Molecular detection of multi-drug resistance genes in *Mycobacterium tuberculosis*

M. PHIL THESIS

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ABSTRACT

The spread of multidrug resistant tuberculosis (MDR-TB) due to emergence of multidrug resistant *M. tuberculosis* isolates has increased worldwide and reached epidemic proportions in many countries. The emergence of MDR-TB has become a major threat for TB control in Bangladesh. MDR-TB is defined as resistant to at least rifampicin and isoniazid, which are the backbone of short-course chemotherapy for tuberculosis. Mutations in the 81-bp core region of *rpoB* were reported to be responsible for resistance in at least 95% of isolates. Resistance to isoniazid is due to mutations at one of two sites, in either the *katG* or *inhA* genes. The culture-based methods for detection of *M. tuberculosis* infection and drug susceptibility testing (DST) usually take more than 1 month due to the slow growth of this bacterium. The use of molecular methods for the identification of mutations in the resistance genes may offer the means for rapid screening of the drug resistance among the *M. tuberculosis* isolates and initiation of early treatment. In this study, we evaluated rapid detection of multidrug resistant strains using the molecular techniques from MTB strains and sputum directly. For this purpose, a total of 55 sputum samples were collected from 55 patients of National Tuberculosis Reference Laboratory (NTRL), Mohakhali, Dhaka- 1212, Bangladesh. Samples were tested for microscopic examination, conventional culture method, drug susceptibility testing, Gene Xpert assay and PCR amplification followed by DNA sequencing. All 55 samples (36 from MTB strain, 19 from direct sputum) were subjected to *rpoB* gene amplification and 21 samples (12 from MTB strain, 9 from direct sputum) for *katG* gene amplification by PCR. Then the samples were subjected to DNA sequencing for *rpoB* and *katG* gene mutation analysis. RIF-associated mutations were detected in (44/55) samples (80%), of which, mutations at codons 531 (54.54%), 526 (12.73%) and 516 (9.09%) were observed. Sixteen samples (76.19%) out of 21 had isoniazid associated mutations at codon 315 in the *katG* gene. Considering conventional DST as gold standard, sensitivity and specificity of DNA sequencing for the detection of Rif- resistance were 98% and 100%; whereas isoniazid resistance were 94% and 100%. Comparison between Xpert MTB/RIF and DST results showed that eight samples were sensitive to rifampicin by Genexpert assay, but not on DST. The reason may be due to the selection of resistant strains in the antibiotic added media for a prolonged incubation period. Therefore, false-positive results of rifampicin resistance were observed in DST.

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ABBREVIATIONS

^{o}C – Degree Centigrade
AFB – Acid-fast bacilli
ag - Attogram
bp – Base pair
CDC – Centers for Disease Control and Prevention
cm – Centimeter
DOTS – Directly Observed Treatment Short course
DNA – Deoxyribonucleic acid
dNTPs – Deoxynucleoside tri-phosphates
fg - Femtogram
g – Gram
h – Hour
INH – Isoniazid
RIF – Rifampicin
SM – Streptomycin
EMB – Ethambutol
IS – Insertion Sequence
kDa – Kilo Dalton
L – Liter
L-J – Lowenstein-Jensen
M – Molar
MDR – Multidrug Resistant
mg – Milligram
mM – Millimolar
mL – Milliliter
MDR-TB – Multidrug Resistant Tuberculosis
NA – Nucleic Acid
NaCl – Sodium Chloride
NALC – N-acetyl-L-cystein
NaOH – Sodium hydroxide
ng - Nanogram
NIDCH – National Institute of Disease of Chest and Hospital
NTRL – National Tuberculosis Reference Laboratory
No. – Number
PCR – Polymerase Chain Reaction
pg – Picogram
pmol – picomole
rpm – Revolutions per minute
WHO – World Health Organization
Z-N – Ziehl-Neelsen
µg – Microgram
µL – Microliter

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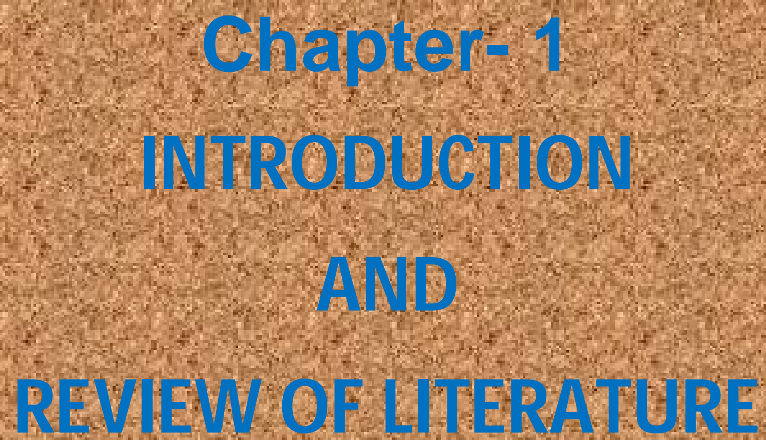

