTP	National Tuberculosis Program
OR	Odds ratios
PCR	Polymerase Chain Reaction
PIMS	Phosphatidylelinositol mannosides
PtPA	Protein tyrosin phosphate

RD	Regions of Difference
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
SIT	Shared international type
SNPs	Single Nucleotide Polymorphisms
SOCS1	Suppressor of cytokine signaling pathway1
STB	Smooth Tuberculosis
TB	Tuberculosis
TbD1	Tuberculosis deletion 1
TLRs	Toll like receptors
VNTR	Variable Number Tandem Repeats
WHO	World Health Organization
WT	Wild type
XDR-TB	Extensively drug resistant TB Abstract

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Chapter1 Introduction

Tuberculosis is an infectious disease mainly caused by bacterium *Mycobacterium tuberculosis*. The causative agent is most commonly transmitted from a patient with infectious pulmonary tuberculosis to other persons by droplet nuclei, which are aerosolized by coughing, sneezing, or speaking. The tiny droplets dry rapidly; the smallest (<10µm in diameter) may remain suspended in the air for several hours and may gain access to the terminal air passages when inhaled (Fauci and Jameson, 2005; Triccas and Counoupas, 2016)

One-third of the world's population infected with TB bacilli and 90% of them were remained latent and 10% of them were developed acute/chronic infection and 20% of the acute/chronic infected cases go extra pulmonary. Out of latent TB cases 5-23% change to active TB through reactivation due to weakening of the immune system by other illnesses, HIV infection and other immune suppressed conditions and with the advent of HIV the risk of infection though increased: about 374,000 deaths are due to TB co-infection (Mesfin *et al.*, 2012; WHO, 2017).

Tuberculosis affects usually the lungs but could go extrapulmonarily to include the lymph nodes, pleura, genitourinary tract, bones and joints, meninges, peritoneum and pericardium. However, virtually all organ systems may be affected (Fauci and Jameson, 2005).

Miliary tuberculosis constitutes 10% of cases of extra pulmonary tuberculosis. It is characterized by the diffuse presence of small nodules throughout the body. The spleen, liver and lung are frequently involved. Tuberculosis lymphadenitis (scrofula) is the most common form of extra pulmonary tuberculosis. It is more common in children and young

adults than in older persons. Pleural tuberculosis constitutes 23% of the cases with extra pulmonary tuberculosis and effusion usually occurs 3 to 6 months after the primary infection. Genitourinary tract tuberculosis is responsible for up to 13% of extra pulmonary disease. It is a late manifestation of the infection. Skeletal tuberculosis is less common and vertebrae are involved in 50% of cases. Tuberculosis meningitis is the most common form of CNS involvement and is localized mainly to the base of the brain. Abdominal tuberculosis frequently affects the peritoneum. Pericardial tuberculosis is usually due to hematogenous spread (Habermann and Ghosh, 2015).

About 15-20% and 2-7% of all TB cases are among the children living in high burden and low burden countries respectively. The most common type of TB disease in children is pulmonary TB (70-80%) and extra pulmonary TB is also common (20-30%) of all child TB cases. It is estimated that over 67 million children are infected with TB and therefore at risk of developing disease in the future and 2 million children are estimated to have MDR (Dodd *et al.*, 2016). Over 1 million children under 15 years fall ill with TB, over 30,000 get infected with MDR strains and 253,000 die of TB annually though preventable and curable disease often missed or over looked due to non specific symptoms and difficult in diagnosis (Graham, 2012; Jenkins, 2016; WHO, 2017).

Despite the fact that TB is a cause for significant childhood mortality and morbidity, TB remains to be neglected. Very little is done to even generate good quality epidemiological data.

The resurgence of TB that led the WHO TB Programme to reassess the TB control strategy in the early 1990s. The implementation of DOTS strategy (1995-2005) and its

successor Stop TB Strategy (2006-2015) has helped countries to improve national TB control programmes (NTPs) and document major success stories in TB control in general (WHO, 2009). Between 2000 and 2015, TB treatment averted 49 million deaths globally. The fact that World Health Organization (WHO) recommended children to be treated within the National TB Programme (NTP) and be notified through their routine reporting systems was also a positive outcome of the global effort to eradicate the disease once for good (WHO, 2016).

Unfortunately, because of most children have paucibacillary TB that is harder to diagnose with sputum smear microscopy i.e. the first 2 years children don't have immunity, even up to six is on development and the TB bacilli extend to cause EPTB which is diagnosed smear negative and not reported, and even not able to expectorate sputum to diagnosis, lack of expertise to diagnose and treat childhood TB, failure to develop new diagnostic protocols, besides diagnostic challenges; children diagnosed with TB are not always reported to national TB programmes because the NTPs focus to adult TB so as to achieve high cure rate thereby decreasing community transmission and the linkages among pediatrics diagnostic clinics and national TB programmes are weak, data from national surveys including children are limited, the optimal dosage of anti-TB drugs for children has been a challenge due to limited information on pharmacokinetics of the drugs and high risk to develop severe forms of the disease and early childhood deaths. Thus, the control strategies could not benefit children as planned. Instead children in developing countries that survived the severe illnesses and often grow as future latent TB cases and continued to serve as reservoirs of TB fueling the adult infection in the society and challenging the end TB strategy (Cui X *et al.*, 2017; WHO, 2014a).

Although TB is one of the leading causes of infectious death and hospitalization among adult, it is much more numbers are expected to occur in children. At the moment only 5% of the cases are reported in children, the rest are waiting for better methods of diagnoses to come yet (CDC, 2014; Graham *et al.*, 2004). The issue of childhood TB diagnosis is not well appreciated, Scientists failed to attain reduction of TB prevalence and transmission by identification of more than 70% active TB cases and treating 85% of them under DOTS treatment regimens (Dangisso *et al.*, 2015; WHO, 2002), one of WHO eradication strategies.

TB programme still lack the capacity to pick adult TB which could worsen the children position in TB transmission (WHO, 2015). From the total estimate of pediatrics TB worldwide 31% are found in African region. Ethiopia being one of the 30 TB high-burden countries is expected to share high burden of childhood tuberculosis. Despite advances in diagnostics, a considerable proportion (43%) of the pulmonary TB cases are still diagnosed clinically rather than bacteriologically (WHO, 2016). This is because of the non specificity of clinical and radiological findings and paucibacillary nature of the disease and the difficulty to get sputum from young children, the efficacy of the diagnostic for childhood tuberculosis is not good enough to show clearly the magnitude of TB in children (Nelson and Wells, 2004). Alternative diagnostic methods for childhood TB are much desired.

Of those children that were eligible to start TB treatment only 7.1% were reported to have been started on TB treatment (WHO, 2016). Using the present diagnostic tools over 95% treatment success rates of childhood tuberculosis with PTB and EPTB are traceable in

developed countries. In developing countries, rates of successful treatment are often much lower (Graham *et al.*, 2004)

Besides these, drug-resistant TB threatens global TB control and remains a major public health concern in many countries. The pattern of drug resistance in children have generally been found to be similar to adult from the same geographic area (Rekha *et al.*, 2011) suggesting whether primary or retreatment MDR TB, they are transmitted to them by adult.

DST methods include both phenotypic (conventional) and genotypic (molecular) testing methods. The molecular assays like Line-Probe Assays for DST is highly sensitive and specific, yield rapid results require less training and infrastructure and can be used more widely (WHO, 2008c; WHO, 2016). Whereas conventional method of DST is slow and cumbersome, requiring sequential procedures for isolation of mycobacteria from clinical specimens, identification of *Mycobacterium tuberculosis* complex, and in vitro testing of strain susceptibility to anti-TB drugs. During this time patients may be inappropriately treated, further continuing the spread and amplification of drug resistant strains thus making development of novel technologies for rapid detection of anti-TB drug resistance a priority in TB research and development in general and in children in particular (WHO, 2008c).

CDC recommends that control of TB in children receive high priority since a case of tuberculosis in an infant and young child is inherently due to recent transmission (Marquez *et al.*, 2012). Analysis of the genetic population structure of *M. tuberculosis* strains isolated from children crucial for tuberculosis control program for better understanding of transmission dynamics and phylogenetic (Borrell and Gagneux, 2011;

Rosales *et al.*, 2010). To this effect, many methods have been developed and tried. Spoligotyping is a widely used molecular method for simultaneous detection and typing of *M. tuberculosis* complex bacteria and providing epidemiologic information on strain identities (Gori *et al.*, 2005), and it has less discriminatory power compared to the restriction fragment length polymorphism (RFLP) typing using insertion element IS6110 (Zanden *et al.*, 2002).

In Ethiopia TB has been recognized as the major public health problem. According to the 2015 health and health related indicators of the FMOH, TB is recognized of the six leading causes of death in Ethiopia (FMOH, 2015). Among smear positive patients registered on DOTS Program only 14% completed their treatment and 64% were cured (WHO, 2008a). Yet, the focus area of National TB control program is in adult cases. It is more recently Ethiopia stipulated Xpert MTB/RIF as the initial diagnostic test for children presumed to have TB, in order to increase bacteriological confirmed pediatric TB cases. Ethiopia like other developing countries, Childhood tuberculosis was not given priority and the health facilities were not adequately organized to give access to the majority of rural community that suffer from limited expertise to diagnosis childhood tuberculosis. Unfortunately, it is well known that testing only one specimen with Xpert in children results in a small proportion (typically 20%) of patients with a positive result (Graham *et al.*, 2004).

Better ways to identify more children are needed. The outcome of children with TB, however, is largely unknown, since children are more likely to have smear-negative PTB

or extra-pulmonary TB (Mun oz-sellart *et al.*, 2009) and even with the available confirmatory diagnosis there is limited study in prevalence, strain type and their drug susceptibility pattern of childhood tuberculosis in Ethiopia.

Objectives

General Objective: The aim of this study is to evaluate modified diagnostic approach and molecular epidemiology of childhood tuberculosis.

Specific Objectives

To assess Childhood tuberculosis suspected cases who visited selected health facilities in Sidama zone, and there by:

- determine prevalence of active tuberculosis
- determine drug susceptibility pattern of *Mycobacterium tuberculosis* isolates
- identify the strains of *M .tuberculosis* in circulation causing tuberculosis in the children population
- improve the detection and confirmation of children with TB through use of pooled gastric aspirate or expectorate, an innovative diagnostic approach

To evaluate the treatment outcome of children with TB registered for treatment in the two Hawassa Public Hospitals

Design of the thesis write up

The thesis comprises of 6 chapters: Chapter 1 Introduction; Chapter 2 Background; Chapter 3 Materials and Methods; Chapter 4 Results; Chapter 5 Discussion; Chapter 6 Conclusion and recommendations

Chapter2 Background

2.1 *Mycobacterium tuberculosis* complex

Tuberculosis (TB) is a disease caused by closely related acid fast bacteria known as the *Mycobacterium tuberculosis* complex (MTBC), with *M. tuberculosis* being the most common infecting species in humans (Brown *et al.*, 2010). MTBC comprises various bacterial species and sub-species sharing 99.9% DNA sequence identity (Brites and Gagneux, 2015) but differing in their primary host range, pathogenicity and other phenotypes (Frothing *et al.*, 1998). MTBC includes human associated pathogens *M. tuberculosis* and *M. africanum* and the primarily animal infecting species *M. bovis* (in cattle), *M. microti* (in voles), *M. caprae* (in goats), and *M. pinnipedii* (in seals and sea lions) (Brites and Gagneux, 2015).

Mycobacterium bovis has a wide animal host range outside the bovine species, and occasionally humans. *M. bovis* has been found to be a source of tuberculosis (TB) in humans infected through drinking contaminated unpasteurized milk or inhaling aerosols produced by diseased farm animals (Mignard *et al.*, 2006). *M. canettii*, is the most recent of the common ancestors. It, with MTBC, causes diseases in humans and is part of the so-called 'smooth TB bacilli' (STB) and unlike MTBC it undergoes frequent horizontal gene transfer and inter-strain recombination (Brites and Gagneux, 2015).

Phylogenetic studies have shown that the MTBC comprises of at least seven distinct lineages distributed in different geographical locations around the world.

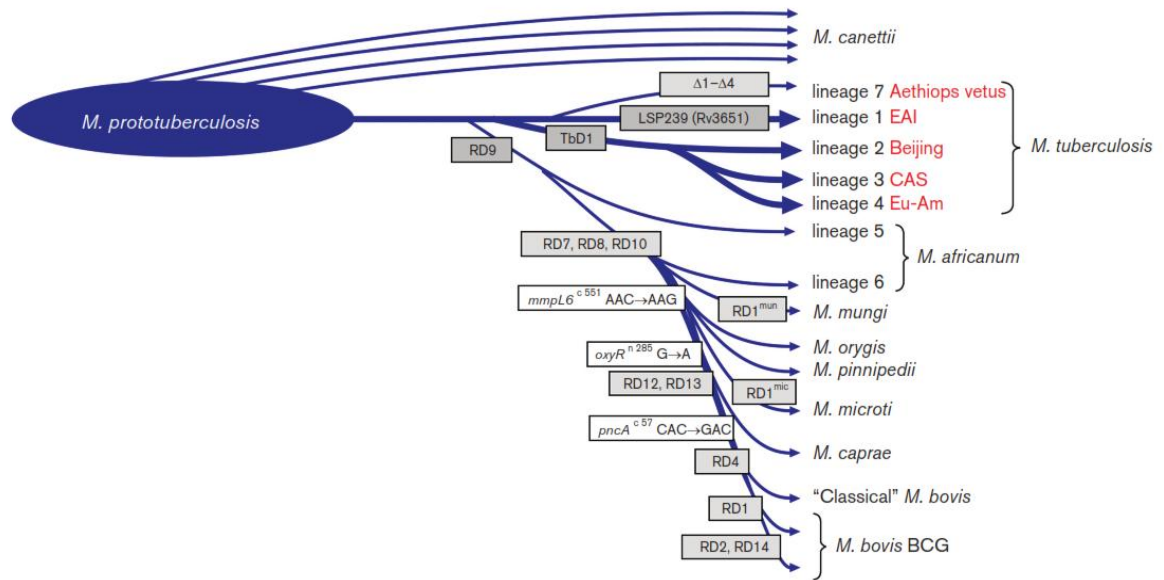


Figure1 Phylogenetic lineage classification based on the presence or absence of regions of difference (RD) and smaller deletions (Nebenzahl-Guimaraes *et al.*, 2016)

Polymorphic genome sites are used for classification of MTBC strains. The polymorphic genome sites of MTBC are divided into three main groups, i.e., single nucleotide polymorphisms (SNPs), long sequence polymorphisms (LSPs), and polymorphisms in repetitive sequences (subdivided into scattered repeat [insertion sequence (IS) and Direct repeat(DR)] and tandem repeat/ direct continuous repeats [Variable Number Tandem Repeat(VNTR)] (Muller *et al.*, 2017).

2.2 Immunology of Tuberculosis

BCG vaccine is the only licensed TB vaccine which is believed to protect Children against miliary tuberculosis, but its efficacy in preventing pulmonary tuberculosis in adults ranges from 0 to 80% (Andersen and Doherty, 2005). Therefore Low protection of BCG vaccine and drug-resistant strains require better anti-*Mycobacterium tuberculosis*

vaccines with a broad, long lasting, antigen-specific response (Kovjazin *et al.*, 2013). It has proven challenging to develop vaccines which drive primarily cellular immune response to *Mycobacterium tuberculosis*. Currently there are most advanced TB vaccine candidates in clinical trials that can be either by improving BCG vaccine or by genetically attenuating *Mycobacterium tuberculosis* (as shown below in Table1).

Table1 Tuberculosis vaccine candidates in clinical trials (Triccas and Counoupas, 2016)

Category	Vaccine	Clinical trial stage	Vaccine Description
Subunit- protein in adjuvant	M72/AS01	Phase IIb	Fusion protein (Mtb39a,Mtb32a) in AS01 adjuvant
	H1/IC31	Phase IIa	Fusion protein(Ag85B,ESAT-6) in IC31 adjuvant
	H4/IC31	Phase II	Fusion protein (Ag85B,TB10.4) in IC31 adjuvant
	H56/IC31	Phase IIa	Fusion protein (Ag85B,ESAT-6,Rv2660c) in IC31 adjuvant
Subunit-viral vectors	ID93/GLA-SE	Phase I	Fusion protein (Rv3619,Rv1813c, Rv3620c,Rv2608) in GLA-SE adjuvant
	MVA85A	Phase IIb	Replication-deficient vaccinia Ankara virus expressing Ag85A
	Ad5Ag85A		Replication-deficient adenovirus-5 expressing Ag85A
Recombinant BCG	AD35,TB-S		Replication-deficient adenovirus-35 expressing Ag85A,Ag85B,TB10.4
	rBCG30	Phase I (discontinued)	Recombinant BCG over-expressing Ag85B
	VPM1002	Phase IIa	Recombinant BCG deleted of ureC gene and expressing L.monocytogenes LLO
Attenuated <i>M.tuberculosis</i>	AERAS-422	Phase I (discontinued)	Recombinant BCG expressing Ag85A,Ag85B,Rv3407
	MTBVAC	Phase IIa	Live attenuated <i>M.tuberculosis</i> deleted of phoP and fadD26 genes
Inactivated Mycobacteria	Dar-901	Phase I	Heat-inactivated Mycobacterium obuense

The immune system is divided into innate and adaptive immunity. The innate immune system provides the first line of host defense against invading microorganisms before the development of adaptive immune responses. For development of protective immunity against TB, immune cells of innate arm and the adaptive arm have crucial role to give protection and only 5-10% of those immunocompetent individuals infected by *Mycobacterium tuberculosis* develop clinical diseases.

Innate Cellular immune response against *M. tuberculosis*

Macrophages

The macrophage plays a dual role in tuberculosis, promoting not only protection against mycobacteria, but also survival of the pathogen. Macrophages inhibit multiplication of mycobacteria but also act in concert with lymphocytes through presentation of antigens to T cells (Liebana *et al.*, 2000).

Alveolar Macrophages engulf the invading *mycobacterium tuberculosis* after recognizing through germline encoded pattern recognition receptors such as the mannose receptor, Fc receptors, complement receptors, Class A scavenger receptors and Toll-like receptor (Yoshikai, 2006).

Precise receptor involved in phagocytic entry may have a major impact on the survival chances of *M. tuberculosis* inside the macrophage. For example, ingestion of particles through Fc receptors results in a respiratory burst and an inflammatory response in macrophages; in contrast, internalization via CR3 receptor prevents the activation of the macrophages (Caron and Hall, 1998).

Lipoarabinomannan (LAM) of *mycobacterium tuberculosis* is well characterized mannose receptor ligand which is abundant and peripherally exposed and contains terminal mannose residues, engage the mannose receptor (MR), a monomeric transmembrane protein with an extracellular domain containing eight carbohydrate-recognition domains characteristics of calcium dependent lectin, on macrophage during phagocytosis.(Ernst, 1998; Torrelles and Schlesinger, 2010). Engagement of the Mannose receptor by mannose-capped lipoarabinomannan (ManLAM) during the phagocytic process is a key step in limiting Phagosome- Lysosome fusion (Kang *et al.*, 2005).

Fc gamma receptors are the pathogen recognition receptors that can be activatory [FcγRIIIa (CD16a), FcγRIIa (CD32a) and FcγRI (CD64a)] or inhibitory [FcγRIIB (CD32b)]. Macrophages through Fc gamma receptor engulf IgG opsonized mycobacteria and entry through this receptor doesn't inhibit fusion of phagosome with lysosome. (Weng and Levy, 2009).

M. tuberculosis can activate the alternative pathway of complement activation and produce C3b and C3bi. Opsonization of *Mycobacterium Tuberculosis* by C3b or C3bi leads to phagocytosis via complement receptors such as CR1, CR2 and CR3. Pathogenic mycobacteria uniquely recruit the complement fragment C2a to form a C3 convertase and generate opsonically active C3b in the absence of early activation components of alternative or classical pathways and the predominant opsonin generated by scavenging C2a is C3b, rather than C3bi, and bind predominantly to CR1 rather than to CR3 or CR4 (Ernst, 1998).

