Comparison of Xpert MTB/RIFAssay with Smear Microscopy and Culture for the detection of Pulmonary Tuberculosis

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted by

Mohammad Tanvir Ahmed

Student ID: 15176002

Department of Mathematics and Natural Sciences

BRAC University

Dhaka-1212, Bangladesh

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Dedicated to

My Parents

Comparison of Xpert MTB/RIF assay with smear microscopy and culture for the detection of pulmonary tuberculosis

Declaration

I here by declare that the thesis entitled '**Comparison of Xpert MTB/RIF assay with smear microscopy and culture for the detection of pulmonary tuberculosis**' is my own work done under joint supervision of **Dr. Sayera Banu**, Senior Scientist and Acting Head, Programme on Emerging Infections, Infectious Diseases Division (IDD), International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) and **Prof. Dr. Naiyyum Choudhury**, former Coordinator of Biotechnology and Microbiology Programms, Department of Mathematics and Natural Science, BRAC University It has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Student name: Mohammad Tanvir Ahmed

ID: 15176002

Signature ……

Supervisor
Prof. Dr. Naiyyum Choudhury
Professor
Department of Mathematics and Natural Science
BRAC University

Signature ……

Supervisor
Dr. Sayera Banu
Senior Scientist and Acting Head
Programme on Emerging Infections
Infectious Diseases Division (IDD)
(icddr,b)

Signature……

CERTIFICATE OF APPROVAL

We here by declare that the thesis entitled '**Comparison of Xpert MTB/RIF assay with smear microscopy and culture for the detection of pulmonary tuberculosis'** is from the student's own work and effort, and all other sources of information used have been acknowledged. This **Thesis** has been submitted with our approval.

SUPERVISOR: Dr. Sayera Banu
Senior Scientist and Acting Head
Programme on Emerging Infections
Infectious Diseases Divison (IDD)
(icddr,b)

Signature:……..

SUPERVISOR: Prof. Dr. Naiyyum Choudhury
Professor
Department of Mathematics and Natural Science
BRAC University

Signature:……

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The Author

Mohammad Tanvir Ahmed

CONTENTS

Abstract

Tuberculosis (TB) remains one of the world's most serious diseases, leading to more than one million deaths each year. The emergence of drug-resistant strains of *Mycobacterium tuberculosis* poses a threat to tuberculosis control programs and often results in high-level of mortality. Current methods of detection and determining anti-mycobacterial susceptibility are time consuming. Xpert MTB/RIF assay, used in this study, is an automated real-time polymerase chain reaction (PCR) system with rapid on-demand, near-patient technology, which can simultaneously detect *M. tuberculosis* and rifampicin resistance status within two hours. In this study we used 145 sputum specimens from suspected TB patients.

All specimens were tested by AFB microscopy, Xpert MTB/RIF assay and conventional culture. Culture positive specimens were further tested for drug susceptibility testing (DST) against four first line anti-TB drugs. Among 145 sputum specimens 20 (13.80%) were positive in AFB microscopy. Among 20 AFB positive cases 8 (40%) were scanty, 3 (15%) were 3+, 4 (10%) were 2+ and 5 (25%) were 1+ respectively. Among 28 GeneXpert positive cases, 5 (17.85%) were very low, 10 (35.71%) were low, 8 (28.57%) were medium, 5 (17.85%) were high detection level respectively. Two isolates were found to be rifampicin resistant in Xpert MTB/RIF assay test. Among the 23 culture positive cases, DST results were available for 20 cases of which 19 were sensitive to all drugs while the remaining 1 was resistant to Isoniazid, Rifampicin and Streptomycin. Between Xpert MTB/RIF assay and smear microscopy sensitivity and speicficity were 100%, Xpert MTB/RIF assay were compared with the conventional culture on LJ media, sensitivity and specificity were 82.61% and 92.62% respectively and smear microscopy were compared with the culture, sensitivity and specificity were 65.22% and 95.90% respectively. From this data we can conclude that Xpert MTB/RIF assay is more useful then other conventional methods.

List of Tables

Abbreviations

Symbols	
%	Percentage
&	and
°C	Degree Centigrade
µg	Microgram
µl	Microliter
gm	Gram
mL	Milliliter
mg	Milligram
General Terms	
AFB	Acid-Fast Bacilli
bp	Base Pair
CDC	Centers for Disease Control
CCD	Centre for Communicable Diseases
cfu	Colony Forming Unit
Ct value	Threshold Crossing Value
DNA	Deoxyribonucleic Acid

dNTPs	Deoxynucleoside tri-phosphates
e. g.	Examplia gratia
et al	And others
HIV	Human Immunodeficiency Virus
kb	Kilo base
kbp	Kilo base pair
MDR	Multi Drug Resistant
MTB	*Mycobacterium tuberculosis*
MTC	*Mycobacterium tuberculosis* complex
PCR	Polymerase Chain Reaction
RIF	Rifampicin
rpm	Rotation Per Minute

sec	Second
TB	Tuberculosis
WHO	World Health Organization
XDR	Extensive Drug Resistance
SPC	Sample Processing Control
PCC	Probe Check Control

Chapter One

Introduction and Literature Review

1. Introduction and Literature Review

1.1 Tuberculosis: A Global burden

Tuberculosis (TB) causes ill-health among millions of people each year and remains one of the major global public health problems. TB ranks as the second leading cause of death from an infectious disease worldwide, after Human Immunodeficiency Virus (HIV). According to WHO (2015) report almost 10.4 million new cases and 1.8 million TB deaths (990,000 among HIV negative people and 430,000 HIV-associated TB deaths). The Millennium Development Goal (MDG) target to halt and reverse the TB epidemic by 2015 has already been achieved. New cases of TB have been falling for several years and fell at a rate of 2.2% between 2010 and 2011.[1]

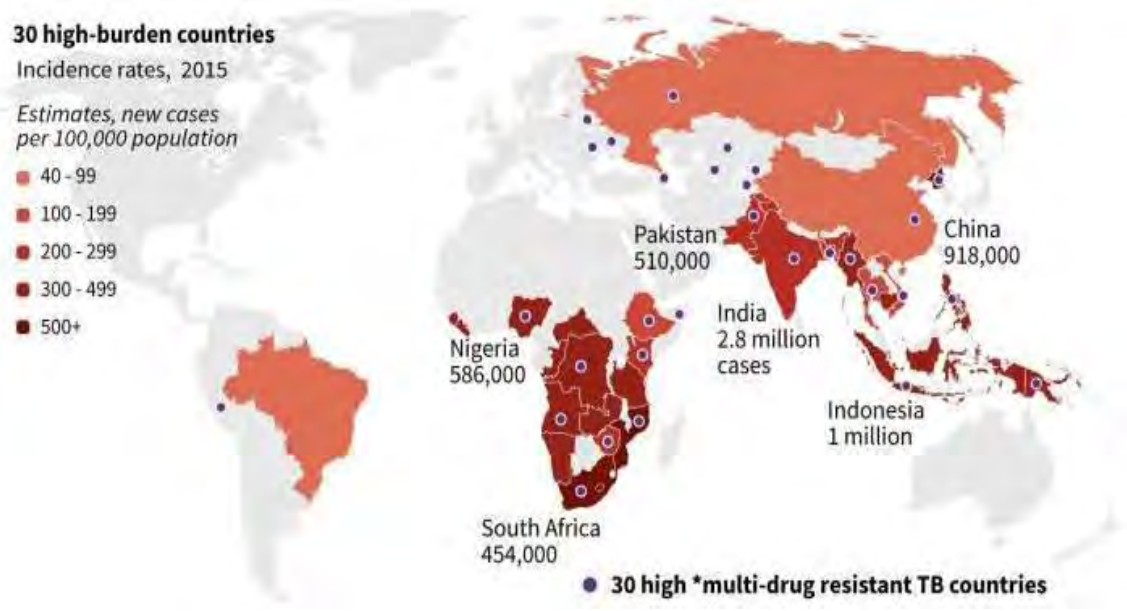


Figure 1.1: Global Map of WHO TB incidence

The TB mortality rate has decreased 41% since 1990 and the world is on track to achieve the global target of a 50% reduction by 2015. Mortality and incidence rates are also falling in all of WHO's six regions and in most of the 22 high-burden countries that account for over 80% of the world's TB cases. In the under-developed countries 95% of infections occur due to pitiable diagnostic and treatment facilities. It is estimated that approximately 70 million people will die from TB within next 20 years and it is because of inadequate measures for the TB control.

1.2 TB situation in Bangladesh

In Bangladesh, the estimated incidence and prevalence rats for all forms of tuberculosis in 2014 were 227 and 404 per 100,000 poupaltion respectively. An estimated 51 per 100,000 population died in the same year. The proportion rate of multidrug- resistant tuberculosis among new TB cases was 1.4% and among re- treatment cases was 29%. According to the global TB report published in the 2015, the absolute numbers and burden severity of TB in Bangladesh is 3.7% per year. Death rate from TB ranks Bangladesh seventh position in the world.

Bangladesh adopted the Directly Observed Treatment Short-course (DOTS) strategy in 1993. Since then, the NTP has expanded to cover nearly all of the country. For many years, Non-Government Organizations (NGOs) have been largely responsible for delivering DOTS services and have had a formal involvement in the NTP since 1994. Their collaboration has been instrumental in promoting DOTS and achieving high DOTS coverage. Participation of NGOs in program delivery continues to be an enormous asset, while the government ensures coordination and sustainability of TB control.

1.3 *Mycobacterium tuberculosis* (MTB): Causative agent of TB

MTB is a slow-growing obligate aerobe. For this reason, in the classic case of tuberculosis, the *Mycobacterium tuberculosis* complexes (MTBC) are always found in the well-aerated upper lobes of the lungs. MTB is fairly large non motile rod-shaped bacterium; rods are 2-5µm in length and 0.2-0.5 µm in width. It has slow generation time of 16 to 20 hours, a physiological characteristic that may contribute to its virulence. Humans are the only reservoir for the bacterium. It can be with stand weak disinfectants and can survive in a dry state for weeks but, spontaneously and can only grow within a host organism. In vitro culture of *M. tuberculosis* took a long time to be achieved, usually 8 weeks.

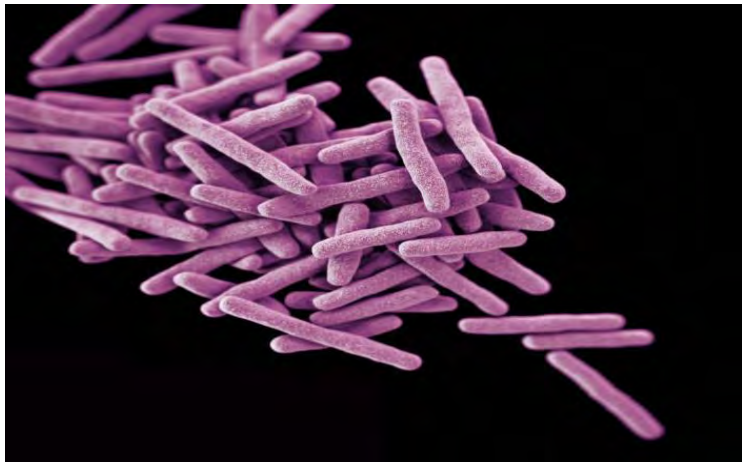


Figure 1.2: Scanning electron micrograph of MTB.

The currently accepted minimal criteria for considering bacteria in the genus Mycobacterium are:

- The acid-alcohol fastness;
- The presence of mycolic acids containing 60-90 carbons, which by pyrolysis are cleaved to C22 to C26 fatty acid methyl esters;
- A guanine plus cytosine (G+C) content of DNA of 61-71 mol% (Levy-Frebault and Portales, 1992), the only exception being *Mycobacterium leprae* with a G+C content of 54 to 57 mol%.

The Greek prefix *myco-* means "fungus," alluding to the way *M. tuberculosis* has been observed to produce the characteristic fungus-like pellicle when grown on liquid media.[2]
Mycobacterium is a genus of Actinobacteria and given its own Mycobacteriaceae family. Actinobacteria includes pathogens known to cause serious diseases in mammals, including tuberculosis (MTB) and the classic Hansen's strain of leprosy (*Mycobacterium leprae*) [3]. The most pathogenic Mycobacteria for man and animal have been grouped under MTBC which till now comprises of following eight members [4, 5].

i. *M. tuberculosis*
ii. *M. africanum*

iii. *M. bovis*

iv. *M. bovis BCG*

v. *M. bovis subsp. Caprae*

vi. *M. canettii*

vii. *M. microti* and

viii. A recently described species *M. pinnipedii [6]* with characteristic animal and/or human epidemiologies [7].
M. tuberculosis, along with *M. bovis*, *M. africanum*, and *M. microti* all cause the disease known as tuberculosis (TB) but *M. tuberculosis* is pathogenic for humans while *M. bovis* is usually pathogenic for animals.

1.4 Evolution of *M. tuberculosis*

Mycobacteria are abundant in soil and water. So, the MTBC probably arose as the result of an ecological niche change that peaked in pathogenicity for mammals and the apparent disappearance of the last free-living ancestor. The nucleotide sequence and 16S rRNA sequence of MTBC is 99.9% similar [8, 9] but differ widely in terms of their host tropisms, phenotypes, and pathogenicity. Assuming that they all are derived from a common ancestor, it is intriguing that some are exclusively human (MTB, *M. africanum*, *M. canettii*) or rodent pathogens (*M. microti*), whereas others have a wide host spectrum (*M. bovis*). The main human-infecting species have been classified into seven spoligotypes: type 1 contains the East African Indian (EAI) and some Manu (Indian) strains; type 2 is the Beijing group; type 3 consists of the Central Asian (CAS) strains; type 4 of the Ghanaand Haarlem (H/T), Latin America Mediterranean (LAM) and X strains; types 5 and 6 correspond to *M. africanum* and are observed predominantly and at very high frequency in West Africa. A seventh type has been isolated from the Horn of Africa [10].