Chapter- 1

INTRODUCTION AND REVIEW OF LITERATURE

1.0 INTRODUCTION AND REVIEW OF LITERATURE

1.1 General Introduction

Tuberculosis infection is a global problem due to the high-risk of human transmission, morbidity and mortality. Nearly two million deaths and more than 9 million new cases are reported each year, making *Mycobacterium tuberculosis* the primary cause of death from a single pathogen (Pheiffer *et al*., 2005). About 80% of the global TB burden is in low-income countries, where pulmonary disease and transmission are serious public health problems (Kent and Kubica, 1985). As per the WHO Global TB Report 2013, there were an estimated 8.6 million incident cases of TB globally in 2012 with 1.3 million deaths (WHO, 2013).

Resistance of the organisms to TB drugs is a major public health problem that threatens the progress made in TB control worldwide. Drug resistance arises due to improper use of antibiotics in chemotherapy of drug susceptible organisms (WHO, 2013). In recent years, drug-resistant TB has emerged, largely due to delays in treatment, gaps in treatment protocol, and ineffective or delayed drug-susceptibility testing (Werf *et al*., 2012). Although major progress has been made to reduce global incidence of drug-susceptible tuberculosis (TB), the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB over the past decade presents an unprecedented public health challenge to which countries of concern are responding far too slowly. Indeed, a recent WHO TB surveillance report indicates the highest global level of drug-resistance ever recorded, which affected disproportionately developing countries with an estimated 440,000 MDR-TB cases worldwide resulting in 150,000 deaths in 2009 (WHO, 2010).

The emergence and spread of drug-resistant strains of *M. tuberculosis*, especially multidrug-resistant (MDR) strains, are serious threats to the control of tuberculosis and comprise an increasing public health problem (Zumia and Grange**,** 2001). Patients infected with MDR strains, which are defined as strains resistant to both rifampin

(RIF) and isoniazid (INH), are difficult to cure and are more likely to remain sources of infection for a longer period of time than are patients with drug-susceptible strains (Zumia and Grange, 2001). Multi-drug resistant tuberculosis (MDR-TB) is defined by the resistance of the bacillus to the most powerful TB drugs, isoniazid and rifampin, while extensively drug-resistant TB (XDR-TB) is also resistant to some second line drugs (WHO, 2009; Abdel and Wright, 2006) .

In a population of *M. tuberculosis*, resistance to anti-TB drugs is due to spontaneous chromosomal mutations that occur at a relatively low frequency, 10−6 to 10−8 mycobacterial replications. Individual nucleotide changes (point mutations) confer resistance to single drugs, and the sequential accumulation of these mutations in different genes involved results in multi-drug resistance (Ozturk *et al.*, 2005; Soini and Musser, 2001). The development of drug resistance is the result of selection of random genetic mutations in genes associated with drug resistance. The mechanism of resistance to RIF involves missense mutations, small deletions or insertions in the *rpoB* gene encoding the β-subunit of RNA polymerase. Studies from diverse countries have shown that 95–96% of all RIF-resistant isolates have mutations within an 81 bp 'core region' of *rpoB* (Bártfai *et al.*, 2001; Cavusoglu *et al.*, 2002; Mani *et al.*, 2001). In contrast, but perhaps not unexpectedly given its highly complex mechanism of action, the mutations causing INH resistance are located in more than three genes and regions (Barry *et al.*, 1998; Slayden and Barry, 2000). Depending on the sample set analysed, it has been reported that 50–95% of INH-resistant strains contain mutations in codon 315 of the *katG* gene encoding the catalase-peroxidase (Mokrousov *et al.*, 2002; Musser *et al.*, 1996; Piatek *et al.*, 2000) and 20–35% harbour mutations in the *inhA* promoter region (Musser *et al.*, 1996; Piatek *et al.*, 2000; Telenti *et al.*, 1997).

As treatment of MDR-TB is difficult and its transmission is rapid, the need for developing a rapid diagnostic test to detect MDR-TB and effectively prevent the spread of MDR-TB in the community is paramount to avoid an epidemic. The gold standard for diagnosing MDR-TB is culture-based drug susceptibility testing (DST). However, the long replication time of *M. tuberculosis* delays the time to reach a diagnosis via DST, and

performing DST requires appropriate laboratory equipment and skilled personnel, making it difficult to adapt DST as a routine lab test. Drug susceptibility testing by the conventional solid medium culture method is highly sensitive and specific but extremely slow, due to the slow growth of *M. tuberculosis*. Liquid culture methods can reduce the turnaround time but require specialized instrumentation and reagents and are not feasible in most resource-limited settings.