Class A scavenger receptors are also important receptors for *M.tuberculosis* on human monocyte-derived macrophages. It is not yet known whether scavenger receptors can activate the cytoskeleton to internalize bacteria or, alternatively, whether scavenger receptors act to bind bacteria but phagocytosis is executed by other receptors (Ernst, 1998).

Toll-like receptors (TLRs) have emerged as an essential family of innate immune pattern recognition receptors which play a pivotal role in host defense against microbes, including pathogenic strains of mycobacteria. TLR ligands specifically promote bacterial phagocytosis, in both murine and human cells, through induction of a phagocytic gene program. Importantly, TLR-induced phagocytosis of bacteria was found to be reliant on

myeloid differentiation factor 88(MyD88)-dependent signaling through interleukin-1 receptor-associated kinase-4 (IRAK-4) and p38 leading to the up-regulation of scavenger receptors. Interestingly, individual TLRs promote phagocytosis to varying degrees with TLR9 being the strongest and TLR3 being the weakest inducer of this process (Doyle *et al.*, 2004; Hart and Tapping, 2012).

Prolonged TLR signaling by *M. tuberculosis* inhibits certain macrophage responses to IFN-gamma, particularly those related to MHC-II antigen presentation. This inhibition may promote *M. tuberculosis* evasion of T-cell responses and persistence of infection in tuberculosis.(Pai *et al.*, 2004).

Once the *Mycobacterium tuberculosis* is internalized to the macrophage, they generally locate themselves in the mycobacterial phagosome. Phagosome derives from the plasma membrane and presents some cell surface receptors (Scott *et al.*, 2003; Trimble and Grinstein, 2007).

In contrast to normal phagocytosis, during which the phagosomal content is degraded upon fusion with lysosomes, *Mycobacterium tuberculosis* (MTB) is able to inhibit phagosomal maturation through secretion of some bacterial components and modulation of host cell intracellular signaling pathways (Kawamura, 2010).

Reversible protein phosphorylation regulates multiple biochemical events. *Mycobacterium tuberculosis* phosphatases play important roles in regulating the pathogen physiology and interference of host signaling. They are also involved in the evasion of host immune response and blockage of the phagosome-lysosome fusion (Teng *et al.*, 2011).

The secreted *Mycobacterium tuberculosis* protein tyrosine phosphatase (PtpA) binds to subunit H of the macrophage vacuolar-H (+)-ATPase (V-ATPase) machinery, a multisubunit protein complex in the phagosome membrane that drives luminal acidification. Furthermore, the macrophage class C vacuolar protein sorting complex, a key regulator of endosomal membrane fusion, associates with vesicular proton-pump adenosine triphosphatase (V-ATPase) in phagosome maturation, suggesting a unique role for V-ATPase in coordinating phagosome-lysosome fusion. PtpA interaction with host V-ATPase is required for dephosphorylation of VPS33B (Vacuolar protein sorting 33 homolog B) and subsequent exclusion of V-ATPase from the phagosome during *Mycobacterium tuberculosis* infection. These findings show that inhibition of phagosome acidification in the mycobacterial phagosome is directly attributed to PtpA, a key protein needed for *Mycobacterium tuberculosis* survival and pathogenicity within host macrophages (Wong *et al.*, 2011).

Phagosomes containing *M. tuberculosis* interact normally with early endosomes but fail to fuse with late endosomes and lysosomes. Several Rab GTPase proteins, in particular, Rab7 associates with the phagosomal membrane and regulates the fusion between late endosomes and lysosomes. The function of Rab7 mediated by the Rab7 effector RILP (Rab7-interacting lysosomal protein). Live mycobacteria express within the macrophage a Rab7 deactivating factor leading to abortion of RILP-mediated fusion with lysosomes (Sun *et al.*, 2007).

Virulence of *Mycobacterium tuberculosis* and related pathogenic mycobacteria requires the secretion of early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 (CFP-10), two small proteins that lack traditional signal sequences and are

exported through an alternative secretion pathway encoded primarily by the RD-1 (region of difference-1) genetic locus. The ESAT-6/CFP-10 secretion system plays a role in preventing phagolysosomal fusion, a novel function that accounts for the ability of bacteria to survive inside host cells. This finding provides a mechanism by which the ESAT-6/CFP-10 secretion system potentiates the virulence of pathogenic mycobacteria (Tan *et al.*, 2006).

SOCS1 (Suppressor of cytokine signaling pathway 1) has been shown to inhibit responses to IFN-gamma. *M. tuberculosis* is a highly efficient stimulator of SOCS1 expression in murine and human macrophages and in tissues from infected mice. Surprisingly, SOCS1 reduced responses to IL-12, resulting in an impaired IFN-gamma secretion by macrophages that in turn accounted for a deteriorated intracellular mycobacterial control. Despite SOCS1 expression, mycobacteria-infected macrophages responded to exogenously added IFN-gamma. SOCS1 attenuated the expression of the majority of genes modulated by *M. tuberculosis* infection of macrophages. Using a conditional knockdown strategy in mice, SOCS1 expression by macrophages hampered *M. tuberculosis* clearance early after infection in vivo in an IFN-gamma-dependent manner. On the other hand, at later time points, SOCS1 expression by non-macrophage cells protected the host from infection-induced detrimental inflammation (Carow *et al.*, 2011).

Coronin-1a (Coro1a), a crucial component of the cytoskeleton of macrophages, promotes the survival of *M. tuberculosis* by blocking the activation of the p38 MAPK pathway and inhibits autophagosome formation (Seto *et al.*, 2012).

The inhibition of phagosome maturation by mycobacteria may be reverted by cytokines, IFN γ is a key cytokine responsible for macrophage activation during *Mycobacterium tuberculosis* infection (de la Barrera *et al.*, 2004).

IFN- γ , released by T cells, induces autophagy, phagosomal maturation, the production of antimicrobial peptides such as cathelicidin, and antimicrobial activity against *Mycobacterium tuberculosis* in human macrophages via a vitamin D-dependent pathway (Fabri *et al.*, 2011).

Autophagy has been linked with innate and adaptive immune responses against *Mycobacterium tuberculosis*, which can survive within macrophages by blocking fusion of the phagosome with lysosomes. Induction of autophagy by the Th1 cytokine IFN- γ enables infected macrophages to overcome this phagosome maturation block and inhibit the intracellular survival of mycobacteria. Conversely, the Th2 cytokines IL-4 and IL-13 inhibit autophagy in murine and human macrophages (Harris *et al.*, 2009).

Macrophages have been shown to kill *Mycobacterium tuberculosis* through the action of the antimicrobial peptide cathelicidin (CAMP), whose expression was shown to be induced by 1,25-dihydroxyvitamin D₃ (1,25D₃) (Sonawane *et al.*, 2011).

Dendritic cells

Dendritic cells (DCs), myeloid or lymphoid origin, are the most potent antigen-presenting cells of the immune system and play a central role in initiating a primary immune response. Dendritic cells (DCs) are crucial in adaptive immunity because they are the only antigen-presenting cells that can present antigens to naive T lymphocytes. They serve as the sentinels that capture antigens in the periphery, process them into peptides

and present these to lymphocytes in lymphoid organs (Adema, 2009; Henderson *et al.*, 1997; Ito *et al.*, 2011; Janelins *et al.*, 2013).

Dendritic cells bind antigens via C-type lectin receptors, Fc γ receptor types I (CD64) and II (CD32) and Toll like receptor (TLR) family of proteins (Different TLRs recognize different pathogen-associated molecular patterns), and internalize them by endocytosis (Adema, 2009; Banchereau *et al.*, 2000).

The distinct pattern of expression of C-type lectins on DCs in situ and their non-overlapping antigens recognition profile hint to selective functions of these receptors to allow a DC to recognize a wide variety of antigens and to process these to induce T cell activation (Engering *et al.*, 2002).

M. tuberculosis endocytosis is carried out through known C-type lectin receptors, such as the dendritic cell-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) (CD209), a calcium-dependent carbohydrate-binding protein with specificity for mannose-containing glycoconjugates and fucose-containing Lewis antigens. Mannosylated moieties of the mycobacterial cell wall, such as mannose-capped lipoarabinomannan (manLAM) or higher-order phosphatidylinositol-mannosides (PIMs) of *M. tuberculosis*, bind to DC-SIGN on immature dendritic cells. This interaction impairs dendritic cell maturation and induce production of the anti-inflammatory cytokine IL-10 (Chan *et al.*, 2010; Ehlers, 2010; Geijtenbeek *et al.*, 2003).

Studies have shown that BCG and *M. tuberculosis* target the DC-specific C-type lectin intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) to infect DC and inhibit their immunostimulatory function. This occurs through the interaction of the mycobacterial mannosylated lipoarabinomannan to DC-SIGN, which prevents DC

maturation and induce the immunosuppressive cytokine interleukin-10(IL-10) synthesis (Gagliardi *et al.*, 2005).

Mycobacteria induce DC maturation through Toll-like receptor (TLR) signaling. DC-SIGN, upon binding of Man LAM, interferes with TLR-mediated signals to cause suppression of protective immunity to TB. (Ehlers, 2010; Geijtenbeek *et al.*, 2003).

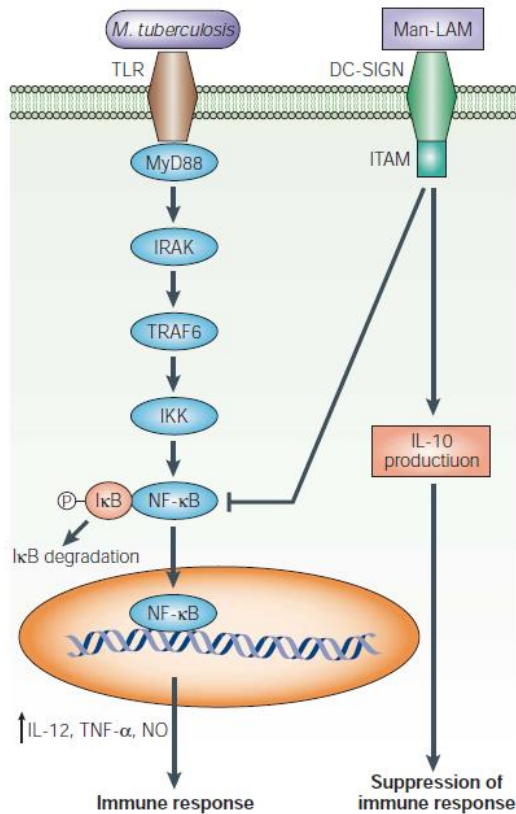


Figure2 Disruption of Dendritic cell signaling pathways by mycobacteria(Geijtenbeek *et al.*, 2003)

Disruption of dendritic-cell signaling pathways by mycobacteria

Dendritic cells (DCs) express DC-SIGN (DC-specific intercellular adhesion molecule-grabbing nonintegrin) and Toll-like receptors (TLRs) on their surfaces. TLRs initiate signaling by binding an adaptor protein, MyD88, which in turn recruits a serine/threonine kinase, IRAK (interleukin-1 receptor-associated kinase). IRAK then associates with the adaptor protein TRAF6 (TNF-receptor-associated factor 6). This leads to the activation of the IKK (inhibitor of κ B kinase) complex, which phosphorylates I κ B, an inhibitor of NF- κ B. Phosphorylation of I κ B causes its degradation and thereby allows translocation of NF- κ B to the nucleus. Binding of *M. tuberculosis* to TLRs results in activation of NF- κ B, which leads to DC maturation and production of immunostimulatory cytokines that activate T cells and mediate killing of pathogenic mycobacteria. However, Man-LAM from pathogenic mycobacteria binds to DC-SIGN and inhibits TLR signaling. This blocks DC maturation, enhances the production of the immunosuppressive cytokine IL-10 and, as a consequence, the activation of T cells is impaired. ITAM, immunoreceptor tyrosine-containing activation motif.

Mycobacterium tuberculosis lipoproteins are an important class of TLR2 ligand.

Mycobacterium tuberculosis lipoproteins agonist activity for both human and murine TLR2 and induced expression of TNF α , IL-10, and IL-12 and also induced dendritic cell maturation as shown by increased expression of CD40, CD80, and class II MHC (MHC-II) (Pecora *et al.*, 2006).

Evidence from *M. tuberculosis* studies suggests mycobacterial proteins activate dendritic cells (DCs) via Toll-like receptor (TLR) 4, eventually determining the fate of immune responses (Byun *et al.*, 2012).

Protection against infection with *Mycobacterium tuberculosis* is preferentially associated with the development of the T helper 1 subset, IFN-gamma production and a cell-mediated response, rather than with T helper 2 cells, Interleukin 4 (IL-4) and antibody production. The type of predominate response depends on mycobacterial components interacting with Dendritic cell. DC can induce production of cytokines associated with a protective immune response when presenting peptides derived from heterogeneous mycobacterial antigens but not when exposed to the single antigen like immunodominant 19 kDa protein (Baird *et al.*, 1995).

DCs are available for immediate interaction with *Mycobacterium tuberculosis* after inhalation of the pathogen. The antimicrobial activity of iDC against intracellular *Mycobacterium tuberculosis* inversely correlated with TNF- α release. Differentiation of iDC into mature DC by addition of TNF-alpha or activation via Toll-like receptors further reduced killing of *Mycobacterium tuberculosis*. Therefore, the maintenance of a pool of DCs at the site of disease activity in tuberculosis, and the maturation of these DC by TNF- α provides a mechanism by which *Mycobacterium tuberculosis* escapes the innate immune system (Buettner *et al.*, 2005).

In murine study showed that DCs may serve as a reservoir for *M. tuberculosis* in tissues, including the lymph nodes and lungs, activation of DCs and Macrophages with INF- γ inhibit the growth of the intracellular bacteria in a nitric oxide synthase-dependent

fashion. However, this activation enables Macrophage to kill the *M. tuberculosis* bacilli whereas the *M. tuberculosis* bacilli in activated DCs were not killed (Bodnar *et al.*, 2001). Mycobacterial growth in DCs is reduced may be due to trafficking between the vacuoles and the host cell recycling and biosynthetic pathways is strikingly reduced which is likely to impair access of intracellular mycobacteria to essential nutrients. This unique location of *M. tuberculosis* in DCs is compatible with their T lymphocyte-stimulating functions, because *M. tuberculosis*-infected DCs have the ability to specifically induce cytokine production by autologous T lymphocytes from pre-sensitized individuals. DCs have evolved unique subcellular trafficking mechanisms to achieve their antigen-presenting functions when infected by intracellular mycobacteria (Tailleux *et al.*, 2003).

DC generated from human peripheral blood by short term culture in medium containing recombinant human cytokines granulocyte-macrophage-CSF and IL-4 were capable of phagocytosing *M. tuberculosis*. Infection of DC with live *M. tuberculosis* bacilli resulted in increased APC surface expression of the co-stimulatory molecules CD54, CD40, and B7.1, as well as MHC class I molecules. In addition, infected DC secreted elevated levels of inflammatory cytokines including TNF- α , IL-1, and IL-12. These data indicate that infection with *M. tuberculosis* results in direct activation and maturation of the DC. In vivo, such activation may facilitate migration of DC to the lymph nodes, and enhance presentation of antigen to T cells, thereby facilitating the induction of the immune response against this pathogen (Henderson *et al.*, 1997).

Dendritic cells are able to present antigens in the context of major histocompatibility complex (MHC) molecules, as well as through the family of CD1 proteins (the isoforms are CD1a,b,c,and d) (Sugita *et al.*, 2000).

Mycobacterially infected DC are particularly potent activators of autologous T cells compared to TNF- α exposed DC and that the resultant T cells are functionally superior (Cheadle *et al.*, 2003).

There are genes that play critical roles in modulating diverse functions of dendritic cells (DCs) during *Mycobacterium tuberculosis* infection. Knockdown of many of these genes resulted in reduced bacterial burden within DCs. These included genes that regulated activation of transcription factors, ubiquitin-specific peptidases, and genes that are involved in autophagy. Knockdown of certain genes increased the expression of IL-12p40 and surface densities of co-stimulatory molecules in an antigen- and receptor-specific manner. Increased IL-12p40 and co-stimulatory molecules on DCs also promoted the development of Th1 responses from a Th2 inducing antigen. Furthermore, modulation of autophagy and oxidative burst appeared to be one of the mechanisms by which these genes regulated survival of *M. tuberculosis* within DCs. Although some genes regulated specific responses, others regulated multiple responses that included IL-12 production, T cell priming, as well as intracellular survival of *M. tuberculosis* (Singhal *et al.*, 2012).

Emerging evidence points to an important role of autophagy in the immune response mediated by dendritic cells (DC) against *Mycobacterium tuberculosis*. a deeper comprehension of the alterations induced by *Mycobacterium tuberculosis* in DC is essential for setting new vaccine strategies. *Mycobacterium tuberculosis* H37Rv impairs autophagy at the step of autophagosome-lysosome fusion. In contrast, neither *Mycobacterium tuberculosis* H37Ra nor BCG strains were able to hamper autophagosome maturation. *Mycobacterium tuberculosis* alters the autophagic machinery

through the ESX-1 system, and thereby opens new exciting perspectives to better understand the relationship between *Mycobacterium tuberculosis* virulence and its ability to escape the DC-mediated immune response (Romagnoli *et al.*, 2012).

Polymorphonuclear neutrophils (PMNs)

Neutrophils Exert Protection in the early tuberculous granuloma. Study in zebrafish infected with *Mycobacterium marinum* showed that Neutrophils are recruited to granulomas by signals from dying infected macrophages and phagocytose infected macrophages and then rapidly kill the internalized mycobacteria through NADPH oxidase-dependent mechanisms. Thus Neutrophils are protective in the early granuloma (Yang *et al.*, 2012).

Human neutrophil peptides (HNPs) belong to a family of antimicrobial and cytotoxic peptides known as 'defensins' which are oxygen-independent antimicrobial peptides in neutrophil granules. HNPs have highlighted their bactericidal action against *Mycobacterium tuberculosis*. HNPs are active against *M. tuberculosis* grown in vitro or within macrophages. HNPs released by neutrophils recruited in the early lesion could attract monocytes to the site and macrophages may in vivo uptake the extracellular HNPs and kill the intracellular pathogens (Fu, 2003).

On the other hand, neutrophils have recently been ascribed a role in the development of the pathology, rather than the protection of the host. The exact role of neutrophils in the pathogenesis of TB is poorly understood. Recent evidence suggests that neutrophils are not simply scavenging phagocytes in *Mycobacterium tuberculosis* infection. Study showed in clinical specimens from patients with active pulmonary TB who underwent lung surgery that neutrophils are the predominant cell types infected with *Mycobacterium*

tuberculosis in patients with TB and that these intracellular bacteria appear to replicate rapidly. These results are consistent with a role for neutrophils in providing a permissive site for a final burst of active replication of the bacilli prior to transmission (Eum *et al.*, 2010).

Natural killer cells

Human Natural killer cells lyse *Mycobacterium tuberculosis*-infected monocytes and alveolar macrophages and up regulate CD8(+) T cell responses and produce IL-22, which inhibits intracellular growth of *Mycobacterium tuberculosis* by enhancing phagolysosomal fusion and IL-15 and DAP-10 elicit IL-22 production by NK cells in response to *Mycobacterium tuberculosis* (Dhiman *et al.*, 2012).

Natural killer cells maintain the frequency of *M. tuberculosis*-responsive CD8+IFN-gamma+ T cells by producing IFN-gamma, which elicits secretion of IL-15 and IL-18 by monocytes. These monokines in turn favor expansion of Tc1 CD8+ T cells. The capacity of NK cells to prime CD8+ T cells to lyse *M. tuberculosis*-infected target cells required cell-cell contact between Natural killer cells and infected monocytes and depended on interactions between the CD40 ligand on NK cells and CD40 on infected monocytes. (Vankayalapati *et al.*, 2004).