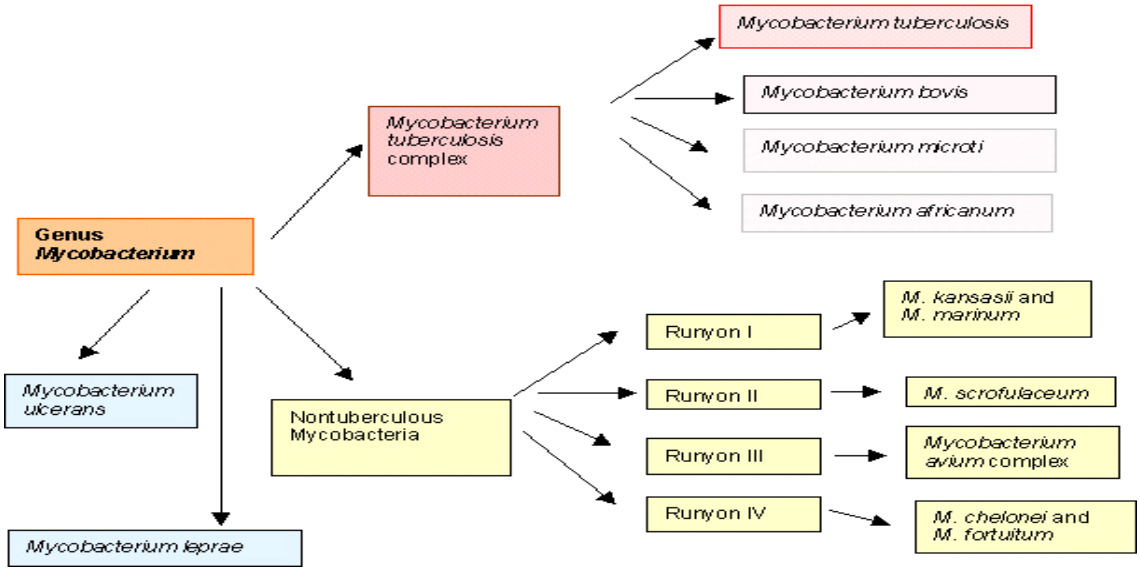


Figure 1.3: Evolution of MTB

It is generally believed that tuberculosis was acquired from cattle following the domestication of livestock at the beginning of the Neolithic period when the hunter–gatherer lifestyle was replaced by agriculture. *M. bovis* was the ancestor of MTB [11]. The most common ancestor of the MTBC evolved ~40,000 years ago [12]. All types of the *M. tuberculosis* began their current expansion about 5000 years ago—a period that coincides with the appearance of *M. bovis*. MTB emerged as a human pathogen as recently as 10000–15000 years ago [8-14]

1.4.1 Scientific classification of *Mycobacterium*

Kingdom: Bacteria

Phylum: Actinobacteria

Order: Actinomycetales

Suborder: Corynebacterineae

Family: Mycobacteriaceae

Genus: *Mycobacterium*

1.5 Properties of *Mycobacterium*

Mycobacterium is classified as acid-fast bacilli because when they are stained by Gram staining, they cannot be decolorized by acid alcohol. Mycobacterium contain high content of mycolic acids, long chain cross-linked fatty acids and other cell-wall lipids in the cell wall that confer it Acid fastness [15]. Mycobacterium is non-motile bacteria, exception is *M. marinum* which has shown to be motile within macrophage [12]. They have an outer membrane [16]. Most of them do not form endospore and they do not have capsules. *M. marinum* and perhaps *M. bovis* have been shown to sporulate [17]. Cell wall of mycobacterium is thicker than in many other bacteria and this is the distinguishing characteristic of all *Mycobacterium* species. Cell wall is hydrophobic, waxy and rich in mycolic acids/ mycolates; glycolipids like the inflammatory molecule lipoarabinomannan and its variants; polypeptides like phenolphthiocerol, which complexes with mycocerosic acid to form the virulence factor phenolphthiocerol-dimycocerosate (PDIM); and polysaccharides such as arabinogalactan and arabinomannan [18]**.** Unlike the other complex members*, M. microti* and *M. bovis* require pyruvate as a growth supplement. There are also differences in the natural resistance to certain antibiotics such as pyrazinamide (PZA), due to a missense mutation in the activating enzyme pyrazinamidase [19] and thiophen-2-carboxylic hydrazide (TCH), as well as in the production of niacin. All virulent members of the complex are capable of withstanding phagocytosis and replicating within macrophages and monocytes.

1.5.1 Cell Wall Structure

The cell wall structure of MTB deserves special attention because it is unique among prokaryotes and it is a major determinant of virulence for the bacterium. The membrane is surrounded, as in almost all bacteria, by a cell wall that protects the cell contents, provides mechanical support and is responsible for the characteristic shape of the bacterium. The wall is constituted by an inner peptidoglycan layer, which seems to be responsible for the shape-forming property and the structural integrity of the bacterium. The structure of this stratum differs slightly from that of common bacteria, as it presents some particular chemical residues and an unusual high number of cross-links. Indeed, the degree of peptidoglycan cross linking in the cell wall of MTB is 70-80 % whereas that in *E. coli* is 20-30 %.

The cell wall complex contains the followings:

- Peptidoglycan (PG)
- Arabinogalactan (AG)
- Mycolic acids and
- Lipoglycans such as phosphoinositolmannosides (PIM), lipomannan (LM) and lipoarabinomannan (LAM). Other cell wall associated lipids include trehalosedimycolate (TDM), trehalosemonomycolate (TMM), sulpholipids (SL), phthioceroldimycocerosate (PDIM) and di-acyl trehalose (DAT).

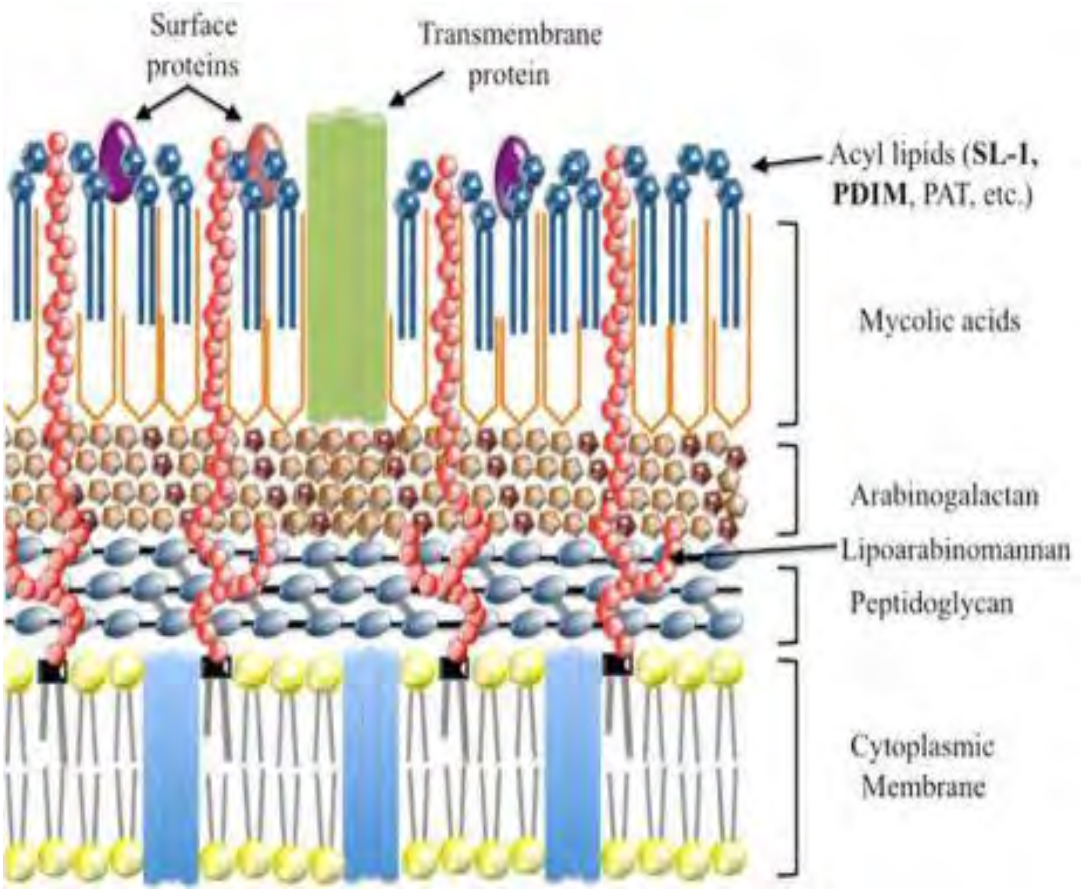


Figure 1.4: Cell wall structure of *M. tuberculosis.* Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of MTB cell wall consists of three major components.

1.5.2 Mycolic Acids

- Unique alpha-branched lipids found in cell walls of *Mycobacterium* and *Corynebacterium.*
- Make up 50% of the dry weight of the mycobacterial cell envelope.
- Form a strong hydrophobic lipid shell around the organism and affect permeability properties at the cell surface.
- A significant virulence factor of MTB which protect mycobacteria from cationic proteins, lysozyme and oxygen radicals in the phagocytic granule.
- Protect extracellular mycobacteria from complement deposition in serum.

1.5.3 Cord Factor

- Most abundantly produced in virulent strains of MTB
- Toxic to mammalian cells

- ➢ An inhibitor of PMN migration.

1.5.4 Wax-D

Wax-D in the cell envelope is the major component of Freund's complete adjuvant (CFA). Finally, the high concentration of lipids in the cell wall of MTB has been associated with some properties of the bacterium such as:

- ➢ Impermeability to stains and dyes;
- ➢ Resistance to many antibiotics ;
- ➢ Resistance to killing by acidic and alkaline compounds;
- ➢ Resistance to osmotic lysis via complement deposition;
- ➢ Resistance to lethal oxidations and survival inside of macrophage

1.6 Genomics of MTB

The genome of MTB was sequenced in 1998 [20, 21]. An integrated approach was adopted for the genome project undertaken with the widely used reference strain MTB H37Rv [22]. Unlike some clinical isolates that often lose virulence after laboratory passage; this strain has retained full virulence in animals since its isolation in 1905. In the early phase of the project, a physical map of the 4.40 Mb chromosome was constructed using PFGE of macro-restriction fragments and this was connected to the gene map by means of hybridization with landmark clones from an ordered cosmid library bearing known sites or genetic markers [23]. Subsequently, an ordered library of Bacterial Artificial Chromosome (BAC) clones was constructed containing large inserts of MTB H37Rv DNA and this enabled near-complete coverage of the MTB H37Rvgenome to be achieved [24].

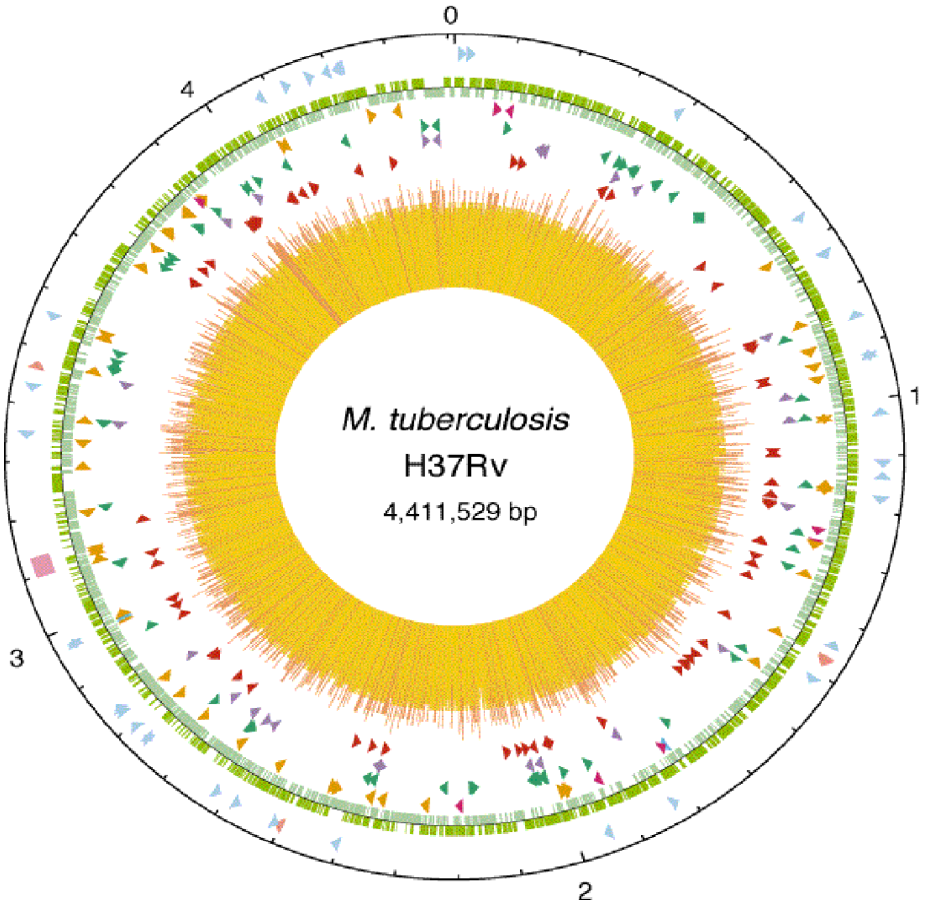


Figure 1.5: H37Rv genome of MTB. The outer circle shows the scale in *M. tuberculosis*, with 0 representing the origin of replication. The first ring from the exterior denotes the positions of stable RNA genes (tRNAs are blue, others are pink) and the direct repeat region (pink cube); the second ring inwards shows the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring depicts repetitive DNA (insertion sequences, orange; 13E12 REP family, dark pink; prophage, blue); the fourth ring shows the positions of the PPE family members (green); the fifth ring shows the PE family members (purple, excluding PGRS); and the sixth ring shows the positions of the PGRS sequences (dark red). The histogram (centre) represents G + C content, with <65% G + C in yellow, and >65% G + C in red (obtained from Software, DNASTAR).

A canonical set of 68 BAC clones carries 98.5% of the genome. Ordered clone libraries, particularly those based on episomal or integrating shuttle vectors [25, 26] are invaluable tools for functional genomics of tubercle bacilli. The minimally overlapping set of BAC clones containing large inserts of MTB H37Rv DNA [24] was of critical importance for the timely completion of the MTB H37Rv genome sequence**.** Over 51% of the genes have arisen as a result of gene duplication or domain shuffling events and 3·4% of the genome is composed of insertion sequences (IS) and prophages (phiRv1, phiRv2). There are 56copies of IS elements belonging to the well-known IS*3*,

IS*5*, IS*21*, IS*30*, IS*110*, IS*256* and IS*L3* families, as well as a new IS family, IS*1535*, that appears to employ a frame shifting mechanism to produce its transposase. IS*6110*, a member of the IS*3* family, is the most abundant element and has played an important role in genome plasticity.

One of the major findings of the MTB genome project was the identification of large gene families, which were either unknown previously or poorly understood. Foremost among these were the novel PE and PPE families, comprising 100 and 67 members, respectively [26, 27] which occupy about 8% of the genome. Members of each family share a conserved N-terminal domain of 110 and 180 amino acid residues, with the characteristic motifs Pro-Glu (PE in single letter code) or Pro-Pro-Glu (PPE) at positions 8–9, or8–10, respectively. The PE and PPE proteins can be divided into subfamilies on the basis of their C-terminal domains; in some cases, these are simple and repetitive in sequence while in others they are of higher complexity. Belonging to the former group are the PE proteins of the PGRS (polymorphic GC-rich sequence) class [27] and the PPE proteins of the MPTR (major polymorphic tandem repeat) class. The PGRS encodes the motif Asn-Gly-Gly-Ala-Gly-Gly-Ala, or variants thereof, while MPTR encodes Asn-X-Gly-X-Gly-Asn-X-Gly. Multiple tandem repetitions of these motifs are found in the corresponding proteins, which are acidic and exceptionally rich in glycine, and at the gene level variations occur in the repeat copy number and sequence thereby accounting for the genomic polymorphisms observed in hybridization patterns obtained with PGRS or MPTR probes [12, 28]. Initially, the PGRS and MPTR sequences were thought to correspond to dispersed tandem repeats or microsatellites but the finding that they were part of coding sequences led to reflection about the functions of these proteins.