New molecular diagnostic methods represent a potentially rapid and sensitive alternative to conventional diagnostics. There has been an increasing interest in the development of rapid molecular methods for detection of those mutations associated with drug resistance to replace the conventional phenotypic drug-susceptibility testing (DST). PCR-based methods with readouts including line probe assays, analysis with DNA microarrays and real-time PCR have been the focus of much recent effort in developing molecular DST methods to determine resistance to RIF and INH (Cirillo *et al.*, 2004; Edwards *et al.*, 2001; El-Hajj *et al.*, 2001). Various molecular methods have been used to identify the mutations in *rpoB*, *katG*, *rpsL*, *rrs*, *embB*, *pncA*, *gyrA*, and other genes (Fluit *et al*., 2001). Among these methods, DNA sequencing is the most direct and reliable for detection of both known and novel mutations. DNA sequencing is considered the gold standard for nucleic acid identification and mutation detection. DNA sequencing technique is a rapid, conclusive and more advantageous technique than the conventional susceptibility testing for detection of rifampicin resistance in terms of the risk involved and time consumption (Deepa *et al*., 2005).

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1.2 Review of Literature

1.2.1 Tuberculosis

Tuberculosis (TB) is a contagious airborne disease caused by the bacteria *Mycobacterium tuberculosis* (MTB). TB is widespread and deadly, and causes the highest number of deaths worldwide. An estimated of 8.7 million of new cases of TB and 1.4 million deaths occurs per year (WHO, 2012). About 90% of those infected with *M. tuberculosis* have asymptomatic, latent TB infections (Skolnik and Richard, 2011), with only a 10% lifetime chance that the latent infection will progress to overt, active tuberculous disease (Arch *et al*., 2009). If effective treatment is not given, the death rate for active TB cases is up to 66% (WHO, 2010). In those with HIV, the risk of developing active TB increases to nearly 10% a year (Arch *et al*., 2009).

1.2.1.1 Causes of tuberculosis

Tuberculosis or TB (short for tubercle bacillus), is a widespread, and in many cases fatal, infectious disease caused by various strains of mycobacteria, usually *M. tuberculosis* (Kumar *et al*., 2007). *M. tuberculosis* is a pathogenic bacterial species in the family Mycobacteriaceae and the causative agent of most cases of tuberculosis (Ismael and Ray, 2004). It was first described on 24 March 1882 by Robert Koch, who subsequently received the Nobel Prize in physiology or medicine for this discovery in 1905; the bacterium is also known as "Koch's bacillus"(Robert Koch and Tuberculosis, 2008).

The *M. tuberculosis* complex includes four other TB-causing mycobacteria: *M. bovis*, *M. africanum, M. canetti,* and *M. microti* (Soolingen *et al*., 1997).

M. africanum is not widespread, but in parts of Africa it is a significant cause of tuberculosis (Niemann *et al*., 2002; Niobe *et al*., 2003). *M. bovis* was once a common cause of tuberculosis, but the introduction of pasteurized milk has largely eliminated this as a public health problem in developed countries (Kumar *et al*., 2007; Thoen *et al*., 2006). *M. canetti* is rare and seems to be limited to Africa. *M. microti* is mostly seen in

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immunodeficient people, although it is possible that the prevalence of this pathogen has been underestimated (Panteix *et al*., 2010).

1.2.1.1.1 General characteristics of *Mycobacterium tuberculosis*

The physiology of *M. tuberculosis* is highly aerobic and requires high levels of oxygen. Primarily a pathogen of the mammalian respiratory system, it infects the lungs (Ismael and Ray, 2004). Because of MTB's large metabolic oxygen requirement, it is typically found in oxygen rich areas such as the respiratory system. Although it generally starts in the lungs, it may spread to other parts of the body via the lymphatic system or blood vessels (Tuberculosis, 2005).

M. tuberculosis divides every 15–20 hours, which is extremely slow compared to other bacteria, which tend to have division times measured in minutes (Escherichia coli can divide roughly every 20 minutes). It is a small bacillus that can withstand weak disinfectants and can survive in a dry state for weeks. Its unusual cell wall, rich in lipids (e.g., mycolic acid), is likely responsible for this resistance and is a key virulence factor (Murray *et al*, 2005).

M. tuberculosis is a fairly large nonmotile rod-shaped bacterium distantly related to the Actinomycetes. Many non pathogenic mycobacteria are components of the normal flora of humans, found most often in dry and oily locales. The rods are 2-4 µm in length and 0.2-0.5 µm in width.