CD1d-restricted invariant natural killer T cells

CD1d-restricted T cells express an invariant V alpha 24 TCR that recognizes the non-classical antigen-presenting molecule CD1d. (Michael *et al.*, 2003). Activation of CD1d-restricted invariant NKT (iNKT) cells requires CD1d expression by infected monocyte-derived cells as well as IL-12 and IL-18. NKT cells secrete IFN-gamma, proliferate, and

exert lytic activity in response to Alpha-galactosylceramide (marine sponge-derived)-pulsed monocyte-derived cells. NKT cells also expressed granulysin, an antimicrobial peptide shown to mediate an antimycobacterial activity through perturbation of the mycobacterial surface. (Gansert *et al.*, 2003).

CD1d-restricted NKT cells are known to play a protective role in the immune responses of mice against a variety of infectious pathogens. However, Study done to examine NKT cell levels and functions in patients with active *M. tuberculosis* infection, the poor proliferative response of NKT cell to alpha-galactosylceramide (alpha-GalCer) observed in active tuberculosis patient compared to latent tuberculosis due to increased NKT cell apoptosis, reduced CD1d expression, and a defect in NKT cells. Notably, *M. tuberculosis* infection was associated with an elevated expression of the inhibitory programmed death-1 (PD-1) receptor on NKT cells, and blockade of PD-1 signaling enhanced the response to alpha-GalCer. Thus In active tuberculosis the number and function of CD1d-restricted NKT cells is reduced (Kee *et al.*, 2012).

Epithelial cells

In response to mycobacteria infection, epithelial expression of Toll-like receptors and surfactant proteins plays the most prominent roles in the recognition and binding of the pathogen, as well as the initiation of the immune response. Moreover, the antimicrobial substances, proinflammatory factors secreted by Alveolar Epithelial cells (AECs) , composed a major part of the innate immune response and mediation of adaptive immunity against the pathogen (Li *et al.*, 2012b).

In vitro study, CpG oligodeoxynucleotides capable of inducing host phospholipase D (PLD) dependent intracellular mycobacterial killing in type II alveolar epithelial cells.

These are the first evidences showing that alveolar epithelial cells may represent efficient effector cells during primary innate antimycobacterial immune response (Greco *et al.*, 2009).

Other study in vitro showed that autophagy signaling pathway prevents apoptosis in type II alveolar epithelial cells infected with *M. tuberculosis* (Guo *et al.*, 2013).

Mast cells

Cholesterol-enriched membrane micro domains (lipid rafts) in mast cells play a role in the uptake of many pathogens. Mycobacteria are one of the intracellular pathogens that utilize lipid rafts in order to invade mast cells. Study showed that the interaction of *M. tuberculosis* (H37Rv strain) with mast cells resulted in changes in the mast cell surface, with formation of pseudopod-like structure and activation with visibly extruded granules. Moreover, infection of mast cells with Mycobacteria induced cholesterol accumulation at the site of bacterial entry and around intracellular mycobacteria. Disruption of mast cells lipid rafts by cholesterol depletion markedly inhibited the mycobacterium entry. Intracellular multiplication of *M. tuberculosis* within mast cells was also observed. Thus, *M. tuberculosis* employs a cholesterol-dependent pathway to infect mast cells, which leads to degranulation and mast cell morphological changes. These results suggest that although mast cells are capable to respond to *M. tuberculosis* infection, entry of mycobacterium through lipid rafts may allow replication within mast cells (Munoz *et al.*, 2009).

The Fc epsilonRI complex forms a high-affinity cell-surface receptor that controls the activation of mast cells and participates in IgE-mediated antigen presentation. Multivalent antigens bind and crosslink IgE molecules held at the cell surface by Fc

epsilonRI. Receptor aggregation induces multiple signaling pathways that control diverse effector responses. These include the secretion of allergic mediators and induction of cytokine gene transcription, resulting in secretion of molecules such as interleukin-4, interleukin-6, tumour-necrosis factor-alpha and granulocyte-macrophage colony-stimulating factor. Therefore, Mast cell confer protection against infection (Turner and Kinet, 1999).

Mast Cells (MCs) express toll-like receptor 2 (TLR2), a receptor known to be triggered by several major mycobacterial ligands and involved in resistance against *Mycobacterium tuberculosis* infection (Carlos *et al.*, 2009).

Mast cells also participate in host defense against *M. tuberculosis* infection through the production and secretion of cytokines and chemokines that play a role in the recruitment and activation of inflammatory cells in murine experimental model of tuberculosis (Carlos *et al.*, 2007).

Acquired Cellular immune response against *M. tuberculosis*

In contrast to innate mechanisms, the specific or adaptive immune response requires the specific recognition of foreign antigens. The innate immune system has a profound influence on the type of acquired immune mechanisms generated, and *vice versa*, the specific immune response executes several of its effector functions via the activation of components of the innate immunity. Specific immune responses can be divided into cell-mediated mechanisms, which include involvement of B-cells for antigen presentation and production of antibody and T-cell activation and effector mechanisms, and the humoral immune response which involve antibody for the immune response.

B cells

B lymphocytes, through a variety of interactions with the cellular immune response, play previously underappreciated roles in shaping host defense against non-viral intracellular pathogens, including *M. tuberculosis*. B cells have a significant impact on the outcome of airborne challenge with *M. tuberculosis* as well as the resultant inflammatory response (Maglione and Chan, 2009). B cells by producing specific antibody against *Mycobacterium tuberculosis* induce complement activation for phagocytosis during infectious process. (Manivannan *et al.*, 2012).

CD4+ T cells

M.tuberculosis in phagosomes is processed via major histocompatibility complex (MHC) class II-mediated antigen processing pathway and antigens of the bacteria are presented to CD4+ helper T cells (Gannage *et al.*, 2013; Ulrichs *et al.*, 1998).

CD4+ T cells can be classified into three subsets according to the patterns of cytokines they produce; Th1 cells produce IL-2 and IFN-gamma, tumor necrosis factor alpha (TNF- α). Th1- type cytokines stimulate macrophages and cell-mediated reactions important in resistance to infection with intracellular pathogens, and in cytotoxic and delayed-type hypersensitivity (DTH) reactions. Whereas Th2 cells are more efficient in mediating antibody production and secrete IL-4, IL-5, IL-6, IL-10 and IL-13. Th1 and Th2 cells are mutually inhibitory to each other; Th2 cells by releasing IL-4 and IL-10 inhibit the production of cytokines by Th1 cells. Whereas IFN- γ , a product of Th1 cells, prevents the activation of Th2 cells. Infection with mycobacteria stimulates macrophage to produce Interleukin-12 (IL-12) which is a multifunctional cytokine acting as a key regulator of cell-mediated immune responses through the differentiation of naive CD4+ T

cells into type 1 helper T cells (Th1) producing interferon-gamma (Darrah *et al.*, 2007; Mendez-Samperio, 2010; Mosmann and Sad, 1996; Romagnani, 1991; Tsuyuguchi, 1995).

Th17 cells are the other subset that produce IL-17, IL-17F, and IL-22 that act as an effector molecule similar to IFN-gamma after *M.tuberculosis* infection to protect human against TB (Korn *et al.*, 2009; Li *et al.*, 2012a).

Regulatory T cells

CD25(+)CD4(+) regulatory T cells specifically express the transcription factor Foxp3 (Sakaguchi *et al.*, 2007). In vitro study of proliferative response of blood lymphocytes under pulmonary tuberculosis condition showed that Regulatory T cells (CD4+CD25+Foxp3+) play a leading role in formation of immune suppression under infiltrative, disseminated and fibrosis-cavernous pulmonary tuberculosis and there was negative correlation between the number of Treg cells and proliferative activity of blood lymphocytes (basal, mitogen- and antigen-induced), which testifies about participation of Treg cells in suppression of lymphocyte proliferation and Th1- and Th2-immune response.(Churina *et al.*, 2012).

CD8+ T cells

CD8+ T cells potentially mediate protection against *M. tuberculosis* infection by producing pro-inflammatory type 1 cytokines such as IFN γ and TNF α act synergistically to activate macrophages, which increase their microbicidal activity large part through the induction of nitric oxide synthase and phagocyte oxidase, which are critical for the production of nitrogen and oxygen radicals capable of killing *M. tuberculosis*. These

cytokines also mediate a variety of immunological functions including inducing class I and II MHC, up regulating CD1d, and modulating the expression of many genes which are essential for the host response to *M. tuberculosis*. The other is by Cytotoxic activity which can be mediated by the release of cytotoxic granules containing perforin and granzymes (The cytotoxic-granule exocytosis pathway is dependent upon perforin, a pore-forming protein, which facilitates the entry of granzymes into the target cell cytosol, where they induce apoptosis) or CD95/CD95L-dependent (Fas/FasL) killing (activated CTL also express CD95L (FasL) which can bind to CD95 (Fas) on the target cell surface and initiate Fas-induced apoptosis) or the production of TNF that induces apoptosis in some cellular targets (expression of membrane TNF or secretion of TNF by CTL can promote TNF α receptor induced death of the target cells) (Philips and Ernst, 2012; Woodworth and Behar, 2006).

Study showed that a subset of CD8⁺ T cells coordinately expresses CC chemokine ligand 5 (CCL5, RANTES), perforin and granulysin, attracts *M.tuberculosis* -infected macrophages and kill the intracellular *M.tuberculosis* (Stegelmann *et al.*, 2005).

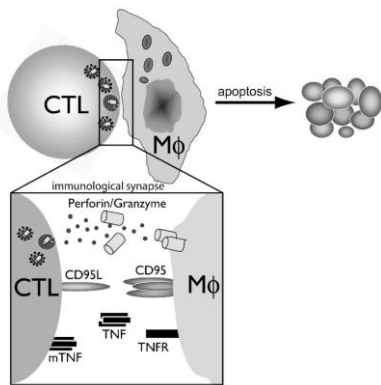


Figure3 Molecular pathways mediate CD8⁺ T cell cytotoxic activity(Stegelmann *et al.*, 2005)

Molecular pathways mediate CD8⁺ T cell cytotoxic activity:

Efficient CTL-mediated lysis of target cells requires cell-cell interaction and CTL activation mediated by signaling via the TCR triggered by recognition of the class I MHC/peptide complex (not shown). T cell activation leads to polarization of the T cell and development of an immunological synapse between the CTL and the target cell. CTL induction of target cell death can be mediated by three distinct molecular pathways. The cytotoxic-granule exocytosis pathway is dependent upon perforin, a pore-forming protein, which facilitates the entry of granzymes into the target cell cytosol, where they induce apoptosis. Although this is the principal mechanism by which CTL kill target cells, activated CTL also express CD95L (FasL) which can bind to CD95 (Fas) on the target cell surface and initiate Fas-induced apoptosis. Finally, expression of membrane TNF or secretion of TNF by CTL can promote TNF receptor induced death of the target cells.

The antigen of intracellular *Mycobacterium tuberculosis* present through MHC I and CD1 to CD8 T cells by dendritic cell using detour pathway, passage of mycobacterial antigen from apoptotic macrophage to non infected dendritic cells that the antigen is exposed for cytosolic pathway on dendritic cells. Thus, cross-priming mediated by apoptotic vesicles is not just a matter of antigen distribution, but an intrinsic immunological function due to the nature of phagosomally located intracellular bacteria (Winau *et al.*, 2004).

CD1-restricted T cells

CD1-restricted T cells can be divided into at least two functional groups: those produce mainly T helper type 1(T_H1) cytokines and are often prominently cytolytic and those that potently produce both T_H1 and T_H2 cytokines. Human foreign antigen-reactive T cells that are restricted by CD1a,CD1b, and CD1c typically produce T_H1 cytokines and are cytolytic (Michael *et al.*, 2003).

CD1-restricted T cells recognize mycobacterial lipids, but their function in human TB is unclear and their ability to establish memory is unknown (Montamat *et al.*, 2011).

$\gamma\sigma$ T cells

Most CD4⁺ and CD8⁺ T cells express T-cell receptors of α and β protein chains. A minor subset of T cells expresses T-cell receptor of γ and σ protein chains ($\gamma\sigma$ T cells). $\gamma\sigma$ T cells tend to distribute in mucosal and epithelial surfaces and contribute to early protection against pathogens invading through epithelium. Human $\gamma(9)\sigma(2)$ T cells (most frequently expressed in human peripheral blood) potently inhibit pathogenic microbes, including intracellular mycobacteria by releasing Soluble granzyme A that triggers TNF- α production by monocytes leading to intracellular mycobacterial growth suppression

with unknown inhibitory mechanism. The inhibitory mechanism was independent of autophagy, apoptosis, nitric oxide production, type I interferons, Fas/FasL and perforin. (Komori *et al.*, 2006; Spencer *et al.*, 2013).

In general, Cells of the innate and adaptive immunity have crucial role either in pathology which favour for the mycobacteria or immunoprotection in favour of the host. Innate cellular immune responses are initiated by germline-encoded pattern recognition receptors (PRRs), which recognize specific structures of microorganisms. In vitro studies showed that the fate of *Mycobacterium tuberculosis* in side innate cells depends on which components of the mycobacterium tuberculosis bind to which receptor of innate cells during the process of phagocytosis. Mycobacteria inside innate cells can be either destroyed by different mechanisms like antimicrobial molecules, via oxygen/nitrogen radicals , lysosomal degradation system or invade the innate cells by inhibition of phagosome maturation, interfering signaling pathway, suppressing the production of Th1 subset cytokines. Innate cells particularly antigen presenting cells such as Macrophage and Dendritic cells can also process and present peptide or lipid antigens of mycobacteria via MHC I/II or CD1 pathway respectively to T cells for initiating specific immune response. There is crosstalk between infected macrophage and uninfected dendritic cells for presentation of antigen via MHC-I and CD1 by detour pathways. Specific immune cells can also directly destroy the mycobacteria through antimicrobial molecules, apoptosis of infected cells particularly by CD8 T cells or by stimulating the production cytokines of Th1 subsets. Also by production of cytokines of Th2 subset they can suppress the immune response that helps for the survival of the mycobacteria.

2.3 Diagnosis of active Tuberculosis

The diagnosis of childhood tuberculosis is complex and most of the new diagnostics for TB are for adults (Cuevas *et al.*, 2012b). Childhood pulmonary TB is under diagnosed, in part due to difficulties in obtaining microbiological confirmation (Nicol and Zar, 2011). The clinical and radiological features of childhood TB are often non-specific and subject to variable interpretation (Swingler *et al.*, 2005).

Recommended approach to diagnosis TB in children Ethiopia

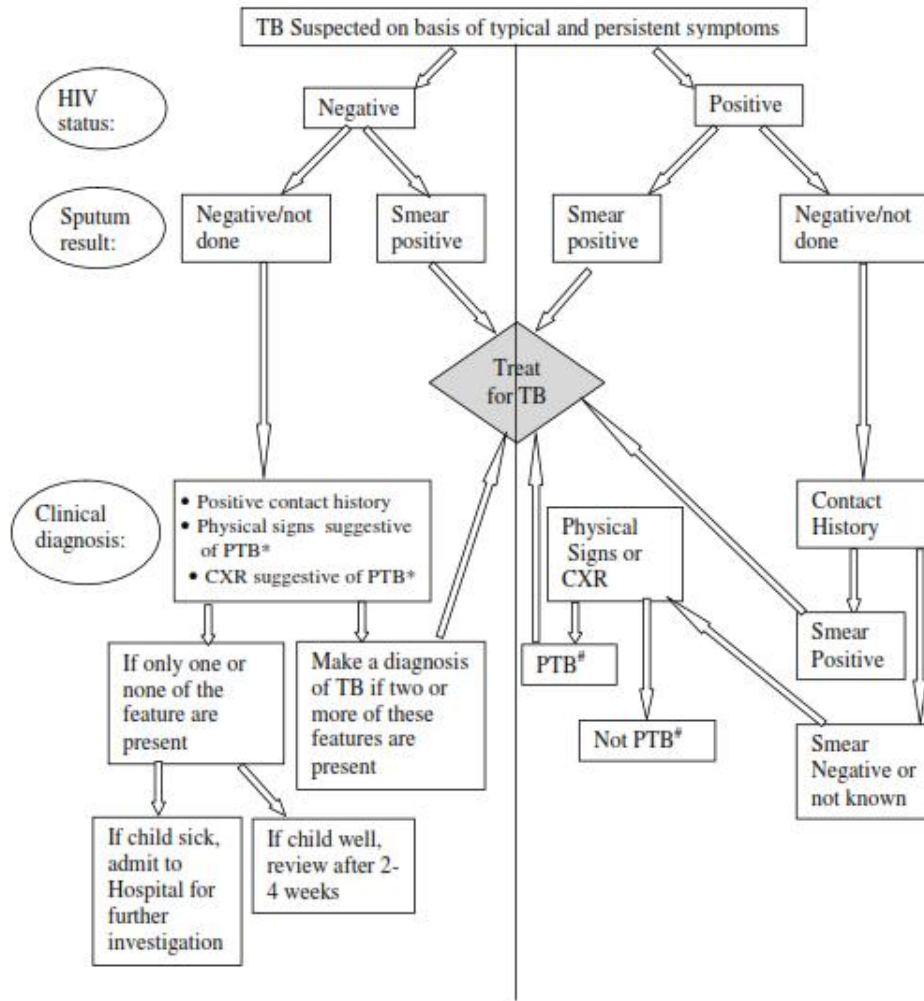


Figure4 Recommended approach to diagnosis TB in children(FMoH, 2012)

If a child is HIV positive and smear negative make a diagnosis of TB by using at least one of the following feature presents- smear positive in contact history or physical signs suggest PTB or CXR suggest PTB

*The abnormalities on CXR suggestive of TB are:

- Enlarged hilar lymph nodes and opacification in the lung tissue
- Miliary mottling in lung tissue
- Cavitation (tends to occur in older children)
- Pleural or pericardial effusion

*The clinical signs suggestive of Pulmonary TB are:

- Conduct through physical examination with special emphasis on weight measurement (look for weight loss or poor weight gain), fever, signs of respiratory distress and chest finding.
- Children can also present with acute severe pneumonia (especially in infants and HIV-infected children) and asymmetrical and persistent wheeze

It can be difficult to clearly define what is “suggestive of PTB” on clinical or radiological findings in HIV-infected children because of clinical overlap between PTB and other forms of HIV-related lung disease. CXR abnormalities of PTB in HIV-infected child are similar to those in HIV-uninfected child.

2.3.1 Clinical diagnostics of TB in children

In most children, TB presents with symptoms of a chronic disease after they have been in contact with an infectious source case. The most common clinical presentation of Pulmonary Tuberculosis in children is persistent respiratory symptoms and poor weight

gain. A child may have nonproductive cough and /or mild wheezes. Pulmonary TB in infants and HIV infected children may present as acute pneumonia (FMoH, 2012).

A retrospective study in the paediatric departments at Danish university hospital from April 2004 to March 2009 aged less than 15 years showed that the cardinal symptoms of childhood tuberculosis were fever, weight loss and cough (Rahman *et al.*, 2012). Other study in approaches to diagnose TB infection and disease in children showed fever (possibly intermittent or low grade), weight loss or failure to thrive, and a persistent cough for >2 weeks are the most important clinical signs for pulmonary tuberculosis (Rigouts, 2009). A retrospective review of clinical course of adolescents (12-18 years old) at a Children's Tuberculosis Clinic in Houston from 1987 to 2012 showed that the most common symptoms were fever (63%), cough (60%) and weight loss (30%), of 79% symptomatic case and 21% were asymptomatic. Only 8% of adolescents with pulmonary TB had hemoptysis (Cruz *et al.*, 2013a). A clinical finding of children with TB involving CNS in a medical center in southern Taiwan showed that the most common presentations were fever (85.7%), signs of increased intracranial pressure (71.4%), drowsiness (64.3%), focal neurological signs (57.1%) and TB contact history was found in 42.5% cases (Cho *et al.*, 2013).