1.6.1 Biology of MTB

The nature of the host-pathogen interaction between humans and MTB is considered to have a genetic component. The information gleaned from the genome sequence provided new and valuable insight into the biology of the tubercle bacillus and highlighted the importance of lipid metabolism to its lifestyle as at least 8% of the genome is dedicated to this activity [27]**.** While the cell envelope of MTB was known to contain a remarkable array of lipids, glycolipids, lipoglycans and polypeptides [29] and the genome sequence revealed many of the genes required for their production; it was a surprise to find numerous genes and proteins that could confer lipolytic functions. Estimates of the concentrations of potential substrates available to a pathogen in host tissues suggest that lipids and sterols are more abundant than carbohydrates [30]. While MTB has the prototype ß-oxidation cycle required for lipid catabolism, catalyzed by the multifunctional FadA/FadB proteins, it also appears to have 100 enzymes potentially involved in alternative lipid oxidation pathways in which exogenous lipids from host cells could be degraded. Such large numbers of lipid-degrading functions have not yet been reported in other bacteria. Although the

presence of a complete network of anabolic systems is in agreement with the notion that the tubercle bacillus has only recently emerged as a human pathogen, and thus had insufficient time to adapt to a new host by shedding biosynthetic genes, it may also indicate that the availability of metabolic precursors is limiting within the phagosome. Support for the latter explanation is provided by the finding that genes for anabolic functions have been heavily conserved in the genome of *M. leprae*, a related, obligate intracellular pathogen, in the face of massive reductive evolution that may have eliminated as many as 2600 genes [31, 32]. MTB genome encodes about 190 transcriptional regulators, including 13 sigma factors, 11 two-component system and more than 140 transcription regulators. Several regulators have been found to respond to environmental distress, such as extreme cold or heat, iron starvation, and oxidative stress [33]. MTB had learned to adapt to the environment by allowing or inhibiting transcription according to its surroundings to extend the duration of surviving in harsh conditions in the host [34]. The genome contains 250 genes involved in fatty acid metabolism, with 39 of these involved in the polyketide metabolism generating the waxy coat [3].There are, however, two additional arguments in favor of MTB recently changing its niche and lifestyle. There are 20 enzyme systems that are predicted to use cytochrome P450 as a cofactor and these are often involved in the degradation of xenobiotics or the modification of organic molecules, such as sterols, by means of their mono-oxygenize activity [35]. These enzymes are common in soil organisms where they enable diverse organic matter to be degraded to yield metabolizable sources of carbon and energy [35, 36]. Both the regulatory networks and the P450 systems have been subject to massive gene decay in *M. leprae* [31]. Recent Genome-wide association studies (GWAS) have identified three genetic risk loci, including at positions 11p13 and 18q11 [37, 38]

1.6.2 Comparative Genomics of MTBC

Several different approaches have been employed to compare the genomes of members of MTBC, extending from various DNA array technologies, which easily identify deletion events but cannot readily uncover insertions [39, 40], to highly sensitive whole-genome sequence comparison which detect the full range of polymorphisms from single nucleotide polymorphisms (SNPs) to gene rearrangements [16, 41, 42] SNPs do occur in the genomes of members of MTC but at a relatively low level for a bacterium of 1 in every 2000–4000bp [8] depending on the species. Some SNPs, like the point mutation the *pncA* gene responsible for pyrazinamide resistance [43] result in phenotypic change but the majority seems to be silent. Many of these studies have compared virulent and virulent strains in the hope of uncovering differences linked to changes in pathogenesis. Most of the insertions result from transposition events, generally involving IS*6110*,

or more rarely from gene duplication. No conclusive evidence in favor of recent horizontal gene transfer occurring in MTBC is available and the closest example of this is provided by the prophage genomes, phiRv1 or phiRv2, respectively [44] corresponding to regions of difference (RD) RD3 or RD11. After the completion of the genome sequence of MTB H37Rv, comparative-genomics approaches greatly enhanced our understanding of the mechanisms of insertion and deletion of DNA and the resulting distribution of variable regions around the genomes of tubercle bacilli [27, 44]. Based on this knowledge, a deletion analysis system has been developed to differentiate the members of the MTBC [41]. There are 20 variable regions, of which 14 regions of difference (RD1 to RD14) were found to be absent from bacillus Calmette-Guérin (BCG) Pasteur relative to MTBH37Rv [27, 39]. Six regions, H37Rv-related deletions (RvD1 to RvD5 and *M. tuberculosis* specific deletion 1 (TbD1), are absent from the MTBH37Rv genome relative to other members of the MTBC. Based on the presence or absence of the TbD1 region, MTB strains can be divided into "ancestral" and "modern" types. The Beijing, Haarlem, and African strains responsible for major epidemics are modern [41, 45].

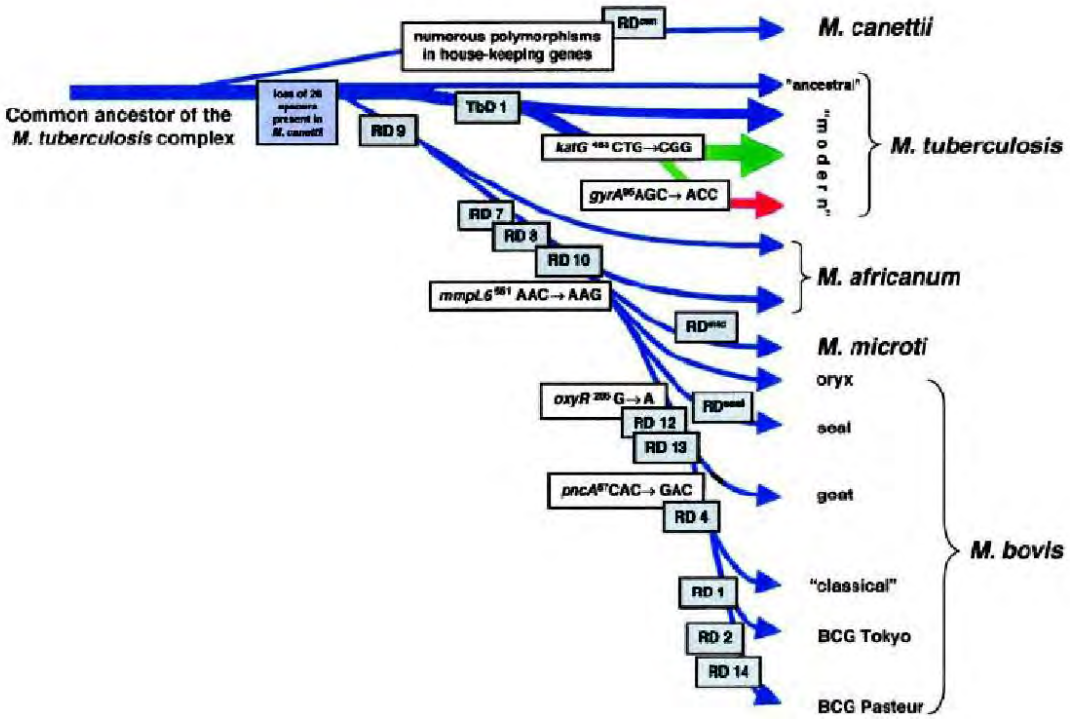


Figure 1.6: Scheme of the proposed evolutionary pathway of the tubercle bacilli illustrating successive loss of DNA in certain lineages (gray boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes. Note that the distances between certain branches may not correspond to actual phylogenetic differences

calculated by other methods. Blue arrows indicate that strains are characterized by *katG*463. CTG (Leu), *gyrA*95 ACC (Thr), typical for group 1 organism. Green arrows indicate that strains belong to group 2 characterized by *katG*463 CGG (Arg), *gyrA*95 ACC (Thr). The red arrow indicates that strains belong to group 3, characterized by *katG*463 CGG (Arg), *gyrA*95 AGC (Ser) as defined by Sreevatsan *et al*., 1997.

1.7 Virulence genes of *M. tuberculosis*

There are many genes of mycobacteria that contribute to the virulence of bacteria. These virulence genes are given below in the table:

Table-1.1: Genes involved in virulence of Mycobacteria

Serial no.	*Gene name*	*Gene number*	Function	Reference
1	aceA	Rv0467	Isocitrate lyase/dormancy	[46]
2	mceD	Rv0170	Cell invasion	[47]
3	cmaA, mmaA4	Rv3392c/Rv0503c/Rv0642c	Mycolic acid bio-synthesis	[48]
4	sigE/sigH	Rv1221/Rv3223c	Sigma factors	[49]
5	Acr	Rv2031c	Growth in macrophages	[50]
6	drrC	Rv2938	ABC transporter	[51]
7	ideR	Rv2711	Iron-dependent repressor	[52]
8	glnA	Rv2220	Nitrogen metabolism	[53]

9	aphC	Rv2428	Oxidative stress defense	[54]
10	KatG	Rv1908c	Catalase/peroxidase	[55]
11	fadD26	Rv2930	Lipid metabolism	[56]
12	fadD28	Rv2941	Mycocerosis acid synthesis	[57]
13	fbpa	Rv3804c	Mycolyl transferase	[58]
14	PKnG	Rv0410c	Phosphorylates the peptide substrate myelin basic protein at serine residues/serine/threonine-protein kinase protein kinase G	[59]
15	Pks2	Rv3825c	Polyketide synthase PKS2	[60]
16	*fadE28*	*Rv3544c*	Acyl-coenzyme A dehydrogenase	[61]
17	*nuoG*	*Rv3151*	NADH dehydrogenase I (chain G) NADH-ubiquinone oxidoreductase chain G	[3]
18	*phoP*	*Rv0757*	Positive regulator for the phosphate regulon, required for intracellular growth	[62]
19	*plcA*	*Rv2351c*	Phospholipase c 1 plca (mtp40 antigen)	[63]
20	*plcB*	*Rv2350c*	Membrane-associated phospholipase c 2 plcb	[63]
21	*plcC*	*Rv2349c*	Intracellular survival, by the alteration of cell signaling events or by direct cytotoxicity/phospholipase c 3 plcc	[63]

22	*plcD*	*Rv1755c*	Intracellular survival, by the alteration of cell signaling events or by direct cytotoxicity/phospholipase c 4 (fragment) plcd.	[63]
24	*mmpL8*	*Rv3823c*	Considered to be involved in the transport of lipids and shown to be required in the production of a sulfated glycolipid, sulfolipid-1	[64]

1.8 Tuberculosis Disease and types

There are two types of TB: latent and active.

1.8.1 Latent TB

Latent TB infection (LTBI) occurs when a person carries the TB bacteria within their body, but do not cause disease because the bacteria are present in very small numbers and are kept under control by the body's immune system [65]. People with LTBI do not feel sick and do not have any symptoms of TB. Most LTBI do not develop active tuberculosis. Only 5% to 10% people with latent TB develop active TB because of their weak immune system. [66]. People who are known to have a higher risk of progressing from latent TB to active Tb include:

- Infants and children aged less than 4 years;
- People infected within the previous two years;
- People infected with HIV;
- People who have certain clinical condition or conditions which compromise their immune system, such as people with diabetes, and people with chronic renal failure.

1.8.2 Active TB

When the TB bacteria have started to multiply and they become numerous enough to overcome the body's immune systems or defenses then active TB or TB disease occurs. Active TB disease usually causes a person to feel ill and in certain circumstances they are able to pass the TB bacteria on to other people [67].

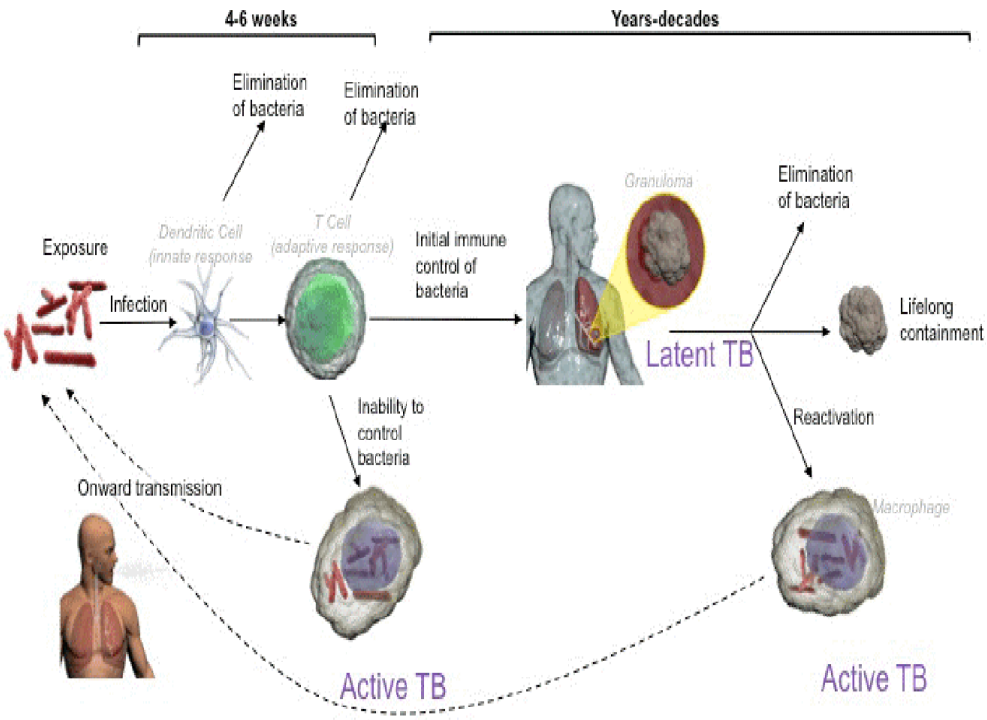


Figure 1.7: Type of tuberculosis infection.

1.9 TB Transmission

MTBis carried in airborne particles, called droplet nuclei of 1– 5 microns in diameter. Infectious droplet nuclei are generated when persons who have active pulmonary or laryngeal TB disease cough, sneeze, shout, or sing. About 40,000 droplet nuclei can be produced by a single sneeze [68]. Depending on the environment, these tiny particles can remain suspended in the air for several hours. MTB is transmitted through the air, not by surface contact. Transmission occurs when person inhales droplets nuclei containing 1-3 viable MTB, and the droplet nuclei travel the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs. The probability of transmission from one person to another depends upon the quantity of the infectious droplets expelled by the patient, the effectiveness of ventilation, the duration of exposure, and the virulence of the MTB strain [69].

1.9.1 TB is not transmitted

- Through food and water
- By kissing

- By skin contact such as shaking hands
- By touching a toilet seat
- Or by sharing a toothbrush

1.9.2 Predisposing factors for TB infection includes

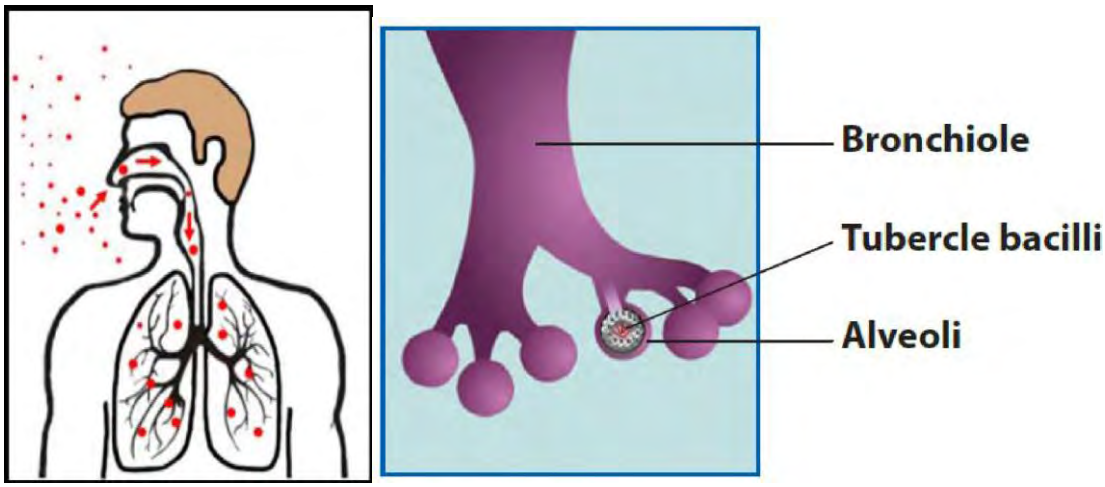
- HIV infection is the first predisposing factor for MTB. Ten percent of all HIV-positive individuals harbor MTB. This is 400-times. The rate associated with the general public
- Close contact with large populations of people, i.e., schools, nursing homes, dormitories, prisons and poor nutrition
- Intravenous drug use and alcoholism.