A cross-sectional analytical study carried out between January 2002 and December 2006 to determine the efficacy of the scoring system, recommended by the Brazilian National Ministry of Health for the diagnosis of pulmonary tuberculosis (TB) in children and adolescents (under 15years of age), regardless of their HIV status involved 239 individuals. The patients were divided into four groups: latent TB group (n = 81); no-TB group (n = 41); TB group (n = 104); and TB/HIV group (n = 13). Reports of fever, cough,

and asthenia and weight loss for at least two weeks were significantly higher in the TB group. The proportion of cases with a history of any contact and household contact with a TB patient was, respectively, 95.0% and 86.1% in the TB group, versus 75.0% and 58.3% in the TB/HIV group (Pedrozo *et al.*, 2010).

2.3.2 Radiologic diagnostics of TB in children

Chest X-ray remains an important tool for diagnosis of PTB in children who are sputum smear negative or who cannot produce sputum. The following abnormalities on Chest X-ray are suggestive of TB: Enlarged hilar lymph nodes and opacification in the lung tissue, Miliary mottling in lung tissue, Cavitation (tends to occur in older children), Pleural or pericardial effusion. The finding of marked abnormality on CXR in a child with no signs of respiratory distress (no fast breathing or chest in-drawing) is supportive of TB (FMoH, 2012).

Chest radiography is useful for the diagnosis of TB in children. In the majority of cases the chest X-ray shows abnormalities suggestive of TB. A retrospective review of adolescents (12-18 years old) showed that the most common radiographic findings were infiltrates (34%), lymphadenopathy (27%), cavitary lesions (26%), pleural effusions (19%) and miliary disease (10%) (Cruz *et al.*, 2013a). Other study on Review of eighty medical records of children with TB in southern Taiwan showed that the major radiological findings were tuberculoma (50%), basilar enhancement (41.6%), infarction (41.6%), hydrocephalus (16.6%), and transverse myelitis (16.6%) (Cho *et al.*, 2013).

To determine the efficacy of the scoring system recommended by the Brazilian National Ministry of Health for the diagnosis of pulmonary tuberculosis (TB) in children and adolescents, regardless of their HIV status, chest X-rays revealed parenchymal alterations

in 75.0% and 53.9%, and combined parenchymal/lymph node alterations in 18.2% and 30.8% in the TB and TB/HIV groups respectively. The mean Brazilian National Ministry of Health system scores in the latent TB, No-TB, TB and TB/HIV groups were, respectively, 24.2, 18.5, 45.3 and 41.5. and The Brazilian National Ministry of Health system scores were significantly higher in the TB and TB/HIV groups than in the other two groups (Pedrozo *et al.*, 2010).

2.3.3 Laboratory diagnostics of TB

It is always advisable to confirm diagnosis of TB in a child using whatever specimens and laboratory facilities are available. Appropriate clinical samples include sputum, gastric aspirates and certain other material (e.g. lymph node biopsy or any other material that is biopsied) (WHO, 2006).

2.3.3.1 Smear microscopy

The acid-fast bacillus (AFB) “smear” using direct microscopic examination remains the initial step for evaluation of TB. The method is inexpensive and fairly rapid. It is the only laboratory diagnostic test for TB used in most of the world. Results should be available within 24 hours of specimen submission. Ziehl –Neelsen (Zn) or Fluorescent (FN) microscopy can be used for detection *Mycobacterium tuberculosis* (Eichbaum, 2002).

The Ziehl-Neelsen (Zn) Microscopy

The Ziehl-Neelsen (Zn) technique is used to stain *Mycobacterium* species unlike most other bacteria, do not stain well by the Gram technique. They can however be stained with carbol fuchsin combined with phenol. The stain binds to the mycolic acid in the mycobacterial cell wall. After staining, an acid decolorizing solution is applied. This removes the red dye from the background cells, tissue fibers, and any organisms in the

smear except mycobacteria which retain (hold fast to) the dye and are therefore referred to as acid fast bacilli, or simply AFB. Following decolorization, the smear is counterstained with malachite green or methylene blue which stains the background material, providing a contrast colour against which the red AFB can be seen (Cheesbrough, 2006). AFB smear microscopy has been estimated to detect 5000-10,000 bacilli per milliliter of sputum (Kubica and Kent, 1985).

Study to optimize Smear microscopy in Children with Tuberculosis in Yemen showed that out of 546 gastric aspirate 17(3%) aspirate were smear positive and out of 329 expectorate sputum 16(5%) were smear positive (Al-Aghbari *et al.*, 2009). A pilot study of same day sputum smear examination in India showed that out of 330 patients 61(18.48%) were smear positivity by standard method and 43/330 (13.03%) were smear positivity by front loading method (i.e. sputum samples are collected and analyzed on the same day) (Myneedu *et al.*, 2011).

Fluorescent (FN) microscopy

Fluorescence microscopy was introduced in the 1930s, in an attempt to improve outcomes of smear microscopy. Fluorochrome dyes are used to stain the smear. A halogen or high-pressure mercury vapour lamp is traditionally used to excite the dye, and make it fluoresce. The sensitivity of fluorescent microscopy is 10 % higher than that of conventional microscopy, and that it remains high even after concentration of the samples. Sensitivity is found to be higher particularly in low grade smear positive sputum. Specificity estimates, however, are similar to conventional microscopy, though turnaround times are shorter. Cost constraints are major issues with fluorescent microscopy. This may be circumvented by the use of light-emitting diodes (LEDs) which

cost less than 10 per cent of a mercury vapour lamp. With a life >50,000 h, it can run on batteries and thus has been used in peripheral areas with definite operational advantages (Desikan, 2013).

2.3.3.2 Culture

Culture techniques have been estimated to detect 10–100 viable mycobacteria per milliliter of specimen (Kubica and Kent, 1985). A positive culture for *M. tuberculosis* confirms the diagnosis of active disease. For culturing of mycobacteria, two types of clinical specimens are considered: contaminated specimens and specimens collected aseptically from normally sterile sites. Sterile specimens can be inoculated directly onto the culture medium. Specimens from non-sterile bodily sites are considered contaminated and therefore require processing before culturing in order to eliminate the associated flora. If not properly eliminated, this flora will overgrow the culture medium long before mycobacteria have the chance to develop visible colonies (Palomino *et al.*, 2007). *Mycobacterium tuberculosis* can be grown in liquid or solid culture.

Processing of samples for culture:

For digestion and decontamination, a maximum of 10 mL sample is taken in a falcon tube. An equal amount of a mixture containing 50 mL of 4% sodium hydroxide, 50 mL of 2.9% sodium citrate and 0.5% N -acetyl cysteine is added to it. The falcon tube is vortexed well and then kept at 37 °C for 20 min. After that, neutralization is done by filling the tube up to 50 mL mark with sterile phosphate buffered saline (pH 6.8). The tube is centrifuged for 20 min at 3000 × g. Supernatant is discarded and the pellet is reconstituted with 1 mL of sterile phosphate buffer, 0.5 mL of this is added to the MGIT

tube and two drops on the LJ media. Samples from sterile sites are inoculated after centrifugation, without digestion and decontamination (Mishra *et al.*, 2016).

Solid Culture

Many different solid media are available for cultivating mycobacteria; they can be either of egg based (such as Löwenstein Jensen slants) or agar based (such as Middlebrook 7H11 agar). After cultures, a variety of methods can be used to differentiate the mycobacteria. These include looking for pigmentation (that may or may not require exposure to light to develop), biochemical testing and immunochromatographic testing. One of the major criteria used to identify isolates is the time required for growth. Fast growers, a group that includes *Mycobacterium fortuitum* and *Mycobacterium chelonae*, generally will grow in less than 1 week. However, *M tuberculosis* requires from 3 to 8 weeks to form a colony on these media (Eichbaum, 2002).

Commonly used LJ media are prepared as follow: fresh eggs, not more than 1wk old, are cleaned by scrubbing with a hand brush in a soap solution, Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them for 15minutes in 70% ethanol. Wash and scrub hands well before breaking the eggs into a sterile flask. Shake the flask by hand to homogenize the eggs. Filter eggs through four layers of sterile gauze into a sterile, graduated cylinder. Homogenized eggs are added to the prepared autoclaved salt solution and 2% malachite green is added then dispensed to screw cap test tube (Kubica and Kent, 1985).

Liquid Culture

The Mycobacterial Growth Indicator Tube (MGIT) assay is rapid liquid culture method. It uses a fluorescence quenching-based oxygen sensor to detect the growth of mycobacteria by measuring oxygen consumption through a UV indicator source. It doesn't use radioactivity for detection (Eichbaum, 2002; Zapata *et al.*, 1999). A BBL MGIT tube (from Becton Dickinson) containing 7 mL modified middle brook 7H9 broth is used. Lyophilized MGIT PANTA (containing polymyxin B, azlocillin, nalidixic acid, trimethoprim, amphotericin B) is reconstituted with MGIT growth supplement (containing oleic acid, albumin, dextrose, catalase, polyoxyethylene stearate), and 0.8 mL of this is added prior to sample inoculation to the MGIT tube (Mishra *et al.*, 2016).

2.3.3.3 GeneXpert

The GeneXpert is Real-time PCR assay having a software-driven cartridge processor and integrated fluorescence-based quantitative thermal cycler that interfaces with single-use disposable cartridges to achieve nucleic acid isolation and quantitative real time PCR. GeneXpert cartridge consists of multiple chambers that are designed to hold the biological sample in lysis buffer; purification and elution buffers; and all RT-PCR reagents, enzymes, and buffers and to retain all sample-processing wastes (Raja *et al.*, 2005). The XpertMTB/RIF© test is utilizing the GeneXpert© platform which detect TB as well as rifampicin resistance in less than two hours with minimal hands on technical time (Blakemore *et al.*, 2010). Resistance to rifampicin almost invariably involves alterations of RNA polymerase, which is encoded by the RNA polymerase subunit gene (*rpoB*). Missense (single or double) mutations, deletions, or insertions within the 81-bp core region of the *rpoB* gene were found to be responsible for rifampicin resistance in

more than 95% of rifampicin -resistant *M. tuberculosis* strains (Van Der Zanden *et al.*, 2003).

The sensitivity and specificity of the Xpert MTB/RIF assay with gastric lavage aspirate and sputum samples of Children were 68.8% and 90.0% sensitivity and 99.3% and 98.5% specificity respectively (Bates *et al.*, 2013). The sensitivity of smear, MODS and Xpert for the diagnosis of pediatric tuberculosis in Viet Nam was 37.9%, 51.7% and 50.0 % respectively. Xpert was significantly more sensitive than smear (Nhu *et al.*, 2013). Other cross-sectional diagnostic study at a tertiary care facility in Uganda showed that the sensitivity and specificity of Xpert MTB/RIF test were 79.4% and 96.5% respectively (Sekadde *et al.*, 2013).

Study compared the diagnostic accuracy of Xpert MTB/RIF to concentrated, fluorescent acid-fast smear with a reference standard of liquid culture in 452 children showed that 6% had a positive smear, 16% had a positive culture, and 13% had a positive Xpert MTB/RIF test result (Nicol *et al.*, 2011). Other finding showed that Xpert MTB/RIF test in 452 hospitalized children suspected of having TB with or without HIV, showed that two Xpert tests doubled the case detection rate compared with two smear microscopy (76% versus 38%), identifying all smear-positive and 61% of smear-negative cases, the specificity was 98.8% (Nicol *et al.*, 2011).

Study on case detection by Xpert showed that the sensitivity of Xpert is 100% of smear positive case and 66.6% of culture positive case (Rachow *et al.*, 2012). Sekadde *et al.*, showed that Xpert MTB/RIF test identified 92.9% smear positive-culture positive cases and 70% smear negative-culture positive cases (Sekadde *et al.*, 2013). Clinical validation

of Xpert MTB/RIF test on 174 gastric aspirates of children showed that the sensitivity and specificity were 81% and 100% respectively (Tortoli *et al.*, 2012)

Study on performance of Xpert MTB/RIF showed that the sensitivity of a single direct Xpert MTB/RIF on culture positive and smear positive cases were 98.2% and culture positive and smear negative cases were 72.5% among patients with smear-negative, culture-positive tuberculosis the addition of a second MTB/RIF test increased sensitivity by 12.6% points and a third by 5.1 % points, to a total of 90.2% (Boehme *et al.*, 2010).

2.4 Diagnosis of Latent Tuberculosis

Latent tuberculosis infection (LTBI) is defined as a state of persistent immune response to stimulation by *Mycobacterium tuberculosis* antigens without evidence of clinically manifested active TB (Mack *et al.*, 2009). LTBI is diagnosed by detection of cell-mediated, delayed hypersensitivity to *Mycobacterium tuberculosis* via the Tuberculin Skin Test (TST) or by interferon gamma release assay (IGRA). The interpretation of the TST or IGRA reaction is based on the likelihood of the person being infected with TB as well as the likelihood of progression to disease if infected.

2.4.1 Tuberculin skin test (TST)

In latent TB infection, the bacteria are made inactive by the body's immune system. The bacteria can remain inactive for many years, perhaps for life. Most people who become infected with TB don't get active TB disease. However, an infected person remains at risk of developing active TB disease at any time. The bacteria can become active and multiply, especially if the immune system becomes impaired. The tuberculin skin test (TST) is used primarily to identify people infected with *Mycobacterium tuberculosis* (MTB). It is used to identify such people because they have a 5 -10% lifetime risk of

developing TB disease. TST should therefore be targeted to those individuals at risk either of acquiring TB infection or of progressing to TB disease, once infected. Purified protein derivative (PPD) consists of bacteria-derived protein without viable organisms and is safe for use in immune compromised persons and in pregnancy. Preventive treatment of people infected with MTB reduces their risk of developing TB disease up to 90%. The tuberculin skin test (TST) is done by injecting 0.1ml of tuberculin-purified protein derivative (PPD) into the skin of the inner surface of the forearm. After measuring two vertical diameters of the skin induration, the average is obtained for interpretation. Indurations are scored as no response or energy (0 mm induration diameter), negativity (0 to 4 mm induration diameter), reactivity (5 to 9 mm induration diameter), and positivity (≥ 10 mm induration diameter) (NSW, 2009; Saffari *et al.*, 2017).

2.4.2 IGRA (Interferon gamma release assay)

The accuracy of IGRAs has been more difficult to assess in children, thus the use of IGRAs in children aged under 5yrs is not recommended but unlike the TST, IGRA tests do not require a return visit and results are less likely to be affected by cross reactivity with BCG or infection due to most non- tuberculosis mycobacteria. IGRA tests can be done by QuantiFERON Gold in tube or TSpot test.

2.4.2.1 QFT-IT Assay (QuantiFERON-TB Gold In-Tube)

QFT-IT is performed as indicated by the manufacturer. Briefly, whole blood is collected in the QFT-IT tubes (Nil Control, TB-Ag and Mitogen) and incubated at 37°C for 16–24 hours. Following incubation, samples are centrifuged and the plasma is used to measure the IFN- γ produced in response to *M. tuberculosis* antigens, phytohaemagglutinin (PHA)

and the negative control. Data are presented as IU/ml of IFN- γ ; the cut-off value for a positive test is 0.35 IU/ml, according to manufacturer's instructions (Sali *et al.*, 2015).

2.4.2.2 TSpot test

The ELISPOT assay (T-SPOT.TB Oxford Immunotec, Oxford, UK) is performed as follow: 8 mL peripheral blood is drawn from each subject into sodium citrate cell preparation tubes for peripheral blood mononuclear cell (PBMC) isolation. Freshly isolated PBMC are resuspended in cell culture medium. A total of four IFN- γ antibody pre-coated wells are required for each patient sample. PBMC are seeded in wells (2.5×10^5 cells/well) containing culture media (negative control), phytohaemagglutinin (PHA; as a positive control), or two different pools of recombinant *M. tuberculosis*-specific antigens A and B (including ESAT-6 and CFP10). Microtiter plates are incubated at 37°C for 16–20 h. Plates are washed with phosphate-buffered saline (PBS) four times. Alkaline phosphatase-labeled mouse anti-human IFN- γ antibodies (Oxford Immunotec) are added to each well and the plates are incubated at 2–8°C for 1 h. After plate washing, substrate solution is added to each well and incubated at room temperature for 7 min. A digital microscope is used to measure the number of spots in each well. If the spot count in the Nil Control well is 0–5, (Panel A minus Nil Control) and/or (Panel B minus Nil Control) ≥ 6 spots, this is regarded as a positive result. If the spot count in the Nil Control well is ≥ 6 spots, (spot count in Panel A and/ or Panel B) $\geq 2 \times$ (spot count in Nil Control), this is regarded as a positive result (Jiang *et al.*, 2016).

2.5 Molecular Typing Techniques for MTBC

Molecular typing of *Mycobacterium tuberculosis* strains has become a valuable tool in the epidemiology of tuberculosis that allows for the analysis and better understanding of

transmission dynamics, genetic phylogeny, detection of laboratory contamination and outbreaks investigation/confirmation, (Allix *et al.*, 2004; Aristimuno *et al.*, 2006; Mathema *et al.*, 2006; Workalemahu *et al.*, 2013).

The basic methodology and usefulness of the most widely used genotyping tools and their molecular stability are described here.

2.5.1 IS6110 RFLP

Repetitive elements were identified in the *M. tuberculosis* genome independently by Eisenach and colleagues, Zainuddin and Dale (Eisenach *et al.*, 1988; Zainuddin and Dale, 1989). The insertion sequence IS6110 was first sequenced and found to be a member of the IS3 family by Thierry and colleagues (Thierry *et al.*, 1990). This group described the insertion sequence as being specific to the *M. tuberculosis* complex, estimated that *M. tuberculosis* possessed between 10 – 20 copies scattered throughout the genome, and hypothesised on its usefulness in epidemiological studies. The insertion sequence was also amplified in clinical samples from patients with suspected and confirmed TB.

The insertion of IS6110 has been shown to modify regulatory function in some strains. Safi and colleagues (Safi *et al.*, 2004) demonstrated that IS6110 can upregulate downstream genes through an outward-directed promoter in its 3' end. Promotor activity was orientation dependent but upregulation was seen in during growth in monocytes and in *in vitro* cultures. This suggests that insertion and deletion of IS6110 may alter the phenotype of the organism as well as being a means by which its evolution may be studied.

Insertion sequences have been used to genotype *M. tuberculosis* in numerous outbreak situations (Cave *et al.*, 1991; Daley *et al.*, 1992; Edlin *et al.*, 1992; Fomukong *et al.*,

1992; Hermans *et al.*, 1990; Mazurek *et al.*, 1991; Otal *et al.*, 1991; van Soolingen *et al.*, 1991) and a consensus was required to allow the standardisation of the methodology that would allow comparison of strain types between centres. Molecular fingerprinting using *IS6110* relies on the cultivation of the organism, genomic DNA extraction, enzymatic restriction, agarose gel electrophoresis, Southern hybridisation and detection of the IS elements with a labelled probe. The three elements that required standardisation were the restriction enzyme, nature of the labelled probe and the molecular size-markers used. In collaboration, researchers who had been instrumental in the discovery and characterisation of *IS6110* concluded that *PvuII* should be used, since *IS6110* possessed only one restriction site for this enzyme (figure5). The probe was designed to bind only to the right of the restriction site in order to allow the visualisation of only a single band per *IS6110* copy (figure5). Finally, it was agreed that a molecular size marker ranging between 0.9 and 10kb be used in addition to a reference strain Mt14323 (van Embden *et al.*, 1993).

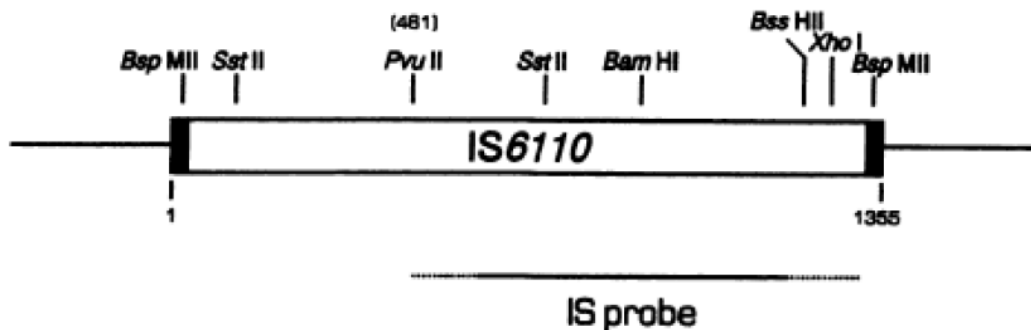


Figure5 Physical map of *IS6110* (van Embden *et al.* 1993).

It was soon noted that some strains possess few copies of *IS6110* and therefore could not be adequately discriminated by this method (van Soolingen *et al.*, 1993).

Isolates that possess fewer than five copies of *IS6110* are termed 'low copy number' strains and are not able to be differentiated by this method. Furthermore, low copy number strains that have been shown to possess the same insertion by sequence analysis have produced different *IS6110* RFLP patterns (Dale *et al.*, 2003). This may be due to polymorphisms elsewhere that insert or delete a *PvuII* restriction site. *IS6110* RFLP offers good discrimination but is slow, labour intensive, and requires large quantities of DNA (>200ng). Mature cultures are required to provide sufficient quantities of DNA, which makes the use of this method limited in real-time outbreak investigation. It is also unable to distinguish strains with few *IS6110* copies (< 5).

2.5.2 Mycobacterium Interspersed Repetitive Units – Variable Number Tandem Repeats (MIRU-VNTR)

Repetitive units of DNA in organisms are well described (Higgins *et al.*, 1988); (Lupski and Weinstock, 1992) and have been used to genotype a large array of bacteria such as *Escherichia coli* (Manges *et al.*, 2009), *Clostridium difficile* (Marsh *et al.*, 2006; van den Berg *et al.*, 2007) , *Bacillus anthracis* (Keim *et al.*, 1999; Le Fleche *et al.*, 2001), *Yersinia pestis* (Klevytska *et al.*, 2001; Le Fleche *et al.*, 2001) , *Listeria monocytogenes* (Lindstedt *et al.*, 2008), *Shigella* spp. (Gorge *et al.*, 2008). Additionally, VNTRs are also used in mapping human genes (Nakamura *et al.*, 1987).

Supply and colleagues identified a novel group of such repetitive units that they named mycobacterial interspersed repetitive units (MIRU). They are mainly intergenic and are distributed throughout the genome. These units were unlike other repetitive units previously described as they contained no obvious palindromic sequences, were direct tandem repeats and were orientated in one direction relative to the transcription of the

adjacent genes. Additionally, approximately half of the MIRUs described contained small ORFs and based on sequence homology of the *M. tuberculosis* sequences at the time, it was estimated that there may be 40-50 such MIRUs per genome (Supply *et al.*, 1997). At approximately the same time, analysis of published sequences by Frothingham and Meeker-O'Connell identified a number of repetitive regions in *M. tuberculosis* H37Rv. Some of these, termed exact tandem repeats A to F (ETR-A to F), were variable between strains (Frothingham and Meeker, 1998).

Further work by Supply and colleagues identified a total of 41MIRU loci when analysing the complete H37Rv genome (figure6) (Supply *et al.*, 2000). These tandem repeats varied between loci and strains and may be amplified with the use of specific PCR primers to each MIRU-flanking region. If a strain lacked a single repeat at a MIRU locus a PCR product would still be generated. The sizing of these PCR products would enable the investigator to identify the number of repeats present at each locus and therefore generate a numerical genotype.

There was similarity between five of these MIRU loci and ETR A-E previously described (Frothingham and Meeker, 1998). The variability between strains of these 41 loci differ and analysis of a set of internationally obtained strains (Kremer *et al.*, 1999) suggested that 12 of these loci exhibited a suitable degree of variability to be used in a genotyping methodology (Supply *et al.*, 2000).

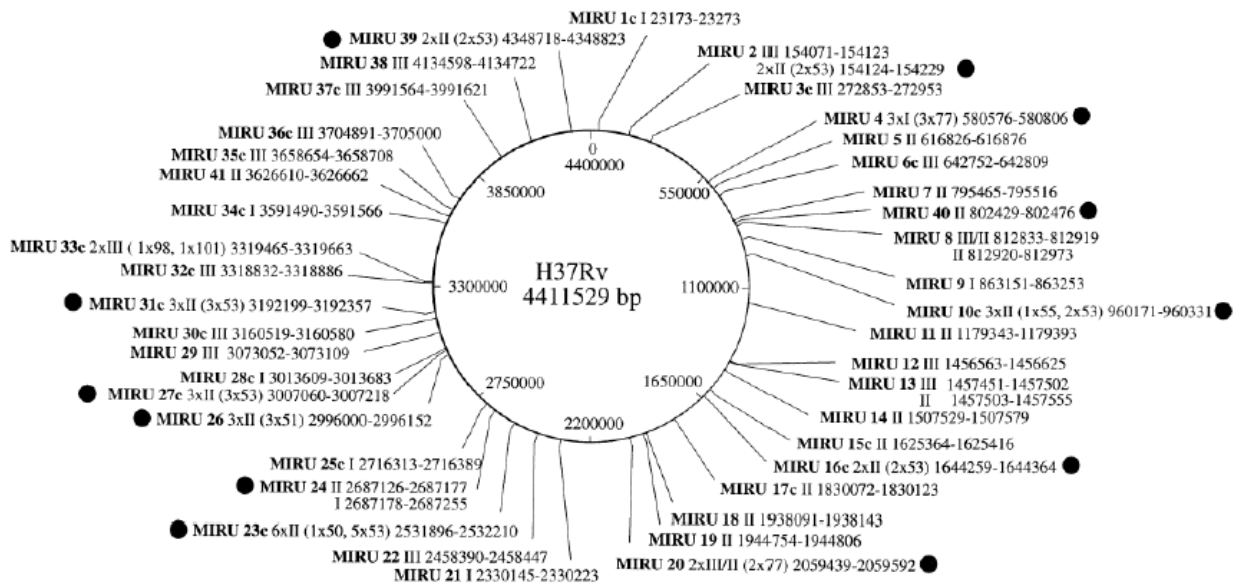


Figure 6 Location of MIRU loci in *M. tuberculosis* H37Rv genome (Supply *et al.*, 2000)

The discriminatory power of this method may be altered by varying the number of MIRU loci included in the analysis. For example, when only five loci were used, Kremer and colleagues found VNTR to be less discriminatory than IS6110 RFLP and spoligotyping (Kremer *et al.*, 1999). Kwara and colleagues analysed 64 *M. tuberculosis* strains clustered into ten groups by IS6110 RFLP (Kwara *et al.*, 2003). Spoligotyping and 12 loci MIRU were used to attempt to further differentiate these clusters. MIRU analysis differentiated these strains to a higher degree than both IS6110 RFLP and spoligotyping. In another study, however, 12 loci MIRU alongside spoligotyping was found to be less discriminatory than IS6110 RFLP (Supply *et al.*, 2006). Five loci VNTR was found to be more discriminatory than IS6110 RFLP in strains with few copies of IS6110 (Barlow *et al.*, 2001). The further discrimination of apparent clusters of high IS6110 copy number strains by 12-loci MIRU has also been described (van Deutekom *et al.*, 2005). The

relative levels of discrimination will of course vary depending on the number, and geographical representation of strains analysed.

As some loci are more variable than others, the discriminatory power of MIRU does not increase in a linear manner with the analysis of additional loci. The more discriminatory a method, the fewer clusters will be generated when strain typing a group of strains.

Various combinations of MIRU loci have been suggested for genotyping of *M. tuberculosis* (Frothingham and Meeker, 1998; Goyal *et al.*, 1994; Kam *et al.*, 2006; Kremer *et al.*, 2005; Le Fleche *et al.*, 2002; Magdalena *et al.*, 1998a; Magdalena *et al.*, 1998b; Mazars *et al.*, 2001; Roring *et al.*, 2002; Roring *et al.*, 2004; Skuce *et al.*, 2002; Smittipat *et al.*, 2005; Supply *et al.*, 2000; Surikova *et al.*, 2005). Supply and colleagues showed by analysing 29 loci that five were unreliable for use in a genotyping methodology. A well characterised set of 90 strains (Kremer *et al.*, 1999) were investigated with the remaining 24 loci (Supply *et al.*, 2006). When using 24 loci, the 90 strains were separated into 89 distinct genotypes – H37Rv and H37Ra were indistinguishable by this method as well as by spoligotyping, and the addition of the five unreliable MIRU loci. Maximum resolution of these 89 genotypes could be achieved by the analysis of only nine loci (MIRU 04, 10, 16, 26 and 40 and VNTR 0577, 2163b, 2165 and 4052), thus indicating that, in this group of strains at least, these nine loci were the most variable. Analysis of nearly 500 isolates identified the loci that demonstrated the most allelic diversity and were most likely to exhibit single, double and triple locus variants (SLVs, DLVs and TLVs). The removal of the loci with the least diversity generated a subset of 15 MIRU loci with 96% of the discriminatory power of the full set

of 24 loci (Supply *et al.*, 2006). It was reasoned that the 15 loci set may be used for epidemiological studies, whilst the 24 loci set would be of use in phylogenetic studies.

The stability of the original 12 MIRU loci (Supply *et al.*, 2000) was assessed by comparing the MIRU genotypes obtained from serial isolates from patients over up to six years (isolates analysed in (Warren *et al.*, 2002)). A total of 123 isolates, representing at least two isolates from 56 patients, were genotyped (Savine *et al.*, 2002). The selected isolates belonged to a variety of distinct IS6110 RFLP families. All 12 MIRU loci were identical within 55 of the 56 serial patient groups. One isolate showed a reduction in one repeat in a single locus. Conversely, 11 of the 56 serial patient isolates showed a variation in their IS6110 pattern. The MIRU genotype for these isolates remained unchanged, suggesting that 12 loci MIRU was more stable than IS6110 RFLP.

MIRU analysis has several technical advantages over IS6110 RFLP. Only small amounts of genomic material are required as the method involves DNA amplification. The assay itself is extremely simple to perform when compared with the involved process of RFLP. Moreover, as a digital genotype is generated, the data may be readily transported and compared in other centres. The method can be simplified even further by the utilisation of multiplex PCR and automated DNA sequencing technologies (Supply *et al.*, 2001). Additionally, the method may be used to genotype *M. tuberculosis* strains that possess both high and low numbers of IS6110.

MIRU-VNTR types align with other phylogenetic markers and assign strains into the large phylogenetic groups and above 90% concordance between MIRU-VNTR types and phylogenetic groups by SNP analysis (Gibson *et al.*, 2005).

2.5.3 Spoligotyping

Spacer-oligonucleotide typing (Spoligotyping) is a PCR-based typing method that relies on identifying polymorphisms in the spacer units in the direct repeat (DR) region of the genome. The DR region comprises multiple, virtually identical, 36bp regions interspersed with non-repetitive spacer sequences of a similar size (Groenen *et al.*, 1993). As a nucleic acid amplification method, small amounts of DNA are required, and as such, spoligotyping has been used to detect and genotype *M. tuberculosis* from paraffin wax-embedded samples (van der Zanden *et al.*, 1998). Examples of spoligotypes are displayed in figure 7.

Variation in these regions was thought to be caused by homologous recombination between adjacent or distinct DRs or by transformation due to the insertion of IS6110, which is almost invariably present in the DR region (Groenen *et al.*, 1993). These direct variant repeats (DVRs) differ between strains, but their order remains conserved (van Embden *et al.*, 2000). The DR region contains over 60 such spacers, but 43 were selected as a basis of this typing methodology. The presence or absence of these spacers is detected by the amplification and subsequent reverse hybridization (Kamerbeek *et al.*, 1997).

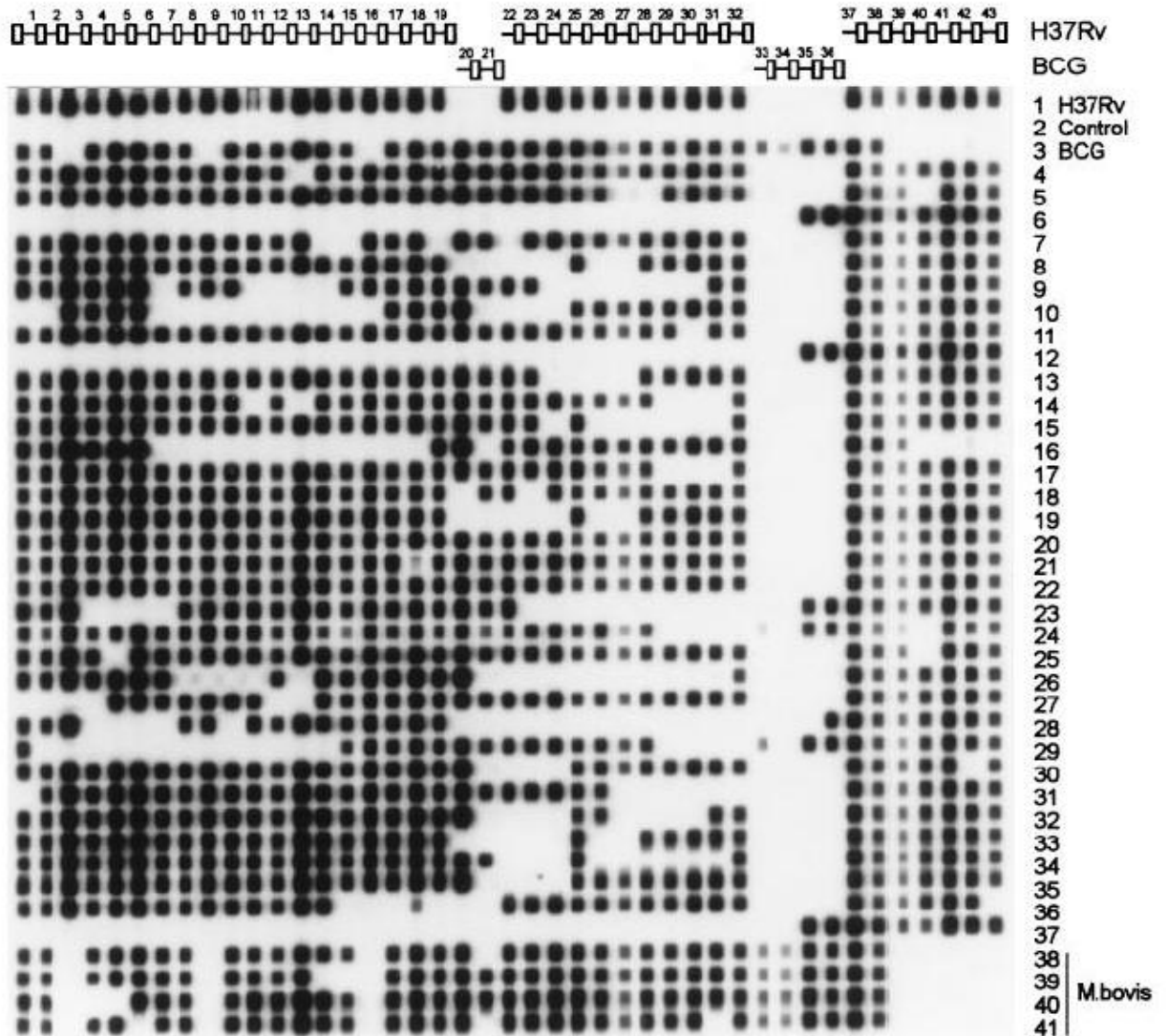


Figure7 Hybridization patterns (spoligotypes) of amplified mycobacterial DNAs of 35 *M. tuberculosis* and 5 *M. bovis* strains. The order of the spacers on the filter corresponds to their order in the genome. Note that the spoligotype of strains 6, 12, and 37 corresponds to that of strains from the Beijing family, as described earlier (Kamerbeek *et al.*, 1997). This technique is widely used in both evolutionary and epidemiological studies. As such, an agreed nomenclature was proposed to allow comparison of genotypes (Dale *et al.*,

2001). Spoligotyping is superior to IS6110 RFLP when studying strains with few copies of IS6110. However, it is less discriminatory than IS6110 typing for all other strains (Kremer *et al.*, 1999; Soini *et al.*, 2001).

The analysis of DVRs by Warren and colleagues revealed that the evolution of a spoligotype occurs by four processes; IS6110-mediated mutation, homologous recombination between repeat sequences leading to DVR deletion, strand slippage during replication leading to duplication of the DVR sequences and point mutation (Warren *et al.*, 2002). Interestingly, it was demonstrated that the insertion of IS6110 into the DR region lead to the failed hybridisation with some DVRs, despite their presence. DR RFLP, together with cloning and sequencing showed that these DVRs were present although spoligotyping indicated their deletion. The authors concluded that as the evolution of spoligotypes was not an independent process, that this method should not be relied upon alone for evolutionary studies in *M. tuberculosis*. They advocate its use alongside other methodologies.

Based on large sequence polymorphism, six main lineages have been described within the *M. tuberculosis* complex (MTBC) affecting humans: Indio-oceanic (IO) (Lineage 1), East-Asian (EA) (Lineage 2), East-African-Indian (EAI) (Lineage 3), Euro-American (EUA) (Lineage 4), West African Lineage I (Lineage 5) and West African Lineage II (Lineage 6) (Gagneux *et al.*, 2006). However, the naming and the grouping systems vary according to the molecular marker and the method of typing used. For example the SpolDB4 system assigns the MTBC organisms into 62 clades/lineages based on the genome variability at the DR locus (e.g. EAI, Beijing, CAS, Haarlem,T, X, AFRI 1 and AFRI 2) (Brudey *et al.*, 2006).

Mycobacteria that cause tuberculosis in mammals form the *Mycobacterium tuberculosis* complex (MTBC) and include *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium microti*, *Mycobacterium caprae*, and *Mycobacterium canettii*. *M. tuberculosis* is the predominant cause of human tuberculosis. Region of difference 9 (RD9) deletion typing is used to detect *Mycobacterium tuberculosis* from other members of *Mycobacterium tuberculosis* complex (Huard *et al.*, 2003).

Genetic typing of MTBC strains isolated from childhood TB patients in Jimma showed that the same lineages that dominate among adults, Lineages 4 and 3 (EUA and EAI or CAS), are prominent in children (Workalemahu *et al.*, 2013).

Paediatric tuberculosis is poorly addressed in Ethiopia and the study on genotype distribution of the causative *Mycobacterium tuberculosis* strains responsible for its spread is scanty

2.6 Treatment of Tuberculosis

The major goals of anti-TB treatment are to cure the patient by eliminating most of the bacilli, prevent the development of drug resistance by using a drug combination regimen, prevent TB relapse by eliminating the quiescent bacilli and, finally, decrease the TB transmission to others.

The poor adherence to the anti-TB therapy represents the main contributory factor to treatment failure and emergence of TB drug-resistance. In order to improve the compliance to the treatment, all guidelines emphasize the widespread use of the directly observed therapy (DOT), a patient-centred strategy in which patients are observed to ingest each dose of anti-TB drugs. All guidelines are also in agreement that TB treatment should include an initial intensive phase, when three to four drugs are used in order to kill

the majority of bacilli and prevent the emergence of drug resistance, and a continuation phase, when fewer drugs are given in order to eradicate quiescent bacilli (Berti *et al.*, 2014).

Mechanism of action of anti TB drugs

The new drugs

The current treatment for drug-resistant tuberculosis (TB) is long, complex, and associated with severe and life-threatening side effects and poor outcomes. For the first time in nearly 50 years, there have been two new drugs registered for use in multidrug-resistant TB (MDR-TB). Bedaquiline, a diarylquinoline, and delamanid, a nitromidoxazole, have received conditional stringent regulatory approval and have World Health Organization interim policy guidance for their use (Brigden *et al.*, 2015).