1.9.3 Risk Factors of Tuberculosis

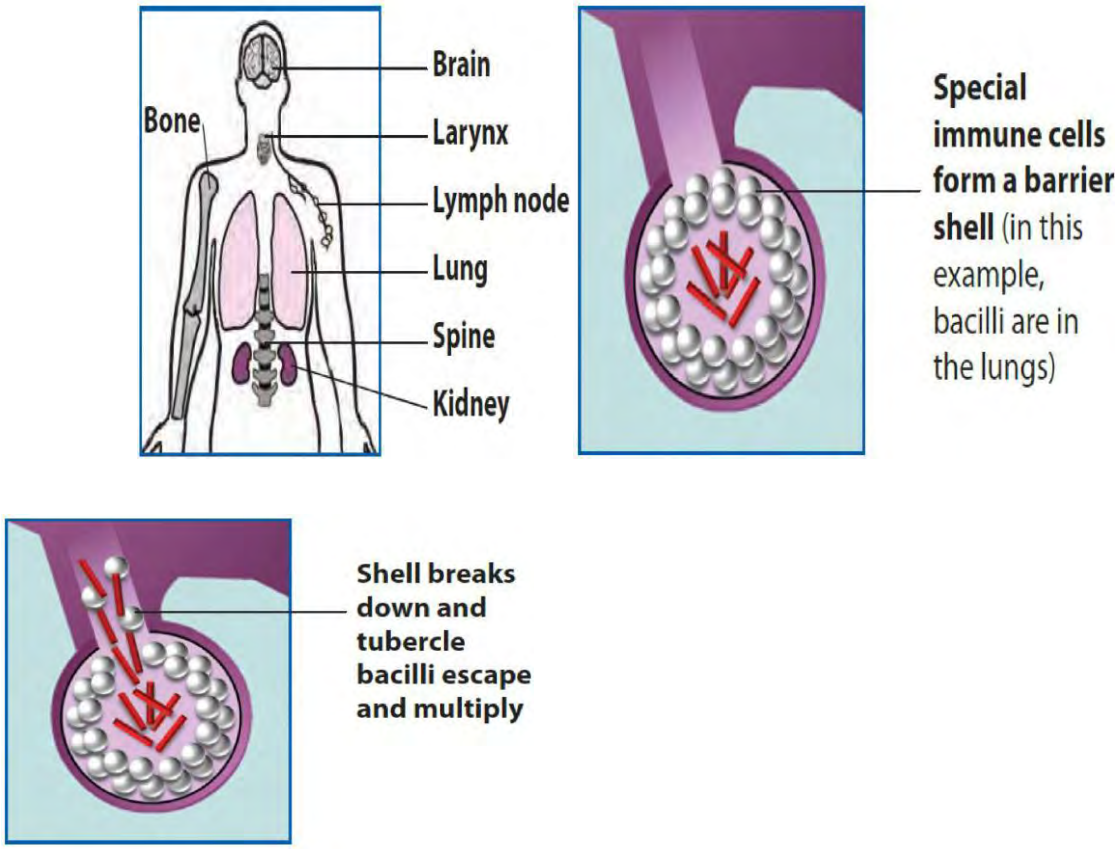
- Age – Extremes of age, young children and elderly adults are both at increased risk;
- Immunodeficiency because of diseases such as HIV;
- Immunosuppression because of medications;
- Overcrowding;
- Contact with a person who has active or latent TB;
- Alcohol intake;
- Diabetes Mellitus;
- Kidney failure etc.

1.10 Pathogenesis

Tubercle bacilli in the form of droplet nuclei are inhaled and reach the terminal alveoli after avoiding entrapment by muco-ciliary clearance mechanism.



Initial infection is most common in the lower lung segments where ventilation is the greatest. The organisms are ingested by alveolar macrophages, but continue to multiply [70]. A mild local reaction with additional macrophages and lymphocytes develops.



A small number of tubercle bacilli enter the bloodstream and spread throughout the body. The tubercle bacilli may reach any part of the body, including areas where TB disease is more likely to develop (such as the brain, larynx, lymph node, lung, spine, bone, or kidney).

Within 2-4 weeks, infected macrophages carry the organism to regional lymph nodes (hilar and mediastinal), where multiplication of the organism continues in presence of a minimal inflammatory response. These macrophages form a barrier shell, called a granuloma, that keeps the bacilli contained and under control (Latent TB).If the immune system cannot keep the tubercle bacilli under control, the bacilli begin to multiply rapidly (TB disease). Bacillemia occurs at 4-6 weeks after inhalation of the organism, resulting extension into the bloodstream from the regional nodes.

1.10.1 Molecular strategies of MTB evasion

MTB has evolved a number of very effective survival strategies, including:

(a) The inhibition of phagosome–lysosome fusion;

(b) The inhibition of phagosome acidification;

(c) The recruitment and retention of tryptophan-aspartate containing coat protein on phagosomes to prevent their delivery to lysosomes; and

(d) The expression of members of the host-induced repetitive glycine-rich protein family of proteins.

1.10.1.1 Inhibition of phagosome– lysosome fusion

Inhibition of growth and killing of intracellular pathogens within the host cell of the macrophage are considered to be dependent on phagosome–lysosome fusion [71]. Individual MTB bud out from the fused phagolysosomes into vacuoles that fail to fuse to the secondary lysosomes and temporary residence within a phagolysosome stimulates a response to the intracellular environment in MTB that facilitates its long-term survival and reproduction. Sulfatides (anionic trehalose glycolipids) of *M. tuberculosis* also have an anti-fusion effect [72]. MTBcan produce ammonia considered to be responsible for the inhibitory effect on phagolysosomal fusion. Ammonium chloride affects the movement of lysosomes by alkalizing the intra-lysosomal compartment [73].

1.10.1.2 Inhibition of phagosomal acidification

The restricted fusogenicity of the mycobacterial vacuole limit the access of lysosomal hydrolases to the bacilli. The absence of a vesicular proton-ATPase pump within mycobacteria results in a lack of acidification of phagosomes [74]. V-ATPase-mediated phagosomal acidification begins at very early stage of phagosome. Mycobacterium containing phagosomes acquire the lysosomal membrane protein LAMP1, but V-ATPase absent on their membrane.

1.10.1.3 Maturation of phagosomes

MTB modifies the maturation of the phagosomal compartment enhances intracellular survival. Maturation of phagosomes leads to the inhibition of phagolysosomal fusion. Moreover, the aberrant expression of Rab5 on the phagosomes containing MTB causes the maturation arrest of

these phagosomes at the early endosomal stage. Phagosomes containing inert particles or avirulent bacteria transiently display Rab5, whereas phagosomes containing virulent MTB exhibit a persistent display of Rab5 [75].

1.10.1.4 Protection against oxidative radicals

The intracellular environment of macrophages is hostile to intracellular bacteria because they produce a vast array of chemicals such as reactive oxygen and nitrogen radicals. The virulent Erdman strain of MTB over expresses a protein that cyclopropanates mycolic acid double bonds, resulting in a ten-fold lower susceptibility to peroxide [76]. The oxyR (i.e. a sensor of oxidative stress and a transcriptional activator that induces the expression of detoxifying enzymes such as catalase/hydroperoxidase) of MTB has numerous deletions and frameshift mutations giving the appearance of a pseudogene [77]. Superoxide dismutases contribute to the pathogenicity of many bacterial species and play an important role in protection against oxidative stress [78].

1.10.1.5 Recruitment and retention of tryptophan-aspartate containing coat protein (TACO) on phagosome wall

TACO/coronin-1 is an actin binding protein known to associate with cholesterol within the plasma membrane [79]. Prevention of bacterial delivery to lysosomes allows the mycobacteria to escape the bactericidal action of macrophages, vitamin D3 and retinoic acid down-regulate TACO gene transcription in a dose-dependent manner [80]. Treatment with vitamin D3 and retinoic acid inhibits mycobacterial entry [81].

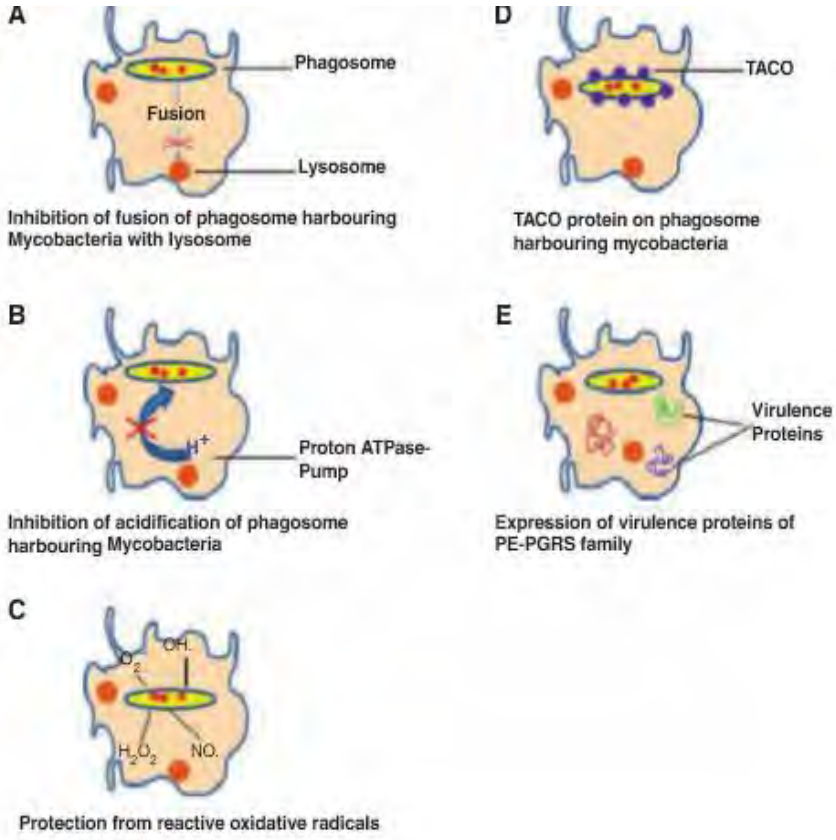


Figure 1.8: Molecular strategies of MTB evasion.

1.11 TB Symptoms

Symptoms of TB depend on where in the body the TB bacteria are growing. TB bacteria usually grow in the lungs resulting pulmonary tuberculosis [68] but can also spread outside the respiratory organs affecting the pleura, central nervous system, lymphatic system, genitourinary system, bones and joints etc. These are collectively denoted as "extrapulmonary tuberculosis" (EPTB). EPTB forms are more common in immunosuppressed persons and in young children. Infectious pulmonary TB may co-exist with EPTB, which is not contagious [82]. A table showing the symptoms associated with pulmonary and EPTB infection is given below:

Table 1.2: Symptoms of pulmonary & extrapulmonary TB

Symptoms of Pulmonary TB	Symptoms of Extrapulmonary TB
• Cough (especially if lasting for 3 weeks or longer) with or without sputum production • Coughing up blood (hemoptysis) • Chest pain • Loss of appetite • Unexplained weight loss • Night sweats • Fever • Fatigue	• TB of the kidney may cause blood in the urine • TB meningitis may cause headache • TB of the spine may cause back pain • TB of the larynx can cause hoarseness • Loss of appetite • Unexplained weight loss • Night sweats • Fever • Fatigue

1.12 Treatment of TB and Drug Resistance

Different categories of drugs are used for TB treatment depending on the severity of the disease. Usually following drugs are used as anti-TB treatment.

1.12.1 First line drugs:

Streptomycin, Isoniazid, Rifampicin, Ethambutol, Pyrazinamide.

1.12.2 Second line drugs:

Kanamycin, Levofloxacin, Ofloxacin, Gentamicin, Amikacin, Capreomycin.

Table-1.3: Mode of action of TB drugs

Compound	**Mode of action**
Isoniazid	Converted to its active form KatG which produces oxidative radicals.
Ethambutol	Inhibits arabinosyl transferases involved in polymerization reaction of Arabinoglycan. Thereby blocking cell wall biosynthesis.
Rifampicin	Binds to RNA polymerase's active center and prevents bacterial RNA from elongating.
Pyrazinamide	Converted to its active form pyrazinoic acid, which disrupts membrane energetics and inhibits membrane transport function. It kills a population of semi dormant tubercle bacilli in acidic pH environment that are not killed by other TB drugs.

For fully susceptible organisms, the following drug regimens are efficacious and can be recommended:

1) Isoniazid (INH) + rifampin (RIF) + pyrazinamide (PZA) daily for 2 months followed by INH + RIF daily or 2-3 times weekly for 4 months. Total duration**:** 6 months

2) INH + RIF + PZA + ethambutol (EMB) or streptomycin (SM) for 2 weeks followed by the same regimen 2 times weekly for 6 weeks, and subsequently INH + RIF 2 times weekly for 4 months. Total duration: 6 months.

3) INH + RIF + PZA + EMB or SM 3 times per week for 6 months

4) INH + RIF daily or 2-3 times weekly after 1 month of daily therapy for 9 months.

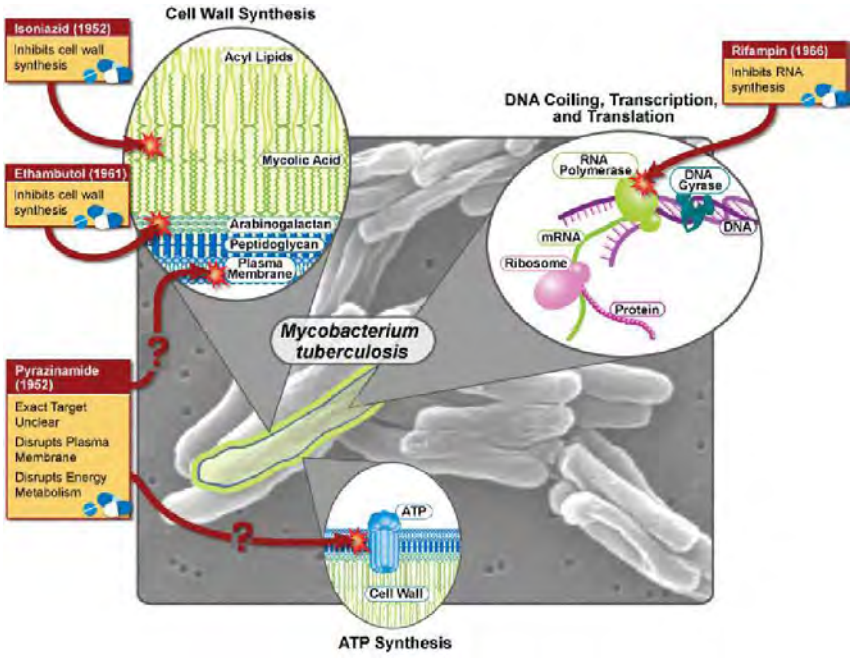


Figure 1.9: Mechanism of action of first line anti-TB drugs

Directly observed therapy (DOT) is mandatory for intermittently administered medication. Unless rates of drug resistance are known to be very low (<4%), regimen 1 should include daily EMB with INH + RIF + PZA for initial therapy. Drug sensitivity data, usually available 2-4 weeks after culture results, should guide the choice of drugs in the continuation phase of therapy. If full susceptibility is documented, EMB or SM can be stopped in Regimen 1 and 2. Four drugs are continued for the entire 6 months period in Regimen 3. Patients with resistance or intolerance to a single drug can usually be treated successfully using combinations of the remaining active drugs, although resistance to RIF often requires more prolonged therapy. Multi-drug resistant (MDR) TB defined as resistance to at least INH and RIF requires prolonged, costly, treatment often with the addition of an injectable drug and more toxic, less effective oral agents.