Delamanid, previously OPC-67863, is a drug of the dihydronitroimidazole class and has potent anti-TB activity. Delamanid is thought to primarily inhibit synthesis of methoxymycolic and keto-mycolic acid, components of the mycobacterial cell wall. As a prodrug, it requires metabolic activation to exert its anti-TB activity (Gler *et al.*, 2012). Bedaquiline, previously called TMC 207, is a diarylquinoline that acts by specifically inhibiting mycobacterial adenosine triphosphate synthase and has a long half-life of approximately 5 months (Matteelli *et al.*, 2010).

The classical drugs

Treatment of drug-resistant TB requires a combination various anti-TB drugs with different mechanisms of action. Traditionally, the classes of anti-TB drugs have been

divided into first- and second-line drugs as briefly explained in Table2 (Godebo *et al.*, 2015)

Table2 some of anti-TB drugs with their category and mechanism of action

Drug name	Category	Route	Chemical Description	Mechanism of action
Isoniazid (INH)	1 st line	Oral	Nicotinic acid hydrazide	Inhibits mycolic acid synthesis
Rifampin/rifampicin (RIF)	1 st line	Oral	Rifamycin derivative	Inhibit RNA synthesis by targeting RNA polymerase
Pyrazinamide (PZA)	1 st line	Oral	Nicotinamide derivative	Inhibits cell membrane synthesis
Ethambutol (EMB)	1 st line	Oral	Ethylene di-imine di 1-butanol	Inhibit cell wall synthesis
Streptomycin(SM)	1 st line	Injectable	Aminoglycoside	Inhibit protein synthesis
Rifabutin	2 nd line	Oral	Rifamycin derivative	Inhibit RNA synthesis by targeting RNA Polymerase
Rifapentine/ (Rfb)				
Amikacin (AM)	2 nd line	Injectable	Aminoglycoside	Inhibit protein synthesis
Kanamycin (Km)	2 nd line	Injectable	Aminoglycoside	Inhibit protein synthesis
Capreomycin(Cm)	2 nd line	Injectable	Cyclic Peptide	Inhibit protein synthesis
Ofloxacin (Ofx)	2 nd line	Oral	Fluoroquinolone	Inhibits DNA synthesis

Levofloxacin(Lfx)	2 nd line	Oral	Fluoroquinolones	Inhibits DNA synthesis and supercoiling by targeting topoisomerase
Moxifloxacin (MFX)	2 nd line	Oral	Fluoroquinolones	Inhibits DNA synthesis and supercoiling by targeting topoisomerase
Ethionamide (Eto)	2 nd line	Oral	Isonicotinic acid derivative	Inhibits mycolic acid synthesis
Protionamide (Pto)	2 nd line	Oral	Isonicotinic acid derivative	Inhibits mycolic acid synthesis
Cycloserine (Cs)	2 nd line	Oral	D-Cycloserine	Inhibits cell wall synthesis
Paraaminosalicylic acid (PAS)	2 nd line	Oral	Para-amino salicylic acid	Inhibit folate biosynthesis

2.7 Strategies for control of Tuberculosis

Major progress in global TB control and expanded access to high-quality tuberculosis care followed the widespread implementation of the DOTS (directly observed treatment, short-course) strategy of 1995 advocated by the WHO has potential impact on epidemiology of tuberculosis through achieving high cure rates and thereby decreasing community transmission. However, global statistics indicated that DOTS alone would not be sufficient to achieve global TB control and elimination. In 2005, the World Health Assembly recognized the need for a new strategy that would build upon and enhance the

achievements of DOTS. The Stop TB Strategy, launched on World TB day in 2006, is designed to meet the TB-related millennium development goal (MDG) as well as the Stop TB partnership targets set for 2015. The Stop TB Strategy underpins the Global Plan to Stop TB 2006–2015. The Stop TB Strategy of 2006 widened its scope to address management of all forms of tuberculosis including HIV-associated and drug-resistant tuberculosis, through engagement of communities, involvement of all care providers, strengthening of health systems, and fostering of research (Uplekar *et al.*, 2015; WHO, 2008b)

The post-2015 global tuberculosis strategy, End TB Strategy, was first launched in 2014 by the World Health Organization is fully in line with the seventeen Sustainable Development Goals one of which (SDG 3) was specifically addressed to health issues and calls for ending the major global epidemics including TB that came into effect since January 2016 and sets ambitious goals for the post-2015 agenda. A 90% reduction in TB-related mortality and an 80% decline in TB incidence within 2030 as well as the abolition of catastrophic expenditures for TB-affected people are the main targets of this strategy. Strong government commitment and adequate financing from all countries together with community engagement and appropriate investments in research are necessary in order to reach these objectives (Raviglione and Sulis, 2016).

Table3 End tuberculosis strategy: vision, targets and pillars and Principles (WHO, 2014b).

Vision	A world free of TB (zero deaths, disease and suffering due to tuberculosis)
Goal	End the global TB epidemic
Targets for 2035	95% reduction in TB deaths (compared with 2015); 90% reduction in TB incidence rate (less than 100 TB cases per million population) ; no affected families facing catastrophic costs due to TB
Principles	Government stewardship and accountability, with monitoring and evaluation; strong coalition with civil society organizations and communities; protection and promotion of human rights, ethics and equity; adaptation of the strategy and targets at country level, with global collaboration.
Pillars and components	<p><i>Integrated, patient-centred care and prevention:</i> i) early diagnosis of tuberculosis including universal drug susceptibility testing, and systematic screening of contacts and high-risk groups; ii) treatment of all people with tuberculosis including drug-resistant tuberculosis, and patient support; iii) collaborative tuberculosis/HIV activities, and management of co-morbidities; iv) preventive treatment of persons at high-risk, and vaccination against tuberculosis.</p> <p><i>Bold policies and supportive systems:</i> i) political commitment with</p>

adequate resources for tuberculosis care and prevention; ii) engagement of communities, civil society organizations, and public and private care providers; iii) universal health coverage policy; and regulatory frameworks for case notification, vital registration, quality and rational use of medicines, and infection control social protection, poverty alleviation and actions on other determinants of tuberculosis.

Intensified research and innovation: i) discovery, development and rapid uptake of new tools, interventions, and strategies; ii) research to optimize implementation and impact, and promote innovations.

Chapter3 Materials and Methods

3.1 Study area

The area the studies were conducted consist of Health centers and Hospitals purposefully selected from those found in Sidama zone in southern Ethiopia. The zone had a population of 3,677,370 people living in 19 Weredas and 3 city administrations. There were a total of 130 Health Centres and 5 Hospitals of which 63 had AFB diagnostic, 3 had GeneXpert and 4 had radiology services.

The study of Prevalence, Strain typing, DST and treatment outcomes however were limited to the two Public Hospitals (Hawassa referral and Adare General Hospitals). Both are located in Hawassa the capital city of SNNPR, Sidama zone. It is located at 1708 m altitude and 275km from Addis Ababa.

Hawassa referral Hospital is a University Hospital which was established as a referral Hospital in 2002 with the objectives of providing medical services to the society and practical trainings to Health science students. The Hospital was estimated to serve a population of about 12,000,000 people having 350 beds.

Adare General Hospital was established as a clinic in 1962 and was serving 2,695 people (the total population of Hawassa city by 1962). The Clinic was then upgraded to Health center as of 1983. The Health center was amongst burdened Health centers in the region. After all this Adare General Hospital was established as Hospital and started serving 341,659 population of Hawassa city in 2011.

3.2 Study design and study subjects

A retrospective data analysis was done on the diagnosis and treatment outcomes of TB positive children under 15 years of age were registered for treatment of TB between

October 2009 and October 2014 at Hawassa referral and Adare General Hospitals in Hawassa. All were put on DOTS.

A prospective cross sectional study was also done on two groups of presumptive TB children under 15 years consecutively enrolled the study facilities during November 2014 – May 2017: the first group was involved in evaluation of modified diagnostic approach and the second group for the study of prevalence, strain typing, and DST. Since the study on both prevalence and evaluation of modified diagnostic approach require large volume of sample, it was impractical to collect sample from a child so that a separate group of study population were used.

The source population of the study was all presumptive TB pediatrics under 15years attending the study facilities.

The study population was all presumptive TB pediatrics under 15years attending the study facilities during the study period.

Children under 15 years who had signs and symptoms of TB (cough for more than 2 weeks duration, unexplained and prolonged fever, unexplained weight losses, or constitutional symptoms in children in contact with an adult with smear-positive TB) who were spontaneously seeking or being referred to the study facilities were included in the study. Children under 15 years who were on anti TB therapy during the study period were excluded from the study.

3.3 Sample size

The sample size (n) was calculated, assuming that the prevalence of culture positive TB in children was 32% (Workalemahu *et al.*, 2013). The expected margin of error (d) was

0.05 and the confidence interval ($Z\alpha/2$) was 95%. The formula used to calculate the sample size is:

$$n = \frac{(Z\alpha/2)^2 * P(1-P)}{d^2} = \frac{(1.96)^2 * 0.32(1-0.32)}{(0.05)^2} = 334$$

Where; n= Sample size; Z= confidence interval; α = level of significance; d= tolerable error; P= prevalence.

With 5% contingency is 350

There were a total of 700 (2n=700) children age under 15 years old with symptoms of tuberculosis that were spontaneously seeking or being referred to the study sites were included. Half of them (n=350) were enrolled to study the prevalence, drug susceptibility and molecular characterization and the remaining half (n=350) were participated to evaluate the modified diagnostic approach of childhood tuberculosis.

3.4 Data collection

Secondary Data collection

Data were extracted from TB unit registers of each study facility by trained nurse professionals by using extraction form which contains demographic information (age and sex), type of TB (smear-positive PTB, smear-negative PTB and Extra PTB), categories of TB (new case, relapse, treatment failure, return after default transfer in and other), HIV sero-status, treatment outcomes (cured, treatment completed, treatment failure, died and transfer out)

Treatment outcomes were evaluated in accordance with the NTLCP (National Tuberculosis and Leprosy Control Program) which is adopted from the WHO (FMoH,

2008) and classified as : cured (finished treatment with negative bacteriology result at the end of the treatment),treatment completed (completed treatment without bacteriology result at the end of the treatment), defaulted (patients who interrupted their treatment for two consecutive months or more after registration), treatment failure (remaining smear positive at five months despite correct intake of medication),died (patients who died from any cause during the course of treatment), transfer out (patients whose treatment result was unknown due to transfer to another health facility), successfully treated (a patient who was cured or completed treatment).

Operational definitions

Successful treatment outcome: Documented outcome as “cured” or “treatment completed”.

Unsuccessful treatment outcome: Documented outcome as “died”, “defaulted”, “treatment failures” and “transfer out”.

Primary Data collection

Data on demographic information, clinical characteristics and laboratory findings were collected using a structured questionnaire

Sample collection and processing for prevalence, strain typing and DST

Three sputum samples (Spot-Morning-Spot) were collected from each child able to expectorate sputum. From younger children unable to provide sputum, gastric aspirate were collected in pediatrics ward on three consecutive mornings after monitoring an overnight fasting by nurses.

This was done by inserting a pediatric nasogastric tube into the stomach immediately after the patients woke up in the morning and attaching a 50ml syringe at the opposite

end. A gastric aspirate was drawn and transferred into a 50 ml sterile falcon tube and an equal volume of PBS (pH = 7.0) was added to maintain the viability of the bacilli (Al-Aghbari *et al.*, 2009).

Smear microscopy was done at the respective Hospitals. Briefly, smears were prepared, air dried and heat fixed by flaming. The slides were then flooded with 1% carbol-fuchsin and heated until steaming but not boiling for 5 minutes. The smears were washed in tap water and decolorized with 3% acid-alcohol for 3 minutes. Finally, the slides were rinsed in a gentle stream of tap water and counter stained with 0.1% methylene blue for 1 minute and washed and air dried. The stained slides were examined with immersion oil objective (Soham *et al.*, 2010). Samples from each child were pooled into a separate sterile falcon tube thereafter transported by triple package within 4 hours to Hawassa Regional Laboratory where TB culture, molecular drug susceptibility test and preparation of killed TB isolates. Killed TB isolates were transported to Aklilu Lemma Institute of Pathobiology (ALIPB) for molecular characterization.

Sample collection and processing for evaluation of modified diagnostic approach

Awareness creation was given to TB focal persons, Health extension workers and health development armies to identify pediatric clinical TB suspected. Training was also given to Pediatricians, Laboratory Professionals and Nurses, that were working in the study facilities. Prior to data collections pre test was done on the standard questionnaires.

After discussion with parents of children on the importance of collection and diagnosis of samples from their children, and getting their consents sputum samples were collected. In brief, two sputum samples were collected, the first sample was collected at spot and the second after one hour of the first from those children that were able to expectorate on two

separate sterile sputum cups and children unable to expectorate sputum were asked to attend the nearby health facility early in the morning and two gastric aspirates were collected by trained nurses. This was done by inserting a pediatric naso-gastric tube into the stomach and using a 50ml syringe a minimum of 30 ml gastric aspirate were drawn twice at one hour interval (Figure8).

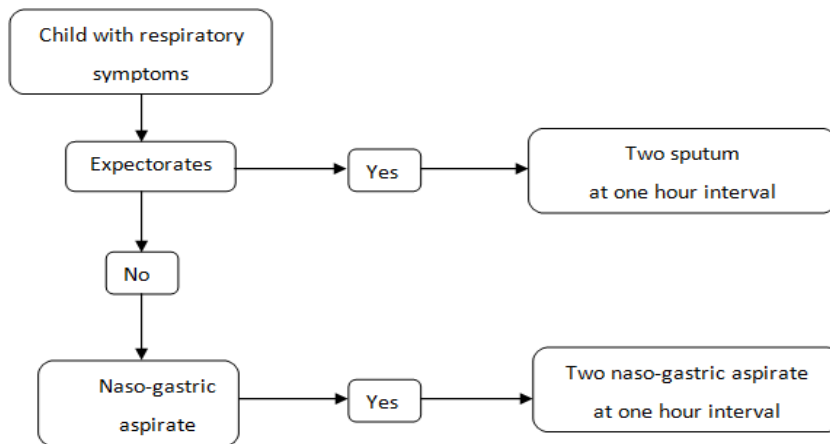


Figure8. Sample collection from children under 15 years with respiratory symptoms of tuberculosis

In case of a child who was not able to expectorate, pooled sample was prepared by transferring half part from naso-gastric aspirate 1 and half part from naso-gastric aspirate 2 into a separate sterile falcon tube by using disposable pipettes and resulted in three samples (aspirate 1, aspirate 2 and pooled sample) (Figure9) whereas a child who was able to expectorate, pooled sample was prepared by transferring half part from expectorate sputum 1 and half part from expectorate sputum 2 into a sterile falcon tube by using disposable pipettes and resulted in three samples (sputum 1, sputum 2 and pooled sample) (Figure10) . After smear examinations of aliquots of the three samples of

each child at microscopy site and the remaining samples were transported to Gene Xpert examination sites using triple package system.

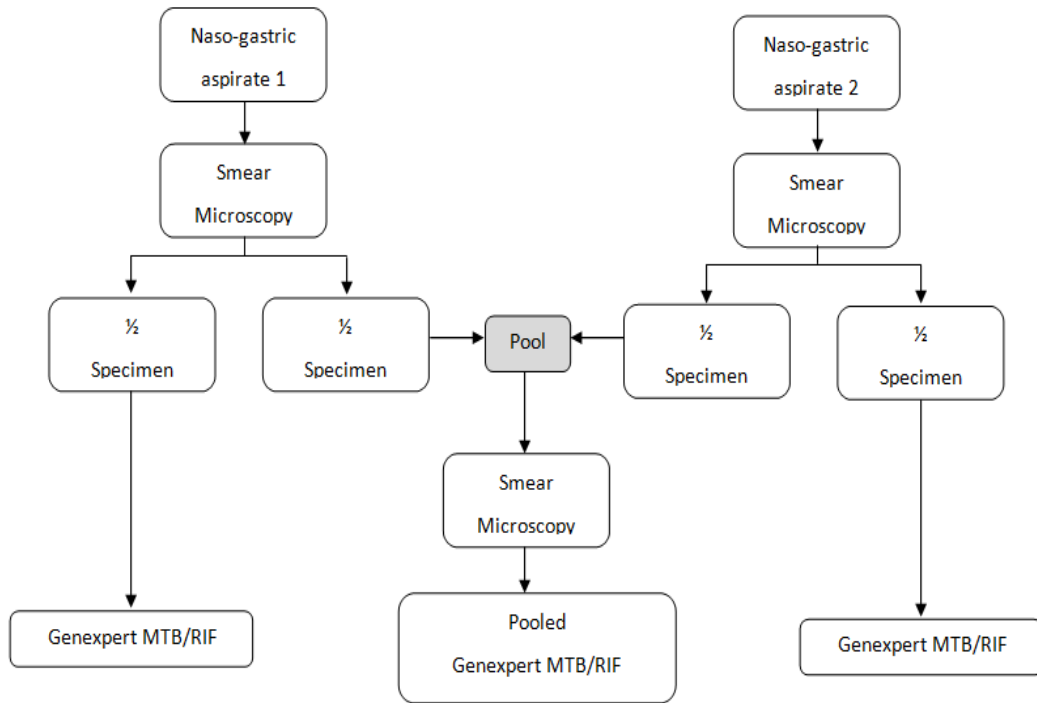


Figure9 Preparation of pooled sample from naso-gastric aspirates collected twice at one hour interval of tuberculosis cases among children under 15 years.

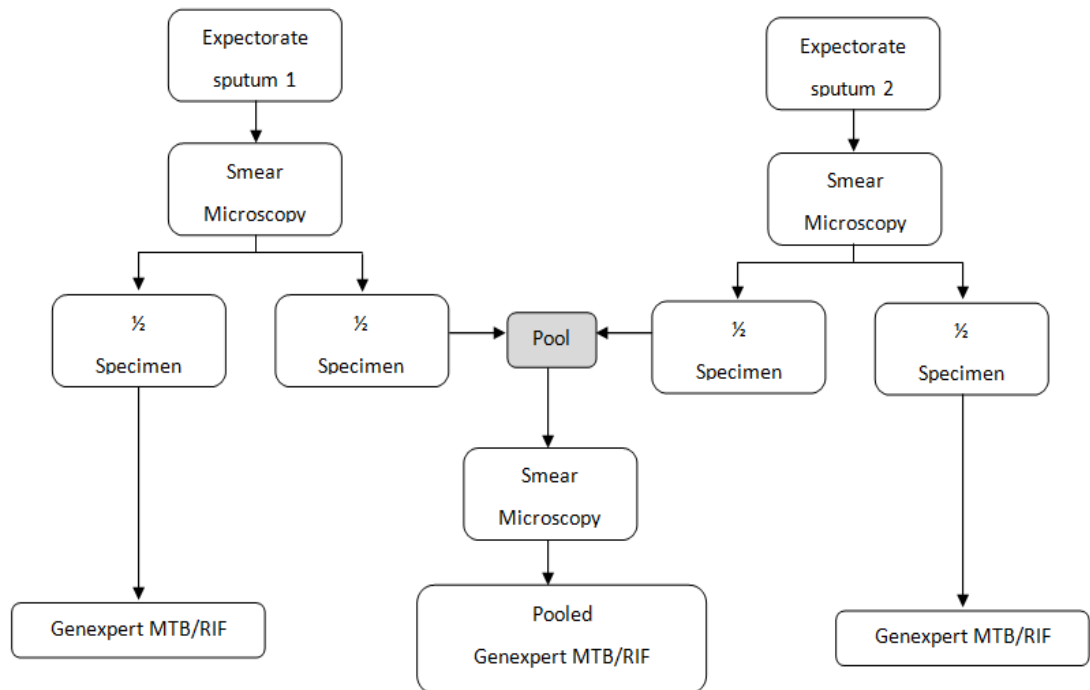


Figure10 Preparation of pooled sample from expectorate sputum samples collected twice at one hour interval of tuberculosis cases among children under 15 years.

3.5 Acid fast microscopy

Acid fast microscopy by Ziehl-Neelsen stain was done for prevalence determination and evaluation of modified diagnostic approach. It was done at the respective Hospitals or Health centers by the method previously described by Soham *et al.*, (2010). Briefly, the smears were prepared, air dried and heat fixed by flaming. The slides were then flooded with 1% carbol-fuchsin and heated until steaming but not boiling for 5 minutes. The smears were washed in tap water and decolorized with 3% acid-alcohol for 3 minutes. Finally, the slides were rinsed in a gentle stream of tap water, and then counter stained with 0.1% methylene blue for 1minute and washed, and air dried. The stained slides were examined with immersion oil objective (Soham *et al.*, 2010).

3.6 Culture and identification

Culture and identification was done for Prevalence determination. Drug susceptibility testing and Strain typing were done from the culture isolates.

The digestion-decontamination procedure of all collected samples was based on the method developed by Kubica et al., (1985). Briefly, a fresh solution of digestant was prepared by adding 0.5 mg of N-acetyl-L-cysteine (NALC) powder to a mixture of a 50ml of 4% NaOH and a 50ml of 2.9% tri-sodium citrate. Digestion and decontamination of specimen was done by mixing an equal volume of fresh digestant and specimen and vortexing it until the specimen and the digestion solution were well homogenized.

This solution was allowed to stand for 15 minutes and neutralized to 50ml mark with phosphate buffer and centrifuged at 3000xg for 15 minutes. The supernatant was then discarded aseptically and the pellet resuspended in 3 ml sterile phosphate buffer saline (PBS). A 100 µl of the suspension was inoculated onto two sterile Lowenstein-Jensen media (LJ) media which were freshly prepared from powdered bases, eggs and pyruvate or glycerol supplement. The inoculated media were then incubated at 37°C in slanted position for at least one week and thereafter positioned upright. The growth of the bacteria was read every week until the 8th week of incubation (Kubica and Kent, 1985).

The isolates from LJ media are confirmed to be MTB complex by testing with SDTBAGMPT64 (Standard Diagnostics, Inc, Korea). The antigen detection in culture isolates has been found to be a highly specific, sensitive and rapid method of confirming MTB isolates (Kumar *et al.*, 2011).

3.7 Drug susceptibility testing (DST)

DNA hybridization technology on nitrocellulose strips was used for Drug susceptibility testing. DST for first line drugs (INH and RIF) and second line drugs (fluoroquinolones (FLQ) (ofloxacin & moxifloxacin) and aminoglycosides (AM)/cyclic peptides (CM) (capreomycin, viomycin/kanamycin and amikacin)) were performed by using GenoTypeMTBDRplus VER 2.0.; Hain Lifescience, Nehren, Germany and GenoType MTBDRsl VER 2.0; Hain Lifescience, Nehren, Germany respectively following the manufacturer's instructions.

DNA extraction for genotyping

Colonies were removed from the surface of LJ medium and suspended in 200 µl of sterile double distilled water and mixed thoroughly and then, the mixture was heated at 80°C for 1 hour in water bath. The suspension was centrifuged at 15,000 rpm for 1 minute, and the supernatant was stored at -20°C until used. DNA extraction for molecular Drug susceptibility testing was performed by GenoLyse® extraction kit (Hain Lifescience, Nehren, Germany) according to the manufacturer's instructions.

3.8 Region of difference (RD) 9-based polymerase chain reaction (PCR)

Identification of *M. tuberculosis* from the other members of *M. tuberculosis* complex species was done using RD9-based PCR. RD9-PCR was performed on heat-killed cells to confirm the presence or absence of RD9 using three primers namely, RD9flankF, RD9 IntR, and RD9 flankR (Parsons *et al.*, 2002). Amplification was done by standard thermo cycler (VWR Thermo cycler, UK). The PCR amplification mixture used consisted of 10 µL HotStar Taq Master Mix (Qiagen, United Kingdom), 7.1 µL distilled water, 0.3 µL of each three primers and 2 µL of DNA template (heat killed cells), giving a total volume of

20 µL. The PCR reaction was heated at 95°C for 15 minutes after which it was subjected to 35 cycles consisting of 95 °C for one minute, 55 °C for one minute, and 72 °C for one minute. Thereafter, the reaction mixture was maintained at 72 °C for 10 minutes following which the product was removed from the thermo cycler and run on agarose gel electrophoresis. For gel electrophoresis, 8 µL PCR products was mixed with 2 µL loading dye, loaded onto 1.5% agarose gel and electrophoresed at 100 V and 500 mA for 45 minutes. The gel was then visualized using a computerized Multi- Image Light Cabinet (VWR). *M. tuberculosis* H37Rv, *M. bovis* Bacille Calmette-Guérin, and water were included as positive and negative controls. Interpretation of the result was based on bands of different sizes, as previously described by Parsons *et al* (Parsons *et al.*, 2002).

3.9 Genotyping

Spoligotyping analysis of the MTB strains was performed essentially as described by Kamerbeek *et al.* (Kamerbeek *et al.*, 1997) . Briefly, genomic mycobacterial DNA was extracted from bacteria by a previously described method (van Soolingen *et al.*, 1991). Oligonucleotides DRa and DRb were used as primers to amplify the whole DR region by PCR. The amplified biotinylated products were hybridized to a set of 43 immobilized oligonucleotides, each of which corresponded to one of the unique spacer DNA sequences within the DR locus. After hybridization, the membrane was washed, incubated with streptavidin-peroxidase conjugate, and finally developed by chemiluminescence method (Amersham Biosciences, Little Chalfont, UK) and by exposure to X-ray film (Hyperfilm ECL, Amersham Biosciences), as specified by the manufacturer. The presence and absence of spacers was visualized on the film as black and white squares, respectively.

Characterized strains of *M. bovis* and *M. tuberculosis* H37Rv were used as positive controls, whereas Qiagen distilled water (Qiagen company, Germany) was used as a negative control.

The hybridization patterns of spoligotyping were converted into binary and octal formats. These binary and octal formats of the strains were entered into query box so that the name of the strains are retrieved from the database if the spoligotype pattern of the strain in question fits the pattern that has already been registered in the SPoIDB4 database (Brudey *et al.*, 2006) and at [http://www.pasteurguadeloupe.fr:8081/SITVITDemo/\(SITVIT1\)](http://www.pasteurguadeloupe.fr:8081/SITVITDemo/(SITVIT1)). If the pattern of the strain in question has not been registered prior, no name was retrieved, and hence the strain was considered as an orphan. Two or more mycobacteria isolates sharing identical spoligotype patterns in the study were identified as clusters, whereas single spoligo pattern were considered as unique strains. Strains matching a pre-existing pattern in the SITVIT2 database were identified with the shared international type (SIT) number. An online tool Run TBLineage http://tbinsight.cs.rpi.edu/run_tb_lineage.html was also used to predict the major lineages using a conformal Bayesian network (CBN) analysis and sub lineage using knowledge based Bayesian network (KBBN).

3.10 GeneXpertMTB/RIF

Evaluation of modified diagnostic approach was done by testing pooled sample which were prepared from half part from first sample and half part from second sample which were collected at 1hour interval from a child. Pooled and individual samples of a child were tested by GeneXpertMTB/RIF (Cepheid, Sunnyvale, CA (USA)) and evaluated the yield of pooled sample against the first or the second sample.

The MTB/RIF test was performed as described previously(Iram *et al.*, 2015). In brief, Sample reagent was added in a 2:1 ratio to unprocessed sample in 15 ml falcon tube and the tube was manually agitated twice during a 15 minute incubation period at room temperature. Then 2 ml of the inactivated material was transferred to the test cartridge by a sterile disposable pipette (provided with kits). Cartridges were loaded into the GeneXpert. The interpretation of data from MTB/RIF tests was software based and not user dependent.

Each Xpert MTB/RIF cartridge includes reagents for the detection of MTB complex and RIF resistance as well as a sample processing control (SPC) to control for adequate processing of the target bacteria and to monitor the presence of inhibitor(s) in the PCR reaction. The Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability (Tenover, 2009).

3.11 Statistical Analysis

The data were checked for completeness and accuracy at collection sites and entered into excel and exported to SPSS version 20 (IBM Corp. NY) for analysis. A p-value <0.05 was considered as statistically significant. Kappa values were done to evaluate the agreement between testing of pooled and individual samples by using Smear Microscopy

& GeneXpert and strength of agreement was evaluated as previously described (Munoz and Bangdiwala, 1997).

3.12 Ethical consideration

Ethical clearances were obtained from Institutional Review Board of Addis Ababa and Hawassa Universities and official permissions from the study sites and Southern Nations Nationalities and People Regional Health Bureau. Written informed assent/consent was obtained from parent/guardian of study participants.

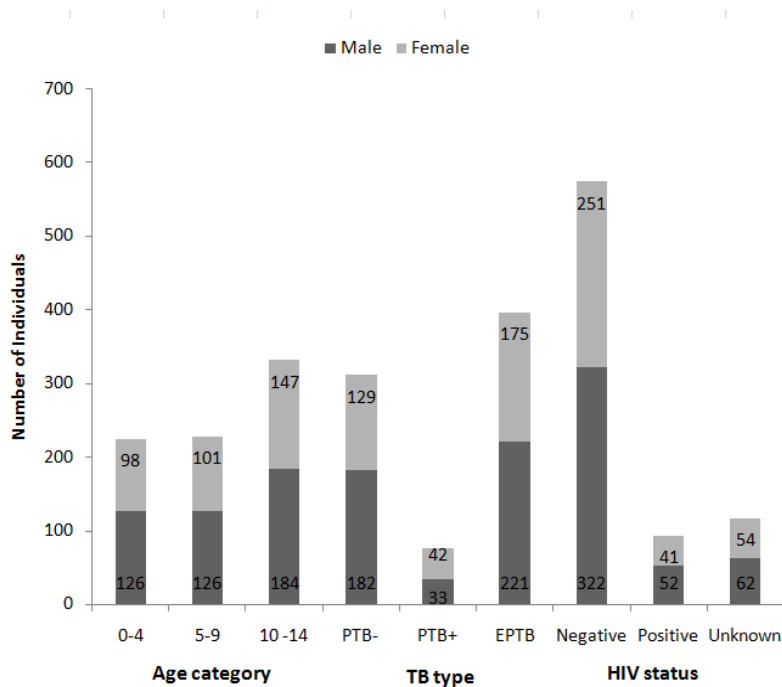
Chapter4 Results

Results in this thesis consist of a five year retrospective study with 782 study population and primary studies with 700 study population (2n=700).

4.1 Retrospective cross sectional study

4.1.1 Patient characteristics

Out of 6928 TB patients diagnosed and registered for DOTS treatment during the 5 year period 782 (11.3%) were children between 3 months – 14 years old, with a mean (SD) age of 7.8(± 4.3). Most of them (762, 97.4%) were new cases. The proportion of TB based on site of infection showed that EPTB and PTB cases were almost equal.



PTB- : Smear negative pulmonary TB, PTB+ : Smear positive Pulmonary TB and EPTB= Extrapulmonary TB.

Figure11 Age, Sex, TB type and HIV spectra among children who visited the two public Hospitals between Oct 2009 – Oct 2014.

Smear Positive PTB is more observable in 10-14 years old and HIV negative children as shown in table4 below.

Table4 Distribution of sex, age and HIV status with types of TB among children with TB (n=782) in two public Hospitals of Hawassa, Oct 2009 – Oct 2014.

Variables	PTB+ N (%)	PTB- N (%)	EPTB N (%)	p-value
Sex				
Male	33(7.6)	182(41.7)	221(50.7)	
Female	42(12.1)	129(37.3)	175(50.6)	0.076
Total	75(9.6)	311(39.8)	396(50.6)	
Age(in years)				
0-4	4(1.8)	114(50.9)	106(47.3)	
5-9	14(6.2)	94(41.4)	119(52.4)	< 0.001
10-14	57(17.2)	103(31.1)	171(51.7)	
Total	75(9.6)	311(39.8)	396(50.6)	
HIV status				
Negative	61(10.6)	208(36.3)	304(53.1)	
Positive	5(5.4)	53(57.0)	35(37.6)	0.004
Unknown	9(7.8)	50(43.1)	57(49.1)	
Total	75(9.6)	311(39.8)	396(50.6)	

PTB- : Smear negative pulmonary TB, PTB+ : Smear positive Pulmonary TB and EPTB= Extrapulmonary TB.

4.1.2 Treatment outcome

From the total of 782 children, successful treatment outcome was recorded for only 310(39.6%) the rest were transferred out 428(54.7%), defaulted 30(3.8%) and depart by death 14(1.8%). Of the 310, 268(86.5%) were labeled as completed treatment and 42(13.5%) were cured.

4.2 Prospective cross sectional study

Two sets (2n=700) of study population under 15 years old suspected for TB were enrolled for the prospective cross sectional study. One set of children (n=350) were used to study prevalence, strain typing and drug susceptibility testing. The remaining set of children (n=350 with 10 (2.9%) non respondent rate) were used to evaluate modified diagnostic approach of childhood tuberculosis.

4.2.1 Prevalence of active tuberculosis

The prevalence as shown in the table4.2 below showed a total of 34 (9.7%) positive cases out of 350 children tested for smear and culture. All smear positives were also culture positive. The sensitivity of smear microscopy compare to culture was 76.5%.

Table5 Prevalence of bacteriological confirmed TB in aspirate or sputum sample collected from children under 15 years (n=350).

Aspirate (n=183)		Sputum(n=167)		Total(n=350)	
Smear	Culture	Smear	Culture	Smear	Culture
15(8.2%)	16(8.7%)	11(6.6%)	18(10.8%)	26(7.4%)	34(9.7%)

Seven isolates grown in TB culture were identified as mycobacteria other than *M.tuberculosis* (MOTT bacilli) by using SD TB Ag MPT64 assay (figure12).



TB Ag MPT64 test positive - confirms presence of *M. tuberculosis* complex, two bands are observed on both control and test area (sample code 13 displayed on table as an example from the total of 34 isolates)

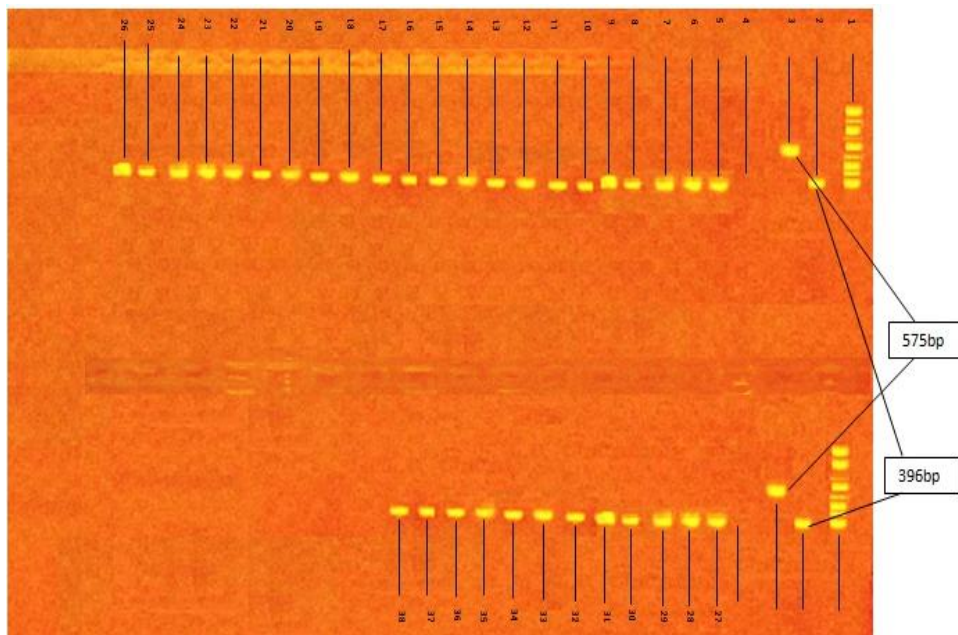
TB Ag MPT64 test negative - confirms presence of mycobacteria other than MTB (MOTT bacilli), only one band is observed on control area (a total of seven MOTT bacilli were isolated with sample codes: 0510, 511, 0550, 0570, 0636, 0727 and 1748)

Figure12. Identification of *M. tuberculosis* complex from Mycobacteria other than *M.tuberculosis* (MOTT) by SD TB Ag MPT64 test.

4.2.2 Strain typing and drug susceptibility testing

Region of difference (RD) 9-based polymerase chain reaction (PCR)

The isolates were analyzed using RD9 PCR and the result indicated that all of the isolates had intact RD9 implying that the isolates were *M. tuberculosis* (Figure13).



Lane 1= DNA ladder; Lane 2 = *M. tuberculosis* H37Rv as 396-bp positive control; Lane 3= *Mycobacterium bovis* as 575-bp positive control; Lane 4 = Negative control (molecular grade H₂O) lane 5-38 are culture isolates of *M. tuberculosis* collected from the study area.

Figure13 Gel electrophoresis banding pattern of PCR based RD9 deletion typing (n=34).

Spoligotyping

DNA extracts from 34 MTB isolates obtained from presumptive TB pediatrics were analyzed using Spoligotyping. Spoligotyping results were obtained for 34 isolates as shown in table6 and 7 and spoligotyping pattern of the isolates were displayed in figure14.



Figure14. Spacers patterns obtained by the spoligotyping of the MTB isolates (n=34). Black squares and white squares showed the presence and absence of spacers respectively.

Table6 Description of 11 shared-types (SITs) of the 29 isolates which have already been registered in the SITVIT2 or SpolDB4 database and the corresponding spoligotyping defined lineages/sub-lineages from a total of 34 *M. tuberculosis* strains isolated in Hawassa, Ethiopia.

SIT	Similar pattern	CBN Lineage	SITVIT2 Lineage/sublineage	Octal number	Binary format
53	10	EA	T	77777777760771	
149	6	EA	T3-ETH	777000377760771	
442	3	EA	T3	777737377760771	
37	2	EA	T3	77773777760771	
463	2	EA	H3	77777777720571	
25	1	EAI	CAS1-Delhi	703777740003171	
52	1	EA	T2	77777777760731	
118	1	EA	T	77776777760771	
428	1	EAI	CAS1-Delhi	703777740003371	
777	1	EA	H3-Ural-1	77777777420771	
2867	1	EA	T2	77777775760731	

Table7 Description of 4 orphan strains with different pattern (n=5) and corresponding spoligotyping defined lineages/sub-lineages recorded among *M. tuberculosis* strains from a total of 34 *M. tuberculosis* isolated in Hawassa, Ethiopia.

SIT	Similar pattern	CBN Lineage	SITVIT2 sublineage	Lineage/	Octal number	Binary format
Orphan	2	EA	T3		00673777760771	
Orphan	1	EA	T3- ETH		777000377761771	
Orphan	1	EA	T3		77773777760770	
Orphan	1	EA	Manu2		77777777742760	

Key: SIT= Spoligotype International Types; EA=Euro-American; EAI=East-African Indian; CBN=conformal Bayesian network;

KBBN =knowledge based Bayesian network.

Genotype MTBDRplus assay

Out of 34 *M. tuberculosis* strains tested for genotypic drug susceptibility testing (DST), one (2.9%) strain was found to be MDR which was resistant to both INH (deletion of KatG WT and mutation at KatG MUT1-which is high level) and RIF (deletion of rpoB WT8 and mutation at rpoB MUT3) (Figure15).



Figure15. Drug susceptibility test results of the isolates (n=34) by GenoType MTBDRplus.

Genotype MTBDRsl assay

The identified MDR case was further tested for pre extensively drug-resistant (pre-XDR) and extensively drug-resistant (XDR) by using Genotype MTBDRsl assay. It was found to be susceptible to second line drugs (figure16).