1.12.3 Multi-drug resistant TB (MDR-TB)

Drug-resistant TB is caused by MTB organism that are resistant to the drugs normally used to treat the disease. Drug-resistant TB is transmitted in the same way as drug-susceptible TB, and is no more infectious than drug-susceptible TB. Sometimes the bacteria become resistant to two or more of the most important drugs, INH and RIF. This is called multidrug-resistant TB, or MDR -TB.

1.12.4 Extensively drug resistant TB (XDR-TB)

Extensively drug-resistant TB (XDR-TB) is a rare type of multidrug-resistant tuberculosis (MDR-TB) that is resistant to isoniazid and rifampin, plus any fluoroquinolone and at least one of three injectable second-line drugs i.e., amikacin, kanamycin, or capreomycin XDR TB is great threat to global health.

1.13 Diagnosis of TB

Diagnosis of TB is important because it provides a great deal of useful information of organism, suggesting potential intervention strategies, including successful treatment of patients on a clinical basis. The steps to diagnose TB infection and disease include:

- ➢ Medical evaluation that includes history and risk assessment
- ➢ The tuberculin skin test
- ➢ Chest x-ray
- ➢ Bacteriological examination.

1.13.1 Medical History

The medical history includes facts about the social, family, medical, and occupational aspects of the patient's life.

The clinician should ask about:

- ➢ Exposure to a person who has infectious TB
- ➢ Any symptoms of TB (productive cough longer than 3 weeks, fever, unexplained weight loss, night sweats)
- ➢ The patient's history of TB infection or TB disease
- ➢ Risk factors for developing the disease (intravenous drug use, immune system condition, diabetes)
- ➢ Country of birth.

1.13.2 Tuberculin skin test

Skin testing is also termed as Mantoux test. The Mantoux test is the preferred type of skin test because it is the most accurate. The tuberculin skin test is used to determine if a person has TB infection. A substance, called tuberculin, is injected into the skin. Tuberculin is purified protein derivative (PPD) which is intra-cutaneously injected in the forearm. Five TU (tuberculin units),

which equals 0.000lmg of PPD, in an O.1 ml volume is injected. Reaction in the skin to tuberculin PPD is initiated when specialized immune cells, called T cells, which have been sensitized by prior infection, are recruited to the skin site where they release chemical messengers called lymphokines. These lymphokines induce induration (a hard, raised area with clearly defined margins at and around the injection site) through local vasodilatation edema, fibrin deposition, and recruitment of other inflammatory cells to the area within 48-72 hours.

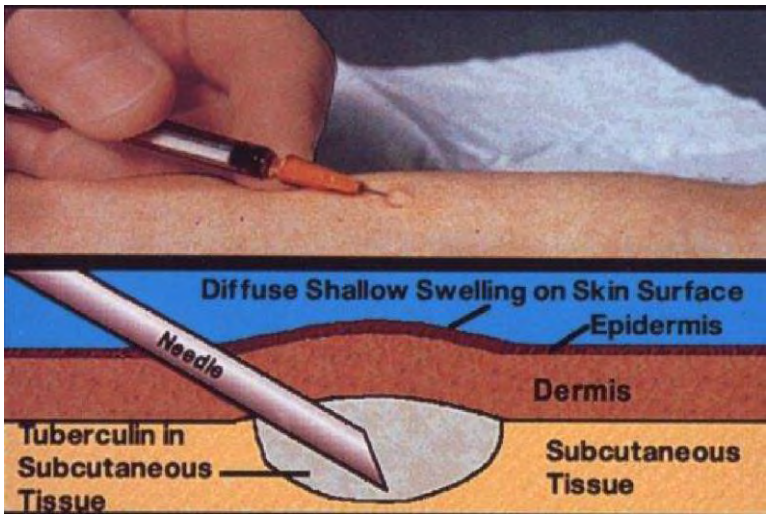


Figure 1.10: Mantoux tuberculin skin test.

The test is considered positive if the diameter of the resulting lesion is 10 mm or greater. The lesion is characterized by erythema (redness) and swelling and induration (raised and hard). 90% of people that have a lesion of 10 mm or greater are currently infected with MTB or have been previously exposed to MTB 100% of people that have a lesion of 15 mm or greater are currently infected with MTB or have been previously exposed to MTB.

1.13.3 Chest X-Ray

The chest X-ray helps the clinician determine any presence of TB or old healed TB disease. Active tuberculosis creates cavities visible in X-rays in the patient's right upper lobe. In active pulmonary TB, infiltrates or consolidations and/or cavities are often seen in the upper lungs with or without mediastinal or hilar lymphadenopathy or pleural effusions (tuberculous pleurisy). However, lesions may appear anywhere in the lungs. In disseminated TB a pattern of many tiny nodules throughout the lung fields [82].

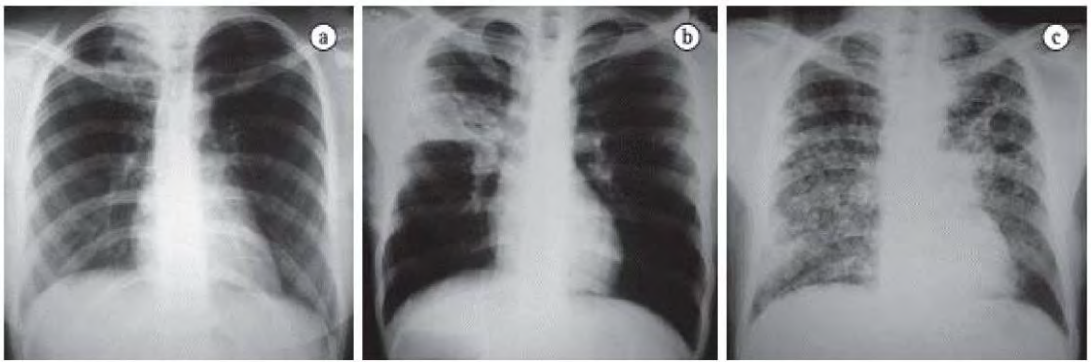


Figure 1.11: Chest X-rays of patients with pulmonary tuberculosis. In a) A 24-year-old male, weight loss in the last three months and current fever 38°C. In b) A 25-year-old female, dry cough for the last three months and recent worsening of health status. In c) A 38-year-old male, previously treated for tuberculosis for the last three weeks.

1.13.4 Bacteriological Examination

Bacteriological examination is performed when patient has TB disease. This is an examination of the secretions from the lungs (sputum), directly under a microscope or by culture methods and indirectly through different molecular techniques.

1.13.4.1 AFB Microscopy

M. tuberculosis is identified microscopically by its staining characteristics. It retains certain stains after being treated with acidic solution, and is thus classified as an "acid-fast bacillus" or AFB (Madison BM, 2001). In the most common staining technique, the Ziehl-Neelsen stain, AFB are stained a bright red which stands out clearly against a blue background. Acid-fast bacilli can also be visualized by fluorescent microscopy, and by an auramine-rhodamine stain. On the basis of the AFB test doctor suggest appropriate treatment.

1.13.4.2 Culture Methods

Solid or liquid media are used in culture methods. Samples of sputum or tissues require initial liquification-decontamination, most commonly using N-acetyl- L-cysteine as a mucolytic in 1% sodium hydroxide solution. Solid culture media are of two general types: agar-based (e.g. Middlebrook 7H11) and egg-based (e.g., Lowenstein-Jensen). The BACTEC radiometric system for culturing mycobacteria is widely used. This liquid culture system generally detects metabolism within 9 to 16 days, depending on the number of organisms in the specimen.

1.13.5 Molecular test

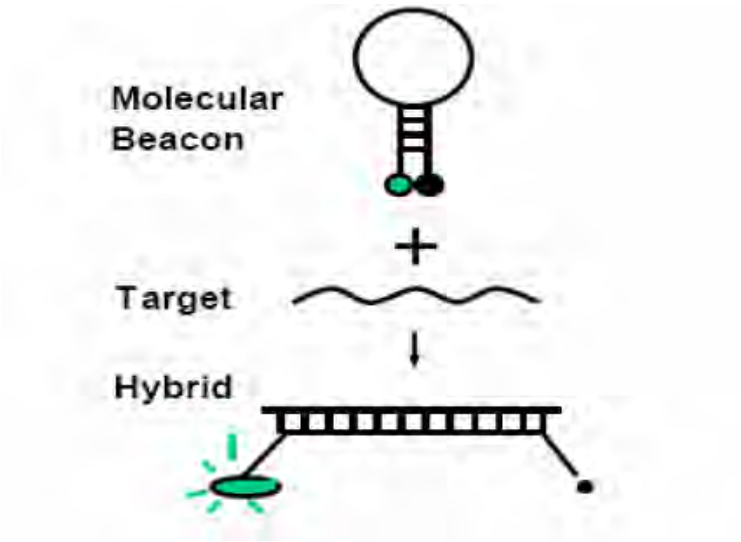
1.13.5.1 Polymerase chain reaction (PCR)

New TB tests are being developed that offer the hope of cheap, fast and more accurate TB testing. Rapid nucleic acid amplification techniques such as polymerase chain reaction (PCR) allow direct identification of *M. tuberculosis* in clinical specimens. Such method detects fewer than 10 organisms in clinical specimens, compared with the 10,000 required for smear positivity. IS*6110* PCR analysis, RD9, TbD1 are most commonly used PCR probes to detect *M. tuberculosis.*

1.13.5.2 Xpert MTB/RIF

The Xpert MTB/RIF is a cartridge-based, automated diagnostic test that can identify MTB and resistance to rifampicin (RIF). The Xpert MTB/RIF detects DNA sequences specific for MTB and rifampicin resistance by real time polymerase chain reaction (RT-PCR). It is based on the Cepheid GeneXpert system, a platform for rapid and simple-to-use nucleic acid amplification tests (NAAT). The Xpert® MTB/RIF purifies and concentrates MTB from sputum samples, isolates genomic material from the captured bacteria by sonication and subsequently amplifies the genomic DNA by PCR and identifies all the clinically relevant rifampicin resistance inducing mutations in the RNA polymerase beta (*rpoB*) gene in the MTB genome in a real time format using fluorescent probes called molecular beacons. Xpert assay uses 3 specific primers and 5 unique molecular probes to ensure a high degree of specificity. The 81-bp *rpoB* hot-spot region was amplified by PCR and DNA sequencing done with specific primers.

rpoB-for 5′ TGGTCCGCTTGCACGAGGGTCAGA-3′ and
rpoB-rev 5′-CTCAGGGGTTTCGATCGGGCACAT-3′[146].


Molecular
Beacon
Target
Hybrid

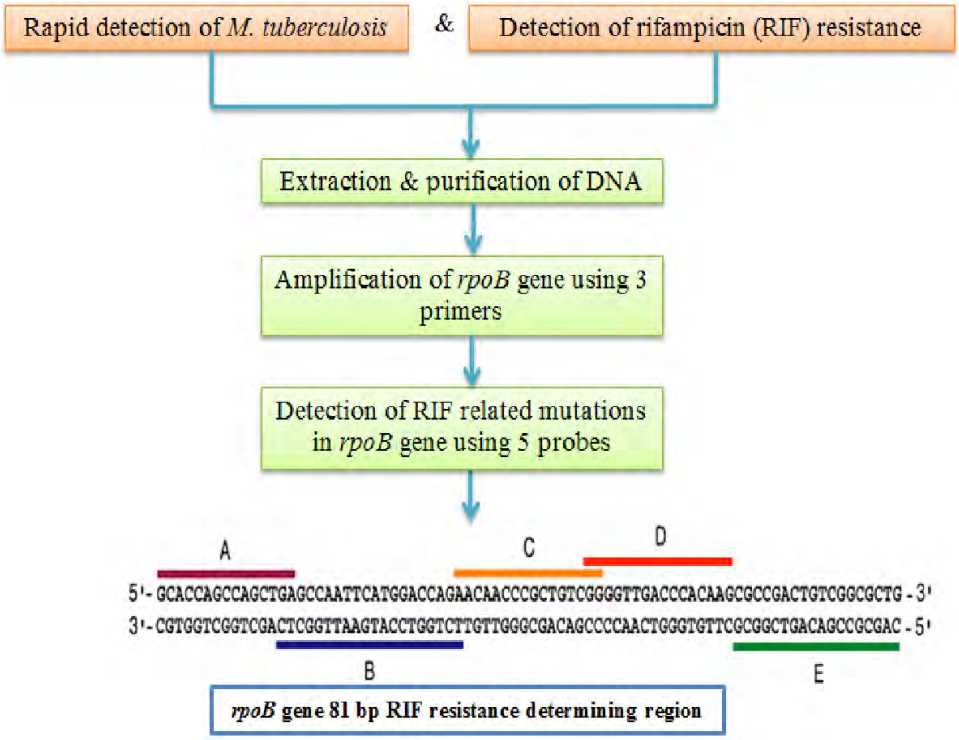


Figure 1.12: Working principle of Xpert MTB/RIF assay.

For mutation analysis, a 437-bp fragment of the rpoB gene was amplified using primers

Results of GeneXpert assay are obtained from unprocessed sputum samples in 90 minutes, with minimal biohazard and very little technical training required to operating. This test was developed as an on-demand near patient technology which could be performed even in a doctor's office if necessary

1.14 Objectives

1.14.1 General objective

To evaluate the most effective method for detection of tuberculosis using pulmonary specimens.

1.14.2 Specific objectives

a) To detect MTB using Xpert MTB/RIF assay, AFB microscopy, conventional culture method.
b) Determination of sensitivity, specificity, positive predictive value and negative predictive value among the methods used in the study.

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Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Study Design

To achieve the objectives of the study, all samples were collected from three TB Screening Centres led by icddr,b in private sector of Dhaka City. Randomly a total of 145 presumptive TB cases were enrolled during the period of May 2016 to April 2017 in this study. Sputum specimens were collected from all the eligible participants. All patients were instructed how collect good specimen through video. A brief study questionnaire was used to collect the demographical and clinical history upon their consent. All collected specimens were transported to Mycobacteriology Laboratory of icddr,b.

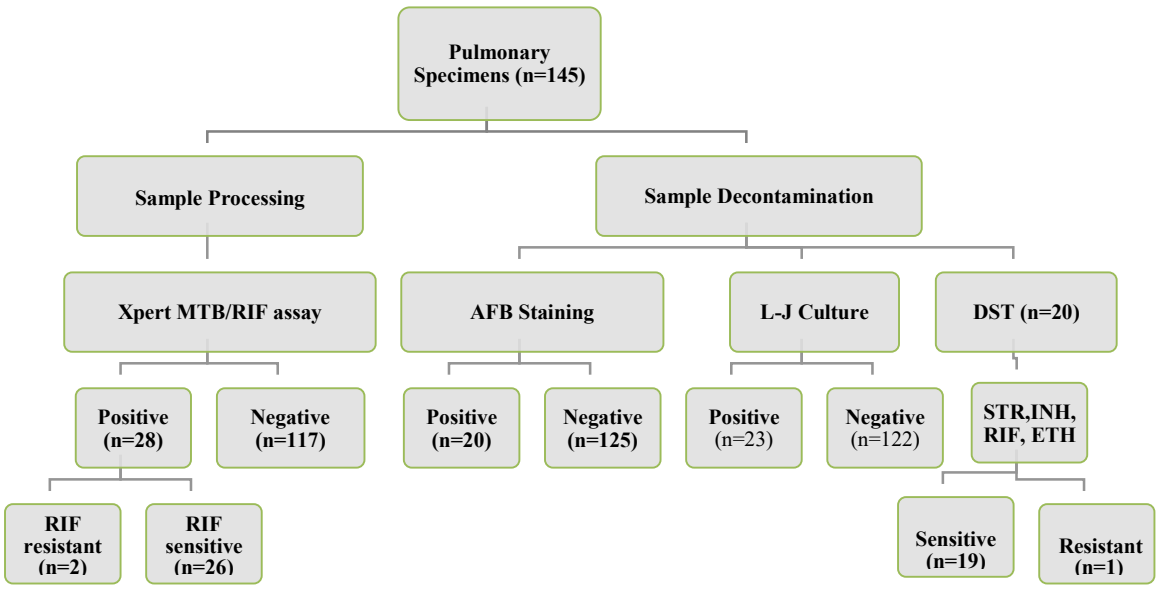

Figure 2.1: Flow diagram of the study procedure.