Figure16. Drug susceptibility test results for second line drugs of the isolate (n=1) by Genotype MTBDRsl assay.

4.2.3 Increasing the detection and confirmation of children with TB

GeneXpert MTB/RIF and Zn microscopy

For evaluation of pooling method out of 350 children, 10 were unable to give two samples with 1hr interval and excluded from the study. The study was done on the remaining 340 children through obtaining 1020 (3x340) first, second and pooled specimens of which 738 (72.4%) were expectorate sputum and 282 (27.6%) were gastric aspirates. GeneXpert MTB/RIF and Zn microscopy test result of first, second and pooled specimens were obtained as shown in table8– 12

Table8 Results of single and pooled sputum samples using Zn microscopy and GeneXpertMTB/RIF

Sputum specimen	Zn Microscopy result			Zn Microscopy additionality		GeneXpertMTB/RIF test result			GeneXpertMTB/RIF additionality	
	Negative	Positive	Total	N ^o	%	MTB-	MTB+	Total	N ^o	%
1 st	244	2	246	2	25	238	8	246	8	28.6
2 nd	243	3	246	3	37.5	237	9	246	9	32.1
Pooled	243	3	246	3	37.5	235	11	246	11	39.3

Table9 Results of single and pooled aspirate samples using smear microscopy and MTB/RIF

Gastric aspirate	Zn microscopy result			Zn Microscopy additionality		GeneXpertMTB/RIF test result			GeneXpertMTB/RIF additionality	
	Negative	Positive	Total	N ^o	%	MTB-	MTB+	Total	N ^o	%
1 st	94	0	94	0	0	91	3	94	3	30
2 nd	94	0	94	0	0	92	2	94	2	20
Pooled	94	0	94	0	0	89	5	94	5	50

Table10 Overall Results of single and pooled samples using Zn microscopy and GeneXpertMTB/RIF

All samples	Zn microscopy result			Zn microscopy additionality		GeneXpertMTB/RIF test result			GeneXpertMTB/RIF additionality	
	Negative	Positive	Total	N ^o	%	MTB-	MTB+	Total	N ^o	%
1 st	338	2	340	2	25	329	11	340	11	28.95
2 nd	337	3	340	3	37.5	329	11	340	11	28.95
Pooled	337	3	340	3	37.5	314	16	340	16	42.1

Table11 Pooled sample against individual sample using GeneXpertMTB/RIF

Expectorate Sputum Sample	Xpert pooled test result (Sputum)			Agreement	Kappa
	Positive	Negative	Total		
Xpert 1 st Sputum	7	1	8	98%	0.73
test result	4	234	238		
	11	235	246		

Xpert 2 nd sputum	Positive	8	1	9	98.4%	0.79
test result	Negative	3	234	237		
	Total	11	235	246		

Gastric aspirate Sample		Xpert pooled test result (Aspirate)			Agreement	Kappa
		Positive	Negative	Total		
Xpert 1 st	Positive	3	0	3	97.9%	0.74
aspirate test result	Negative	2	89	91		
	Total	5	89	94		
Xpert 2 nd aspirate	Positive	2	0	2	96.8%	0.56
test result	Negative	3	89	91		
	Total	5	89	94		

Table12 Pooled sample against individual sample using Zn Microscopy

Expectorate Sputum Sample		Pooled smear test result (Sputum)			Agreement	Kappa
		Positive	Negative	Total		
1 st smear test result	Positive	2	0	2	99.7%	0.80
	Negative	1	337	338		
	Total	3	337	340		
2 nd smear test result	Positive	3	0	3	100%	1.00
	Negative	0	337	337		
	Total	3	337	340		

Chapter5 Discussion

A retrospective study was done to assess treatment outcome of 782 children under 15 years diagnosed as TB based on clinically and/or laboratory investigations and registered for treatment in two public Hospitals of Hawassa, Oct 2009 – Oct 2014.

A prospective study with two set of study population under 15 years was also done to study prevalence, strain typing, drug susceptibility testing with one set of study population (n=350) and evaluation of modified diagnostic approach of childhood tuberculosis with remaining set of study population (n=350 with 10 (2.9%) non respondent rate) consecutively attending selected health facilities in Sidama zone, November 2014 - August 2016. Two set of study population were used since it was difficult to collect adequate sample from a single child for the prevalence and method evaluation study.

The highest proportion (11.3%) of children with TB register in Hawassa referral and Adare General Hospitals which is in agreement with a previous study done in Mizan-Aman General Hospital, Southwest Ethiopia (10.8%) (Wegderese *et al.*, 2014), Debre Berhan Hospital, Northern Ethiopia (11%) (Tefera *et al.*, 2016) and Dilla University Referral Hospital, Southern Ethiopia (13.5%) (Gebrezgabiher *et al.*, 2016). However, the proportion of EPTB in our study was higher compared to Addis Ababa, Ethiopia (6.6%) (Hailu *et al.*, 2014); Lagos, Nigeria (6.3%) (Adejumo *et al.*, 2016) and Western Ethiopia (7.2%) (Ejeta *et al.*, 2015). High proportion of childhood TB finding in this study suggests a high frequency of recent transmissions and a low control of the disease in this setting.

The proportion of smear positive tuberculosis (PTB+) account 9.6% out of all forms of tuberculosis which is comparable with a previous studies done in Mekelle, Northern Ethiopia (7.1%) (Daemo and Kelbore, 2016), Addis Ababa (8.6%) (Tilahun and Gebre-Selassie, 2016), Southeast Ethiopia (12.8) (Ramos *et al.*, 2010) and much lower than study done in Iran (41.2%) (Alavi *et al.*, 2015). The majority (80.6%) of children with PTB were smear negative PTB which is in agreement with previous reports in Southern Ethiopia (74.2%) (Mun oz-sellart *et al.*, 2009), Addis Ababa (83.1%) (Tilahun and Gebre-Selassie, 2016) and Mekelle, Northern Ethiopia (77.8%) (Daemo and Kelbore, 2016). This is mainly because most children present with non cavitory lesions, low bacillary load and they are mostly diagnosed based on clinical and radiologic evidence because of the difficulty of expectorating sputum for smear diagnosis. Although in this study more than half of the children had smear-negative PTB, there is evidence of increment of smear positive PTB with increased age of children from 1.8% in under 5 years old to 17.2% in the age group of 10 – 14 years old ($p < 0.001$) this could be due to diagnosis is difficult in early age and the increased immunity and formation of lung cavitory lesions with increased age.

The proportion of EPTB in our study (50.6%) was much higher compared to reports from Southern Ethiopia (24.8%) (Munoz-Sellart *et al.*, 2009), Lagos, Nigeria (9.2%) (Adejumo *et al.*, 2016) and Kilimanjaro (24.8%) (Mtabho *et al.*, 2010). The higher proportion of Children with EPTB in this study is comparable to those of other studies, such as Beijing, China (Wu *et al.*, 2012) ; Mekele, Ethiopia (Daemo and Kelbore, 2016), and Kinshasa, Democratic Republic of Congo (Aketi *et al.*, 2016) in which $>50\%$ of children had EPTB. This high proportion of EPTB

in children may be due to their immature immune systems: TB often progresses rapidly from infection to disease and affects extra pulmonary sites.

The overall HIV status was known for 85.2% of children with TB of which TB-HIV co-infection was found to be 14.0% which is comparable with study finding in Kinshasa, Democratic Republic of Congo (9.7%) (Aketi *et al.*, 2016) but lower than studies in Addis Ababa, Ethiopia (26.8%) (Hailu *et al.*, 2014) and Lagos, Nigeria (29%) (Adejumo *et al.*, 2016) and higher cases of smear positive TB were recorded in HIV negative than among HIV positives ($P < 0.004$), due to TB-HIV co infected patients may lack cavitation and granuloma formation and have negative smear findings.

In this study, the overall treatment success rate among all children with TB was 39.7 % which is comparable to study at Gondar University Hospital (29.5%) (Tessema *et al.*, 2009) however much lower than studies in Addis Ababa (85.5%) (Tilahun and Gebre-Selassie, 2016); Tigray (84%) (Berhe *et al.*, 2012); Kilimanjaro (73%) (Mtabho *et al.*, 2010); Iran (91.7%)(Alavi *et al.*, 2015) and Beijing (81.6%)(Wu *et al.*, 2012). The high rate of transferred cases (54.7%) registered in the study areas contributed to the low treatment success rate.

The prevalence of smear positive pediatric tuberculosis (7.4%) in this observation is congruent with results of studies performed in Sudan (8.1%)(Elhassan *et al.*, 2015), Vietnam (8%)(Giang do *et al.*, 2015), Yemen (8.5%) (Al-Aghbari *et al.*, 2009), India (13%) (Tiwari *et al.*, 2015). Higher prevalence of smear positive pediatric tuberculosis were reported in study in India (42.4%) (Haritha and Shailaja, 2017), Benin (12.5%) (Kalu *et al.*, 2014) and Ethiopia (17.8%)

(Workalemahu *et al.*, 2013). Lower prevalence of smear positive pediatric tuberculosis were reported in Vietnam (1.4%) (Tran *et al.*, 2013) and India (2%) (Raizada *et al.*, 2015).

The prevalence of culture confirmed pediatric tuberculosis (9.7%) was comparable with study in Peru (10%) (Oberhelman *et al.*, 2010) and Uganda (9%) (Wampande *et al.*, 2015), Houston, Texas (11%) (Cruz *et al.*, 2013b) and the prevalence in the present study was lower than studies reported in Sudan (16.2%) (Elhassan *et al.*, 2015), Ethiopia (24.6%) (Workalemahu *et al.*, 2013), Benin (36.9%) (Kalu *et al.*, 2014) and Peru (32.9%) (Montenegro *et al.*, 2003). Compared to our study lower prevalence of culture confirmed pediatric tuberculosis were reported in Mozambique (1.4%) (Elisa *et al.*, 2017), Bangladesh (3.5%) (Chisti *et al.*, 2015), Vietnam (6.2%) (Tran *et al.*, 2013), India (3%) (Jain *et al.*, 2013).

Variation in magnitude of pediatric tuberculosis could be a reflection of variation in prevalence of adult tuberculosis.

TB in children is often missed or overlooked due to non-specific symptoms and difficulties in diagnosis. This has made it difficult to assess the actual magnitude of the childhood TB epidemic, which may be higher than currently estimated. The present study supported that the routinely used diagnostic method (smear microscopy) had lower sensitivity, 76.5% (95% CI, 62% - 89.2%) compared to culture method to detect pediatric TB cases. The routinely used smear microscopy diagnostic technique under estimate burden of pediatric tuberculosis that childhood tuberculosis could be given little attention by National TB control programme.

In the present study 350 samples were taken from presumptive TB pediatric cases that attended the two public hospitals in Hawassa during the study period and 34 of the samples were found to

be positive for *M. tuberculosis*. Using different diagnostic methods: by first inoculating either sputum or gastric aspirates on LJ media and the isolates were further confirmed to be *M. tuberculosis* complex by using immune-chromagrapic testing (SB TB Ag MPT64 assay) and finally RD9 based PCR testing was done to confirm the isolates were *M. tuberculosis*.

M. tuberculosis isolates were further spoligotyped for strain typing. An online tool Run TB-Lineage http://tbinsight.cs.rpi.edu/run_tb_lineage.html was used for grouping lineages using a conformal Bayesian network (CBN) analysis and sub lineage using knowledge based Bayesian network (KBBN).

The isolates were analyzed using RD9 PCR and the result indicated that all of the isolates had intact RD9 implying that the isolates were *M. tuberculosis*. This finding was consistent with different studies done on molecular characterization (Getahun *et al.*, 2015; Tewodros *et al.*, 2014; Workalemahu *et al.*, 2013).

A total of 15 different types of spoligotype patterns were identified. Out of 15 patterns, 11 patterns (85.3% of the isolated strains) matched with the patterns registered in the SITVIT2 or SpolDB4 database while the remaining 4 patterns did not match with the patterns registered in the SITVIT2 or SpolDB4 database. Similar studies that have been conducted in Ethiopia, and thus all the geographic regions of the country have not been covered and as a result all the circulating strains of *M. tuberculosis* have not yet been registered to the SITVIT2/ SpolDB4 database.

Genotypic strain clustering was used as a proxy for recent transmission and can help to evaluate TB control programs (Mathema *et al.*, 2015). Clustering rate in our study was 73.5% which was

consistent with study in Egypt (74.8%) (Helal *et al.*, 2009) and Houston, Texas (75%) (Marquez *et al.*, 2012).

The clustering rate in our study was lower than study in Zimbabwe (84.1%) (Easterbrook *et al.*, 2004), Honduras (84%) (Rosales *et al.*, 2010), Ethiopia (83.9%) (Fantahun *et al.*, 2013).

Lower clustering rate was observed in various studies compared to ours, like studies in Jimma, Ethiopia (14.3%) (Workalemahu *et al.*, 2013), South Africa (33%) (Dijk *et al.*, 2016), Northwest Ethiopia (45.1%) (Tessema *et al.*, 2013), Southwest Ethiopia, Gambella (57.5%) (Asebe *et al.*, 2015). Although spoligotyping has less discriminatory power in classifying strains, the finding of many isolates clustering in the same pattern could suggest the presence of an on-going transmission of *M. tuberculosis* infection in the study area and the isolates that do not belong to any cluster are often assumed to hesitantly indicate the reactivation of latent infection.

The *M. tuberculosis* isolates in the present study were belonged to Euro-American and East-African-Indian. The dominant lineage was Euro-American Lineage consisting 94.1% of the isolates. The dominancy of this lineage was recorded in other studies in Ireland (Fitzgibbon *et al.*, 2013) and Ethiopia (Asebe *et al.*, 2015; Getahun *et al.*, 2015; Workalemahu *et al.*, 2013).

The dominantly identified strains in the present studies were SIT 53, SIT 149, and SIT 442 consisting of 10 isolates, 6 isolates, 3 isolates, respectively. Similar to this study, SIT 53 and SIT 149 were dominantly isolated by previous studies (Belay *et al.*, 2014; Getahun *et al.*, 2015; Workalemahu *et al.*, 2013).

Out of 34 *M. tuberculosis* strains tested for genotypic DST, one (2.9%) strain was found to be MDR. The MDR case in our finding is under Euro-American lineage which is similar to finding

in central Ethiopia that the identified MDR cases were under Euro-American lineage (Bedewi *et al.*, 2017).

The agreements of Xpert pooled test result with Xpert 1st (Kappa=0.73) and with Xpert 2nd (Kappa=0.79) of sputum sample and with Xpert 1st (Kappa= 0.74) of aspirate sample were substantial whereas the agreement of Xpert pooled test result with Xpert 1st (Kappa=0.56) of aspirate sample was moderate and almost perfect agreement was observed among pooled smear result with 1st(Kappa=0.80) and 2nd (Kappa=1.00) smear results (Munoz and Bangdiwala, 1997).

The current pooling approach that we implemented in selected facilities in Sidama Zone is advantageous as it is powerful to identify the positive cases. Compared to the pooling method proposed by Abdurrahman and colleagues (Abdurrahman *et al.*, 2015) that involves pooling of four sputum samples and subsequent diagnosis to fish out the positive case by case, the current pooling method can single out a case through repeated sampling of a single child's sputum or gastric aspirate. Moreover, the repeated sampling is done in one hour interval minimizing waste of time and energy of parents/guardians to repeatedly bringing the children to the center. Overall, the current approach of pooling within an hour is more convenient in our setting for effective diagnosis of the children.

Smear positive TB case was not identified among the first, second and pooled gastric aspirates compared to 1-13% smear positive TB identified in other studies (Al-Aghbari *et al.*, 2009; Cruz *et al.*, 2013b; Gomez *et al.*, 2000; Parashar *et al.*, 2013). No detection of TB bacilli in gastric aspirate smears in our study could be due to small proportion of gastric aspirate sample to the total of sputum and gastric aspirate samples collected and also gastric aspirate usually contains

low bacilli load and AFB smear of gastric aspirate is a relatively insensitive (Elhassan *et al.*, 2015; Kalu *et al.*, 2014).

Although sputum smear microscopy can give better positive yield compared to gastric aspirate smear microscopy (Berggren *et al.*, 2004), in our study the proportion of sputum smear positive in the first and second samples were low in agreement with findings in other similar study done in Yemen for the first (4%) and second (5%) sputum samples respectively (Al-Aghbari *et al.*, 2009).

Same day smear microscopy for diagnosing TB in the case of suspected tuberculosis seems not a promising step towards improving the quality of smear examination (Myneedu *et al.*, 2011), In our study Xpert MTB/RIF detects more positive than smear microscopy similar to other studies (Ciftci *et al.*, 2011; Geleta *et al.*, 2015). Because of the highly sensitive nature of Xpert MTB/RIF ranging from 95.6% to 99.1% (Bojang *et al.*, 2015; Ciftci *et al.*, 2011; Singh *et al.*, 2015) ,it overcame the dilution effect of pooling of the two serially collected samples that a 13.2% incremental yield obtained in our study on pooled samples which were made by pooling two samples collected by front loading approach (i.e. one hour interval) compared to testing each of the first or the second sample. A major impediment to treatment of TB is a diagnostic process that requires multiple visits (de Cuevas *et al.*, 2016). This data indicates that the strategy of collecting specimen in one hour interval and pooling and then testing once reduced diagnostic visits and could improve compliance in submitting samples and collecting result. It can also decrease the laboratory workload and hospitalization and hence improve the quality of work in settings with high TB burden like Ethiopia.

Confirmatory diagnosis of childhood TB is a medical challenge and the diagnosis of childhood TB is complex and most of the new diagnostics for TB are for adults (Cuevas *et al.*, 2012a). However, in this study we found that pooling of two consecutive samples collected at one hour interval and testing using Xpert MTB/RIF gave much better yield with short turnaround time compared to the routine diagnostic approach.

Chapter6 Conclusion and recommendations

6.1 Conclusion

In this study, the lower prevalence of bacteriologically confirmed pediatric pulmonary tuberculosis could be due to the less sensitive nature of the smear microscopy and culture diagnostic tools rather than the real reduction in prevalence of pediatric tuberculosis in the study area. National TB Control Programme relying on sputum smear microscopy for diagnosis are at risk of under-diagnosing and underestimating the burden of TB in children.

The high clustering rate obtained from analysis of the genetic population structure of *M. tuberculosis* strains isolated from children suggested similarity with that of adults, indicating an on-going and active transmission of *M. tuberculosis* from adults to children in Ethiopia. The same-day approach and the use of a single cartridge for testing multiple samples of a single child using Xpert MTB/RIF reduce the number of visits required for diagnosis, save resources for the health system and the patient, and ultimately through pooling of samples and using GeneXpert MTB/RIF increase the sensitivity so as to improve case detection, confirmation and treatment.

6.2 Recommendations

1. There should be linking mechanism among liaison offices of health facilities to put transfer out cases on treatment so as to reduce TB transmission
2. GeneXpert MTB/RIF should be used in large scale for testing pooled sample to increase the detection and confirmation of children with TB and to clearly show real burden of childhood TB
3. Routine drug-susceptibility testing is necessary to optimize the management of childhood tuberculosis.
4. Further studies recommended using molecular methods with a better discriminatory power such as single nucleotide polymorphism (SNP) typing and MIRU-VNTRs, which allow splitting certain spoligotyping clusters in smaller sub clusters to differentiate new strains from mixed or polyclonal infections.
5. Active childhood TB case finding should be expanded to improve the low case detection and poor outcome of childhood TB
6. Gastric aspirate should be used as routine sample as sputum sample to childhood diagnostic methods to increase cases of tuberculosis.

Limitations of the study

1. The study did not compare with culture method during method evaluation of pooled sample by using GeneXpert MTB/RIF. Therefore, it is difficult to calculate sensitivity, specificity, positive predictive value and negative predictive value.

2. The study was conducted only on two public hospitals, except for method evaluation study, so that it is difficult to generalize the findings to the general population.

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Appendix:

Summary of the study