2.2 MTB detection by Xpert MTB/RIF assay

The Xpert MTB/RIF is a cartridge based nucleic acid amplification test, automated diagnostic method that can identify MTB DNA from concentrated sediments as well as rifampicin resistance associated mutations of the *rpoB* gene from TB suspected patient. The Xpert MTB/RIF assay technique is based on heminested PCR technology uses 3 specific primers and 5 unique molecular probes to ensure a high degree of specificity. This assay targets the *rpoB* gene, which is critical for identifying mutations associated with rifampicin resistance. The real-time PCR assay consists of a single-use multi-chambered cartridge preloaded with the buffers and reagents required for sample processing, amplification, and detection.

2.2.1 Operational procedures of Xpert MTB/RIF assay

2.2.1.1 Sample processing

Samples were processed according to manufacturer instruction (Cepheid Gene Xpert®System, CA, USA). Briefly, 1.5 ml of the sample was transferred to a new 15 ml centrifuge tube. The centrifuge tubes were labeled with individual patient identification number. Sample reagent buffer at 2:1 (v/v) ratio (Appendix II) was added to 15 ml centrifuge tube containing the sample and hand mixing was performed by inverting the tube for 20 times in different intervals. Samples were then incubated for 15 minute at room temperature accompanied with another hand mixing after 8 min incubation for 10 times.

2.2.1.2 Preparing the cartridge and sample loading

Each cartridge (Appendix II) was labeled with individual sample Identification number (ID). It should be concerned not to put the label on the lid of the cartridge or obstruct the existing 2D barcode on the cartridge. The sterile transfer pipette which provided by the manufacturer was used, the liquefied sample was aspirated into the transfer pipette until the meniscus is above the minimum mark.

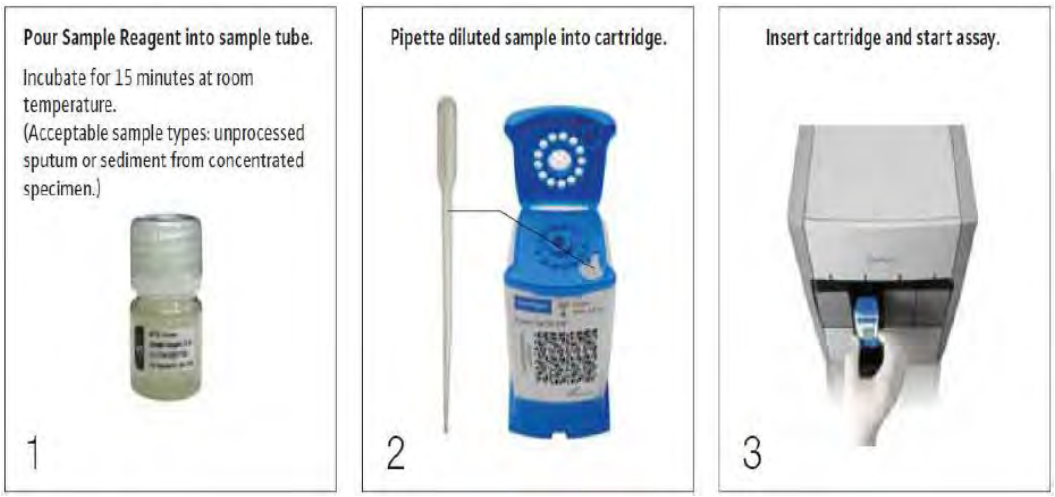

Figure 2.2: Cartridge preparation and sample loading on Xpert MTB/RIF platform.

The cartridge lid was opened and the sample was transferred into the open port of the Xpert MTB/RIF cartridge. It was dispensed slowly to minimize the risk of aerosol formation. Then the cartridge lid was closed. It was made sure that the lid snapped firmly into place.

2.2.1.3 Performing the test

Before starting the test, it was ensured that the system was equipped with the GX 2.1 software, and the Xpert MTB/RIF assay was imported into the software. The following steps are involved to perform the test:

- The computer was turned on, and then the GeneXpert® Dx system was turned on.
- On the Windows desktop, the GeneXpert® Dx shortcut icon was double-clicked.
- The GeneXpert® Dx System software was logged on using user name and password.
- In the GeneXpert® Dx System window, Create Test button was clicked.
- When the Scan Cartridge Barcode dialog box was appeared the barcode on the Xpert MTB/RIF cartridge was scanned. The Create Test window appeared. Using the barcode information, the software automatically filled the boxes for the following fields: assay, reagent Lot ID, cartridge serial number, and expiration date were selected.
- In the sample ID box, the sample ID was scanned or typed. It was made sure that the correct sample ID was typed. The sample ID is associated with the test results and is shown in the "View Results" window and all the reports.
- Then "Start Test" button was clicked.
- When the instrument module door was opened with the blinking green light and then the cartridge was loaded.

- After loading the cartridge the door was closed. When the test was started, the green light stops blinking. The light was turned off after finishing the test.
- It was waited until the system releases the door lock at the end of the run, then the module door was opened and the cartridge was removed.
- Used cartridges were disposed in appropriate specimen waste containers according to Bio-safety regulation.

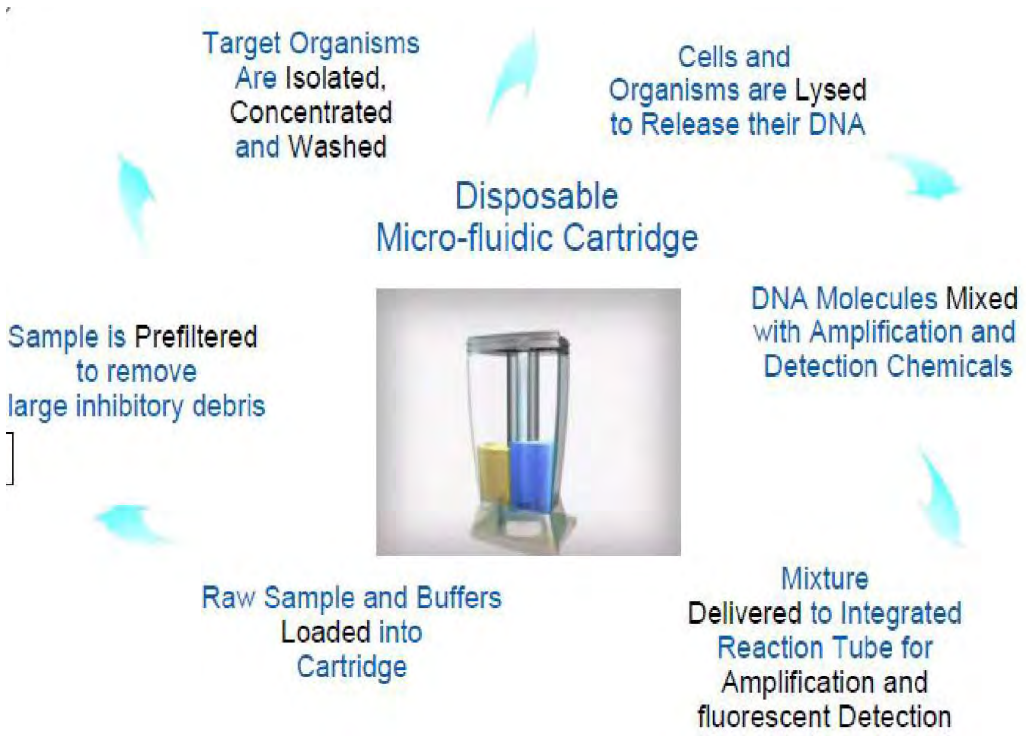

Figure 2.3: Workflow of Xpert MTB/RIF assay

2.2.1.4 Interpretation of the test results

The primers in the Xpert MTB/RIF assay amplify a portion of the *rpoB* gene containing the 81 base pair "core" region. Five differently colored fluorogenic nucleic acid hybridization probes, called molecular beacons, interrogate the entire 81-bp core. Each molecular beacon was designed to be so specific that it does not bind to its target if the target sequence differs from the wild-type *rpoB* sequence by as little as a single nucleotide substitution. Since molecular beacons fluorescence only when they are bound to their targets, i.e. wild-type *rpoB* sequence, the absence of any one of the five colors in the assay differentiate between the conserved wild-type sequence and mutations in the

core region that are associated with RIF resistance. The results are interpreted by the GeneXpert® Dx System from measured fluorescence signals and embedded calculation algorithms and are displayed in the "View Results" window, as indicated below.

Figure 2.4: Detection of MTB by Xpert MTB/RIF assay**.**

Table 2.1: Xpert MTB/RIF Assay Results and Interpretations

Result	Interpretation
MTB DETECTED	The MTB target is detected within the sample:
Rif resistance DETECTED	A mutation in the *rpoB* gene has been detected. SPC: NA (not applicable). An SPC signal is not required because MTB amplification can compete with this control. Probe Check (QC1 and QC2): PASS. All probe check results pass.

MTB DETECTED; Rif resistance NOT DETECTED	The MTB Target is detected within the sample: -A mutation in the *rpoB* gene has not been detected. -SPC: NA (not applicable). An SPC signal is not required because MTB amplification can compete with this control. -Probe Check (QC1 and QC2): PASS. All probe check results pass.
MTB DETECTED; Rif resistance INDETERMINATE	The MTB target is detected within the sample: -A mutation in the *rpoB* gene could not be determined due to insufficient signal detection. -SPC: NA (not applicable). An SPC signal is not required because MTB amplification can compete with this control. -Probe Check (QC1 and QC2): PASS. All probe check results pass.
MTB NOT DETECTED	The MTB target is not detected within the sample: -SPC: PASS. The SPC met the accepted criteria. -Probe Check (QC1 and QC2): PASS. All probe check results pass.
INVALID	The presence or absence of MTB cannot be determined. The SPC does not meet the acceptance criteria, the sample was not properly processed, or PCR was inhibited. Repeat the test. -The presence or absence of MTB cannot be determined. -MTB INVALID: The presence or absence of MTB DNA cannot be determined. -SPC: FAIL. The MTB target result is negative, and the SPC Ct is not within valid range. -Probe Check (QC1 and QC2): PASS. All probe check result pass.

2.3 Decontamination and processing of the samples

All specimens were processed with N-Acetyl-L-Cysteine (NALC)-Sodium Hydroxide (NaOH) method which is widely used and recommended procedure for MTB identification [83, 84]. Briefly, samples were decontaminated and digested with equal volume of 2% NaOH and 1.45% Sodium citrate along with 0.5% NALC. The specimens were then neutralized with phosphate buffer saline (PBS; P^H 6.8) and centrifuged at 3000 rpm for 15 min at 4°C. The pellet was re-suspended in 1.5 mL of PBS and inoculated on Lowenstein-Jensen (L-J) slants. The L-J slants were incubated at 37°C for six to eight weeks.

2.4 Smear preparation and Ziehl-Neelsen staining procedure

2.4.1 Slide identification

For smear preparation the slides were identified by numbering according to the patients identity that already present in the collection tubes.

2.4.2 Smear preparation

After decontamination, the pellets were taken for smear preparation. An wire loop take a loop of sample from the pellet and spread evenly as possible over an area of 3 cm by 2 cm.

2.4.3 Fixation

Fixation was necessary to kill the tubercle bacilli, and to make the smear stick to the slide better. Fixation was usually done by passing the completely dry smears three times through the blue part of the flame of a spirit lamp. Over heating may damage the AFB and even the slide.

2.4.4 Staining of the fixed slides

For staining the slides, the following reagents were used. All the reagents were prepared in the laboratory.

2.4.4.1 Carbol fuchsin solution:

Carbol fuchsin solution was prepared by dissolving 3.0 g of basic fuchsin in 100 mL 96% ethanol; this was solution (A). Phenol solution was prepared by dissolving 5 g of phenol crystals in 100 mL of distilled water; this was solution (B). For 100 mL of carbol fuchsin solution 10 mL of solution (A) was mixed with 90 mL of solution (B).

2.4.4.2 De-coloring agents: 25% solution of H_2SO_4 was used as de-coloring agents.

2.4.4.3 Counter stain: Methylene blue was used as counter stain.

For staining, the heated fixed slides were arranged on a staining rack over a sink. The smear was flooded with carbol fuchsin. The carbol fuchsin was heated by using a metal stick with a piece of cotton wool , which was lighted after putting a bit of burning spirit on it and stopped when the first vapor was seen and kept it for 5 minutes. The slides were then washed with distilled water. The washed slides were decolorized with 25% H_2S0_4 for 2 to 3 minutes. Again the slides were washed with distilled water. After washing the slides were counter stained with Methylene blue and kept at room temperature for 2 to 3 minutes. After washing with distilled water, the slides were dried by electrical heater.

2.4.5 Technique for AFB-Microscopy

The fixed smears were examined with 10X eyepieces and 100X oil immersion objective. Immersion oil was applied by letting a drop fall on the smears without touching the smear to avoid possible contamination. The slides were examined from one end to other and checked each field for red-shaped bacilli. Presence or absence of bacilli was recorded for each field. If present, number of bacilli was counted. When no acid-fast bacilli were observed in 100 fields, the smear was considered as negative. When only few bacilli (1-9) were found per 100 fields, then the examination was continued up to 200 fields. On the other hand, if one or more acid-fast bacilli were found in each field, then the examination was stopped after 20 fields. How the slides are graded from negative to highly positive are shown in the table 2.2.

Table 2.2: Explanation of AFB results and grading

Number of AFBs seen	Result and grading	Number of fields to be examined
None in 100 fields	Negative	100
1-9 in 100 fields	Scanty	200
10-99 in 100 fields	1+	100
1-10 per field	2+	20
> 10 per field	3+	20

2.5 Recovery of MTB by culture method

MTB can be recovered by conventional culture method using solid media (egg based Lowenstein- Jensen (L-J) selective media.

2.5.1 Preparation of egg based L-J media

2.5.1.1 Preparation of homogenized eggs solution

Fresh hen eggs were cleaned with soap solution and soaked in 70% ethanol for 30 minutes. The eggs were broken and the whole of each egg (yellow and white) was taken into a sterile flask. The eggs were homogenized by using a magnetic stirrer for at least 30 minutes and filtered through the four layers of sterile gauze into a sterile cylinder.

2.5.1.2 Preparation of salt solution

For preparation of salt solution Lowenstein- Jensen base (dehydrated) was used (Appendix 1). It is always stored below 30^{o}C.

Salt solution was prepared by dissolving 6.2% of Lowenstein-Jensen base with 100 ml dH2O containing 2 ml of glycerol. The salt solution was autoclaved in 121 to 124 ^{o} C for 15 minutes, and finally cooled to 45 to 60 ^{o} C.

The L-J media was prepared aseptically by mixing salt solution with egg suspension at 9:1 ratio. The L-J media (5.0 mL) were transferred into universal container or culture bottle. The bottles were slanted by inspissations at 85^{o} C for 50 minutes and subsequently incubated with loosen cap at 37^{o}C for at least 48 hours to check the sterility. Then the prepared L-J bottles were stored in the incubator by keeping the caps tighten enough to prevent evaporation (when not immediately used).

2.5.2 Inoculation of samples into L-J media

The pellet, which was previously processed from the sputum, was taken with a wire loop and carefully inoculated into LJ media. The inoculated culture bottles were incubated at 37 ^{o} C for eight weeks in a vertical position for the better development of individual colonies.

2.5.3 Observation of the cultures

After inoculation, the cultures were observed every week for any growth of mycobacterium. The incubation periods were 4 to 6 weeks. Sometimes incubation periods may increase up to 10 weeks, because some mycobacterium grows very slowly. The sample was considered as positive when buff colored small colonies grew in L-J medium. Close inspection, preferably with a hand lens and under a good bench lamp, is necessary to reveal very small colonies or the effuse growth, which are

characteristics of some environmental mycobacterium. Contaminated cultures (e.g. growth of moulds, and also those in which the medium has liquefied or turned a dark green) were discarded.

2.6 Drug Susceptibility Test

2.6.1 Drug susceptibility testing

The phenotypic DST (conventional DST) was performed according to proportion susceptibility method described previously [85]. For each batch, sensitive strain, H37Rv, from American Type Culture Collection, and a laboratory defined RIF-R isolate (SB256) were tested for quality control. An isolate was considered as sensitive if the colony ratio of drug-free media and drug containing media is less than 1% where as if the ratio is equal or above 1%, the isolate considered as resistant.

2.6.2 Preparation of dilution of colonies

One third parts of 3 mm wire loop colonies from well developed cultures were taken and transferred into a bottle (bottle 1) containing 0.2 mL of distilled water and 5-6 glass beads. Then vortex for 30 seconds to produce a uniform suspension and1.8 ml of sterile distilled water was added to the suspension and stands for 15-20 minutes at room temperature to allow the coarser particles to settle down. From this suspension 10 fold dilution was made by carefully adding 0.2ml to 1.8ml sterile distilled water (S1,) Three further serial dilutions 10^{-2} (S2), 10^{-3} (S3) and 10^{-4} (S4) were prepared in a similar manner and then inoculated one loopful (3 mm external diameter) on drug free and drug containing LJ slopes and observed for 4 weeks.

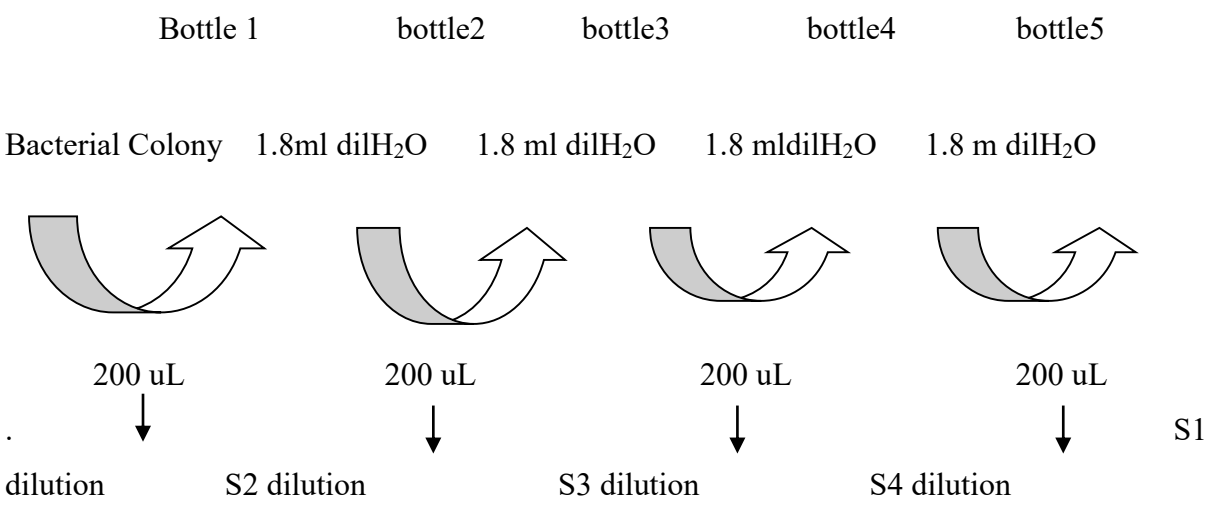

Figure 2.5: Serial dilution of colonies from culture

The susceptibility against drugs was calculated in the following way:

$$\frac{\text{The number of mycobacterial colonies grown on the drug containing LJ slants}}{\text{The number of mycobacterial colonies grown on the drug free LJ slants}} \times 100$$

When the calculated value is less than 1, the isolate was considered as sensitive and if the value was greater 1, the isolate was considered as resistant.

Chapter Three

Results

3. Results

A total of 145 sputum specimens were collected from three TB screening centers in Dhaka and different methods were used for TB diagnosis.

3.1 Demographic Characteristics of all patients

Among the enrolled participants, 98 (67.59%) were male and remaining 47 (32.41%) were female. Mean age of the participants were 42.78±1.43 years. More than half of the participants (55.86%) were form 16-45 years age group (Table 3.1)

Table 3.1: Demographic Characteristics of all patients

Categories	Number of participants (N)	Frequency (%)
Sex		
Male	98	67.59
Female	47	32.41
Age (Years)		
0-15	1	0.69
16-30	44	30.34
31-45	37	25.52
46-60	40	27.59
> 60	23	15.86

3.2 AFB microscopy

The presence of acid-fast bacilli in a smear reflects disease severity and patient infectivity. Therefore, it is important to record the number of acid-fast bacilli on each smear. After collection, the samples were decontaminated and processed according to standard protocol for smear preparation. The Z-N staining was performed and the slides were examined under bright field microscope for the presence or absence of acid-fast bacilli.

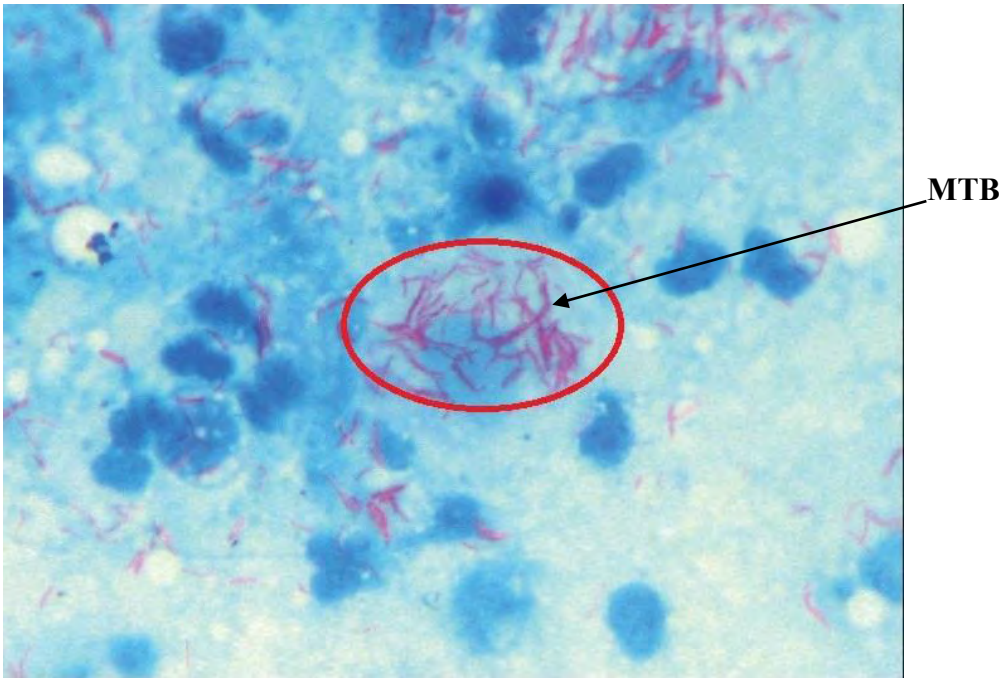

Figure 3.1: Ziehl-Neelsen staining of MTB.

Microscopic examination revealed that 20 (13.80%) of 145 participants were positive for AFB and the remaining specimens were negative. Based on the number of bacilli load positive specimens were categorized into four groups. Among the positive cases, 5 (25%) were 1+, 4 (20%) were 2+, 3 (15%) were 3+ and 8 (40%) were scanty positive. Overall AFB results are summarized in table 3.2.

Table 3.2: Microscopic observation of AFB

AFB Microscopy	Number of cases (%)
AFB status (n=145)	
Negative	125 (86.20)
Positive	20 (13.80)
AFB grading (n=20)	
1+	5 (25.00)
2+	4 (20.00)
3+	3 (15.00)
Scanty	8 (40.00)

3.3 Detection of MTB by Xpert MTB/RIF Assay

All the specimens were tested by GeneXpert MTB/RIF assay. Among 145 cases, 28 (19.32%) were found to be positive for MTBC and remaining 117 (80.68%) were negative. Again, based on bacterial load in the specimen, Xpert assay result can be differentiated as four groups (high, medium, low and very low). Among 28 MTB positive cases, 10 (35.71%) specimens contained "low" and 8 (28.59%) contained "Medium" level of bacilli. The remaining 10 cases were grouped to "Very Low" and "High" with 5 cases each.

Xpert assay can also tell us about the rifampicin susceptibility status (Resistant or Sensitive). Among the 28 positive cases, only two cases were resistance to rifampicin (7.15%) and remaining 26 were susceptible (Table 3.3).

Table 3.3: Analysis of MTB detection and bacterial load by Xpert MTB/RIF assay

Xpert MTB/RIF assay result	**Number of cases (n)**	**Frequency (%)**
MTB Detection (n=145)		
Positive (MTB detected)	28	19.31
Negative (MTB not detected)	117	80.69
MTB load (n=28)		
Very Low	5	17.85
Low	10	35.71
Medium	8	28.57
High	5	17.85
Rifampicin susceptibility		
Resistance	2	7.15
Sensitive	26	92.85

3.4 Assessment of culture in L-J Media

Processed specimens were inoculated into L-J slants and incubated at 37^{o}C for at least 8 weeks. Incubated L-J slants were examined once every week for contamination as well as for growth of visible mycobacterial colony. Samples were considered culture negative when no visible mycobacterial colony was observed onto the L-J slants within 8 weeks of incubation. The L-J slants those showed characteristic buff colored colony of mycobacteria (Fig-3.2b indicated by arrow) were recorded as culture positive.

Figure 3.2 (a) Culture negative (b) Culture positive

Among 145 samples, only 23 (15.86%) samples were found to be positive and 122 (84.14%) samples were negative and 5 (3.45%) were showed contamination on L-J. The overall culture results are shown in Figure-3.3

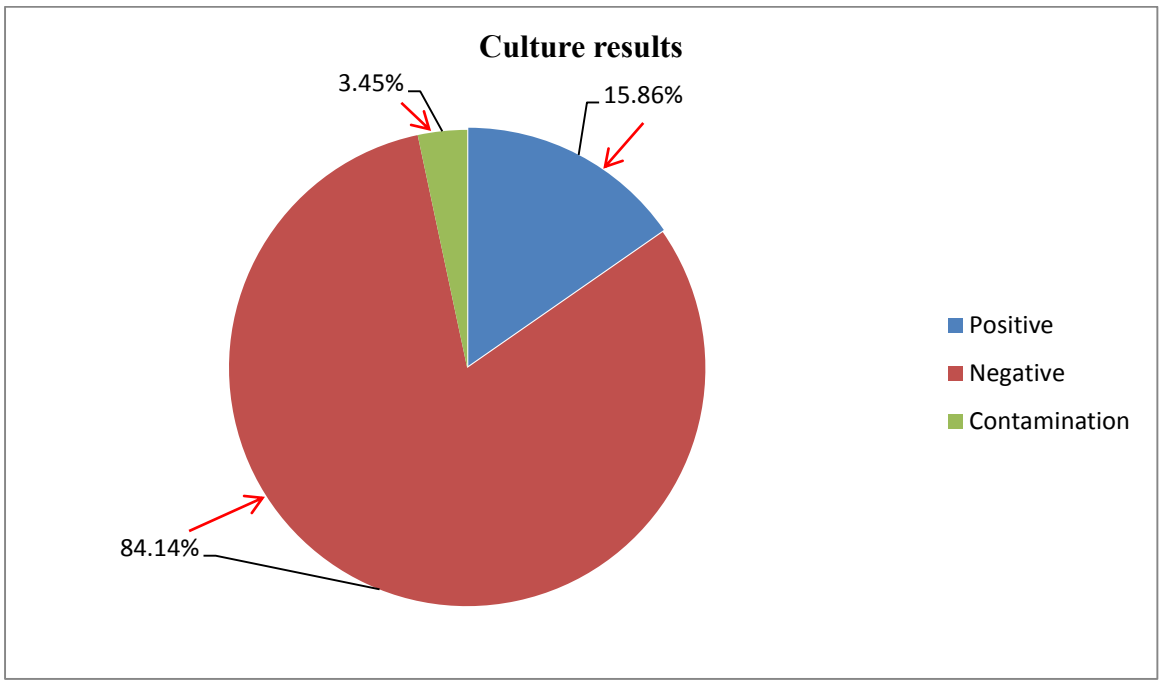

Figure 3.3: Assessment of culture.

3.5 Drug susceptibility testing

Drug susceptibility testing was performed against four first line anti-TB drugs. Among 23 culture positive cases, conventional drug susceptibility testing drugs was possible for 20 culture positive isolates. Out of these 20 cases 19 were sensitive to all the four anti TB-drugs tested. One case was found resistant to INH, RIF and STR but sensitive to ETH. The drug susceptibility patterns are shown in Table-3.4.

Table 3.4: Drug susceptibility patterns among the isolates

Drug susceptibility (n=20)	**Sensitive (%)**	**Resistant (%)**
Streptomycin	19 (95)	1 (5)
Isoniazid	19 (95)	1 (5)
Rifampicin	19 (95)	1 (5)
Ethambutol	20 (100)	0 (0)

3.6 Performance of different TB diagnostics methods

Different tests were performed for the diagnosis of TB among suspected participants and different tests showed different positivity rate. The overall positive results on different tests are shown in Table 3.6. Among 145 participants, GeneXpert test showed highest number (28) of positive among the tests performed. Twenty three were positive on LJ culture and 20 were AFB microscopy.

Among 28 GeneXpert positive cases 26 were RIF sensitive and two were RIF resistant. Among the rifampicin resistant cases, one was found sensitive in conventional DST and other one was negative on culture.

Table 3.5 Diagnostic performance of different TB diagnostics method used

Serial No.	Culture	GX Result		AFB	DST				DST summary
		GX	RIF		STR	INH	RIF	ETH	
1	+ve	+ve (High)	S	2+	S	S	S	S	All Sensitive
2	+ve	+ve (High)	S	2+	N/A	N/A	N/A	N/A	Not available
3	+ve	+ve (Low)	S	-ve	S	S	S	S	All Sensitive
4	+ve	+ve (M)	R	Scanty 3	S	S	S	S	All Sensitive
5	+ve	+ve (M)	S	1+	S	S	S	S	All Sensitive
6	+ve	+ve (Low)	S	Scanty 5	S	S	S	S	All Sensitive
7	+ve	+ve (M)	S	Scanty 3	S	S	S	S	All Sensitive
8	-ve	+ve (M)	S	3+	N/A	N/A	N/A	N/A	Not available
9	-ve	+ve (Low)	S	Scanty 5	N/A	N/A	N/A	N/A	Not available
10	+ve	+ve (High)	S	2+	N/A	N/A	N/A	N/A	Not available
11	+ve	+ve (Low)	S	Scanty 2	S	S	S	S	All Sensitive
12	+ve	+ve (M)	S	Scanty 4	S	S	S	S	All Sensitive
13	+ve	+ve (M)	S	3+	S	S	S	S	All Sensitive
14	+ve	+ve (M)	S	1+	S	S	S	S	All Sensitive
15	+ve	+ve (M)	S	1+	S	S	S	S	All Sensitive

16	-ve	+ve (Low)	S	Scanty 2	N/A	N/A	N/A	N/A	Not available
17	+ve	+ve (Low)	S	1+	S	S	S	S	All Sensitive
18	-ve	+ve (Low)	S	1+	N/A	N/A	N/A	N/A	Not available
19	+ve	+ve (High)	S	3+	S	S	S	S	All Sensitive
20	+ve	+ve (High)	S	2+	S	S	S	S	All Sensitive
21	-ve	+ve (Low)	S	Scanty 2	N/A	N/A	N/A	N/A	Not available
22	+ve	+ve (v.Low)	S	-ve	S	S	S	S	All Sensitive
23	-ve	+ve (v.Low)	S	-ve	N/A	N/A	N/A	N/A	Not available
24	+ve	+ve (Low)	S	-ve	S	S	S	S	All Sensitive
25	-ve	+ve (v.Low)	S	-ve	N/A	N/A	N/A	N/A	Not available
26	-ve	+ve (v.Low)	S	-ve	N/A	N/A	N/A	N/A	Not available
27	+ve	+ve (Low)	S	-ve	S	S	S	S	All Sensitive
28	-ve	+ve (v.Low)	R	-ve	N/A	N/A	N/A	N/A	Not available
29	+ve	-ve	ND	-ve	N/A	N/A	N/A	N/A	Not available
30	+ve	-ve	ND	-ve	R	R	R	S	STR, INH,RIF Resistant & ETH sensitive

31	+ve	-ve	ND	-ve	S	S	S	S	All Sensitive
32	+ve	-ve	ND	-ve	S	S	S	S	All Sensitive

3.7 Determination of sensitivity, specificity, PPV, NPV among the tests performed

The following formula was used for calculating sensitivity, specificity, PPV and NPV.

Table 3.6: Sensitivity and Specificity formula.

	Positive result (gold standard)	**Negative result (gold standard)**
Positive result (test)	True positive	False positive
Negative result (test)	False negative	True negative

Sensitivity: Sensitivity is the proportion of positive samples that are currently identified as positive. Sensitivity can be determined by the following equation,

Sensitivity= True positive / (False negative + True positive)

Specificity: Specificity is the proportion of negative samples that are currently identified as negative. It can be determined by the following equation.

Specificity=True negative / (False positive + True negative)

Positive predictive value is the probability that subjects with a positive screening test truly have the disease.

Negative predictive value is the probability that subjects with a negative screening test truly don't have the disease.

3.8: Comparison of Xpert MTB/RIF assay with AFB smear microscopy

In comparison with Xpert MTB/RIF and smear microscopy, all the smear positive cases (n=20) for MTB had a positive Xpert MTB/RIF test and all smear negative cases were Xpert negative. So the overall sensitivity and specificity of the Xpert MTB/RIF to detect smear positive samples were 100% (Table 3.8). The positive predictive value (PPV) and negative predictive value (NPV) were also 100%.

Table 3.7 Comparison between Xpert MTB/RIF assay and smear microscopy

Xpert MTB/RIF	AFB microscopy		Sensitivity	Specificity	PPV	NPV
	Positive	Negative				
Positive	20	0	100%	100%	100%	100%
Negative	0	125				

3.9 Comparison of diagnostic performance of Xpert MTB/RIF and AFB microscopy with conventional culture

The performance of Xpert MTB/RIF and smear microscopy were compared with the conventional culture on LJ media. Using MTB culture as the reference standard for active pulmonary tuberculosis, the sensitivity and specificity of the Xpert MTB/RIF were 82.61% and 92.62% respectively, corresponding to a PPV of 67.36% and NPV of 96.83% (Table 3.9).

Table 3.8 Comparison of diagnostic performance of Xpert and LJ culture

Xpert MTB/RIF	MTB culture		Sensitivity	Specificity	PPV	NPV
	Positive	Negative				
Positive	19	9	82.61%	92.62%	67.86%	96.58%
Negative	4	113				

The sensitivity and specificity were 65.22% and 95.90% respectively for smear microscopy, corresponding to a PPV of 75.00% and NPV of 93.60% (Table 3.10) compared to culture.

Table 3.9 Comparison of diagnostic performance of AFB smear microscopy and LJ culture

Smear Microscopy	MTB culture		Sensitivity	Specificity	PPV	NPV
	Positive	Negative				
Positive	15	5	65.22%	95.90%	75.00%	93.60%
Negative	8	117				

Chapter Four

Discussion

4. Discussion

Since the beginning of mankind, tuberculosis is an important concern of public health issue and remained untreatable for long time. Now treatments have been introduced for TB but delay of diagnosis makes it difficult to give proper treatment at right time. In Bangladesh more than 209,138 new cases, including 79% sputum smear-positive pulmonary TB cases and 45 per 100,00 population TB-related deaths occur annually [1]. The most important causes of this have been assigned to improper diagnosis of the disease.

We compared the sensitivity, specificity, PPV and NPV of different detection method. Sensitivity is the proportion of true positives that are correctly identified by a diagnostic test. It shows how good the test is at detecting a disease. The sensitivity of Xpert MTB/RIF assay 100% means that there is 100% of chance, the patient with the certain disease will be identified as positive. The results of this study demonstrated that the necessity and importance of the identification of the most effective MTB detection method that is used in diagnosis of PTB samples. The frequency of positivity of different detection method with PTB specimens was different.

AFB smear microscopy despite of being the available screening tool, it carries the risk of false negative results and incompetency to discriminate between drug susceptible and drug resistant strains of MTB. However, smear microscopy is time and labor intensive, requires specialized technicians and has limited sensitivity. In addition, smear microscopy displays limited specificity because AFB staining cannot distinguish between MTB and non-tuberculus mycobacteria. Meanwhile, culture being the gold standard for detecting MTB, proceeds for weeks up to months to yield results, and depends on sophisticated laboratory facilities and skilled technicians [86, 87]

The new molecular technique Xpert MTB/RIF assay is a useful approach for rapid detection of MTBC from clinical specimens, it has been introduced for the rapid detection of tuberculosis as well as rifampicin resistance which was endorsed by WHO in December 2010 [88]. Xpert MTB/RIF system have changed the field of TB with rapid diagnosis combined with high sensitivity and specificity results.

In this study we have compared the performance of the Xpert MTB/RIF assay with AFB microscopy and culture to determine the first-line test for tuberculosis diagnosis and evaluated the diagnostic yield of Xpert MTB/RIF assay. In our study, we found that all smear positive specimens were positive in Xpert MTB/RIF assay, which indicates 100% concordance in smear positive case detection by Xpert MTB/RIF assay. The sensitivity and specificity of the Xpert MTB/RIF in response to smear positive cases were 100%. The positive predictive value (PPV) and negative predictive value (NPV) were also 100%. It is also found that Xpert MTB/RIF assay detects 8 extra

specimens as positive which were found negative in smear microscopy. Similar findings were also found in a study conducted by Opota et. al. [89], In their study, they found 11 extra smear-negative but Xpert MTB/RIF-positive patients with a significant transmission potential and the negative predictive value was also 100%. Another study conducted by Agrwal et al. and they found the sensitivity of the Xpert assay was 86.8% with a specificity of 93.1% [90].

In the current study, culture on LJ medium was considered as the gold standard for detecting MTB. In our study, 28 specimens were Xpert positive, among them 19 were found to be positive on L-J method, with the sensitivity and specificity of 82.61% and 92.62%, PPV and NPV of 67.86% and 96.58% respectively. Moreover, we found 4 extra positive cases in LJ culture which were negative in both Xpert and AFB microscopy. These four cases were confirmed as MTB by PCR. This may be attributed to paucibacillary nature of pulmonary specimens with uneven distribution of the bacilli and formation of clumps. Moreover, during NALC-NaOH decontamination process, there are more chances of killing of viable bacteria as compared to sample processing for Xpert assay in which better homogenization and liquefaction of samples is achieved[83]. In a study conducted in Egypt, the cohort of MTB patients recorded the sensitivity of Xpert in detection of smear positive, culture positive TB was 100% and in smear negative, culture positive TB was 66.6% while its specificity in both was 100% [91]. Even with Versa TREK considered by El Hossary, as the gold standard test, the author reported the superior sensitivity of Xpert MTB/RIF to detect MTB in smear positive specimens compared to Versa TREK (100% versus 52.3%) in Saudi Arabia [92]. Since 2010, the WHO recommended the Xpert MTB/RIF(Cepheid) assay as a diagnostic tool, being reported in a multi-country study to have sensitivity of 98.2% among smear-positive, culture positive patients and 72.5% among smear negative, culture positive patients on a single direct Xpert MTB/RIF test compared to repeated smears and culture results [93]. Compared to smear microscopy and L-J culture out of 23 culture positive only 15 were positive in smear microscopy where sensitivity and specificity was 65.22% 95.90% respectively.

In this study, we also compared the conventional DST results and Xpert MTB/RIF regarding rifampicin resistance. In conventional DST, we found 19 isolates were rifampicin sensitive which were also sensitive in Xpert MTB/RIF assay indicating 100% concordance**.** In Xpert MTB/RIF assay rifampicin resistance was detected in 2 cases (7.15%), among them one was found to be sensitive in conventional DST and the other case was culture negative because during the specimen collection this patient was undertaking the anti-TB treatment. Among the culture positive cases, we found STR, INH & RIF resistant in one case which was negative in both GeneXpert and AFB smear.

In the current study, on the basis of time benefit analysis, Xpert MTB/RIF had the least turnaround time, it took about two hours. On the other hand, ZN smear takes more time with poor sensitivity. Meanwhile the gold standard, LJ culture took about 4–8 weeks with time consuming and laborious procedure. Additional 4-6 weeks require for the conventional DST. From the current study it is recognized that Gene Xpert testing may have improved case detection and time to appropriate treatment for drug-resistant cases, thereby potentially reducing transmission of drug-resistant disease, similar findings were also observed in several studies [94]. Moreover, in the present study we found that, additional cases were detected while using the other conventional methods (AFB smear and culture). These results implied that GeneXpert does not eliminate the need of conventional microscopy, culture and drug conventional susceptibility testing those are required to monitor the progression of treatment and to detect resistance to drugs other than rifampicin [90].

Chapter Five

Concluding Remarks

5. Concluding Remark

Inclusion, we found that the Xpert MTB/RIF assay is the most effective detection method for the rapid diagnosis of MTB from the pulmonary tuberculosis specimens. The automated Xpert MTB/RIF assay showed higher sensitivity and specificity than AFB smear microscopy in respiratory samples. Although culture is considered as a gold standard method but as it takes days to weeks to come positive and simultaneous detection of rifampicin resistance is not possible with it. On other side GeneXpert can be a useful diagnostic method in patients of suspected pulmonary tuberculosis, especially in the smear negative cases. Due to its rapidity and simultaneous detection of rifampicin, it would be very helpful for the anti-TB treatment in MDR patients. Accurate and early diagnosis of mycobacterial infection is important for its effective management. In the present study, Xpert MTB/RIF assay was proven an effective tool in the diagnosis of MTBC. As it require short period of time and cost effective, so it could be very beneficial in the rapid diagnosis for routine purpose in developing countries like Bangladesh.

Chapter Six

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Appendix-I

Unless otherwise mentioned, all instruments involved in media preparation were sterilized by autoclaving at 121° C for 15 minutes at 15 lbs pressure. The media used in this thesis have been given below:

Composition of Lowenstein - Jensen base. (Source: Becton Dickinson)

Ingredients	Amount (g)
Bacto Asparagine	3.6
Monopotassium phosphate	2.4
Magnesium sulfate	0.24
Magnesium citrate	0.6
Potato flour	30
Malachite green	0.4

Appendix-II

Solutions and Reagents used

1. NaOH-NALC

Composition	Amount
Sodium Hydroxide (NaOH)	2%
Sodium Citrate	2.9%
N-acetyl-L-Cysteine (NALC)	0.5%

2. Phosphate Buffer Sulphate (PBS)

Composition	Amount
Sodium Hydrogen Phosphate	0.97%
Potassium Hydrogen Phosphate	0.90%
pH should be adjusted at 6.8	

3. Normal Saline

Composition	Amount
Sodium Chloride (NaCl)	0.9%

4. Ziehl-Neelsen Staining

Carbol Fuchsin (1.0 L)

Basic Fuchsin	3.0 g	A
96% ethanol	100.0 mL	

Dissolved basic fuchsin in ethanol

Phenol crystal	5.0 g	B
D/W	100.0 mL	

Combine 10.0 mL of solution A with 90.0 mL of solution B and store in another bottle. Label bottle with name of reagent as well as preparation and expiry date. Keep in a cool place for up to three months.

25% H_2SO_4

H_2SO_4 (Concentrated) 25.0 mL

D/ W 75.0 mL

Methylene blue (100.0 mL)

Methylene blue 0.3 g

D/ W 100.0 mL

Dissolve 0.3g of Methylene blue chloride in 100 mL of distilled water. Label bottle with name, date of preparation and expire. Store at room temperature for up to three months.

5. **<u>The Xpert MTB/RIF kit (CGXMTB/RIF-10) contains the followings:</u>**

Xpert MTB/RIF cartridges with integrated reaction tubes: 10

Bead 1 (freeze-dried): 2 per cartridge

• Primers
• Probes
• KCl
• MgCl2
• HEPES, pH 8.0
• BSA (bovine serum albumin)

Bead 2 (freeze-dried): 2 per cartridge

• Polymerase
• KCl
• MgCl2
• dNTPs
• HEPES, pH 7.2
• BSA (bovine serum albumin)

Bead 3 (freeze-dried): 1 per cartridge

• Sample Processing Control (SPC) ~2000 non-infectious sample preparation control spores

Reagent 1 (Tris Buffer, EDTA, and surfactants): 4 mL per cartridge

Reagent 2 (Tris Buffer, EDTA, and surfactants): 4 mL per cartridge

Sample Reagent (Sodium Hydroxide and Isopropanol**): 10 x 8 mL bottles**

Appendix III

Instruments & Apparatus

The important instrument and apparatus used through the study are listed below:

Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Bacticinator	Germany
Bio Safety Cabinet	ESCO Class II Type A2, USA
Centrifuge	Thermo Fisher Scintific, USA
Centrifuge tube (50mL)	Eppendorf, Germany
Culture bottle	Japan
Electric balance,	OHASU, USA
Freezer (-20°C)	SHARP, New Jersey
GeneXpert MTB/RIF system	Capheid, Switzerland
Incubator	Japan
Microcentrifuge tube	Eppendorf, Germany
Micropipettes	Eppendorf, Germany
pH meter	Eppendorf, Germany
Refrigerator (4°C)	Vest frost
Slide Warmer	Premiere
Sterilizer, Model no: NDS-600D	Japan
Vortex mixer	WiseMix, USA