1.2.1.1.1.1 Cell Wall Structure

The cell wall structure of *M. tuberculosis* deserves special attention because it is unique among procaryotes, and it is a major determinant of virulence for the bacterium. The cell wall complex contains peptidoglycan, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of MTB's cell wall consists of three major components, mycolic acids, cord factor, and wax-D (Alderwick *et al*., 2007; Brennan, 2003).

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Mycolic acids are unique alpha-branched lipids found in cell walls of Mycobacterium and Corynebacterium. They make up 50% of the dry weight of the mycobacterial cell wall. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic Acids are thought to be a significant determinant of virulence in MTB. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule (Alderwick *et al*., 2007).

Cord factor (trehalose 6, 6'- dimycolate; TDM) is a surface glycolipid, which causes MTB to grow in vitro in serpentine cords. Cord factor is an inhibitor of PMN (Polymorphonuclear neutrophil) migration and is also toxic to mammalian cells. It is most abundantly produced on the surface of virulent strains of MTB while avirulent strains do not have cord factor (Asano *et al*., 1993).

1.2.1.1.1.2 Genome structure

Genome of *M. tuberculosis* has been sequenced with hopes of gaining further understanding of how to defeat the infamously successful pathogens. The widely used reference strain *M. tuberculosis* H37Rv was firstly sequenced in 1998 (Cole *et al*., 1998).

Genome size of *M. tuberculosis* is 4,411,522 base pairs long with ~4000 genes distributed fairly evenly between the two strands and accounting for >91% of the potential coding capacity, 6 pseudogenes, and a relatively high G+C content of 65.6%. At 4.4 Mbp, *M. tuberculosis* is one of the largest known bacterial genomes, coming in just short of *E. coli*, and a distant third to *Streptomyces coelicolor*.

Genes were classified into 11 broad functional groups and, today, precise or putative functions can be attributed to 52%, with the remaining 48% being conserved hypotheticals or unknown (Camus *et al*., 2002). 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 56 copies of IS elements belonging to the well-known IS3, IS5, IS21, IS30, IS110, IS256 and ISL3 families, as well as a new IS family, IS1535, that appears to employ a

frameshifting mechanism to produce its transposase. IS*6110*, a member of the IS3 family, is the most abundant element and has played an important role in genome plasticity (Cole, 2002). About 250 genes are involved in fatty acid metabolism, with 39 of these involved in the polyketide metabolism generating the waxy coat. Such large numbers of conserved genes show the evolutionary importance of the waxy coat to pathogen survival. Approximately 10% of the coding capacity is taken up by two clustered gene families that encode acidic, glycine-rich proteins. These proteins have a conserved N-terminal motif, deletion of which impairs growth in macrophages and granulomas (Glickman and Jacobs, 2001).

1.2.1.1.1.3 Staining characteristics

MTB is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. If a Gram stain is performed on MTB, it stains very weakly Gram-positive or not at all (cells referred to as "ghosts").

Using histological stains on expectorated samples from phlegm (also called "sputum"), scientists can identify MTB under a regular (light) microscope. Since MTB retains certain stains even after being treated with acidic solution, it is classified as an acid-fast bacillus (AFB) (Kumar *et al*., 2007; Madison, 2001). The most common acid-fast staining techniques are the Ziehl–Neelsen stain, which dyes AFBs a bright red that stands out clearly against a blue background (Medical Laboratory Science: Theory and Practice, 2000). Other ways to visualize AFBs include an auramine-rhodamine stain and fluorescent microscopy.

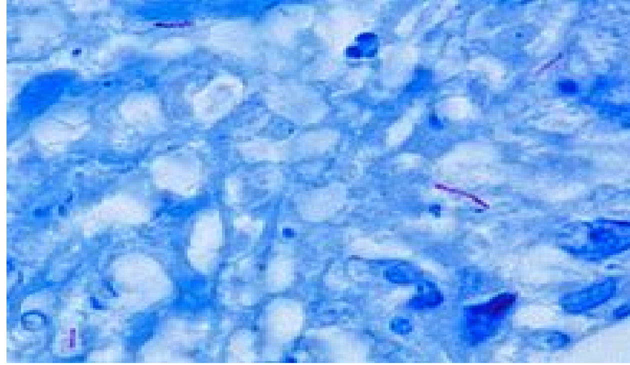

FIG 1.1 *M. tuberculosis* (stained red) in tissue (blue)

1.2.1.1.1.4 Growth characteristics

MTB are aerobes. Their reproduction is enhanced by the presence of 5-10% CO_2 in the atmosphere. They are grown on culture media with high lipid content, e.g. Lowenstein-Jensen (LJ) medium. The generation time of TB is approximately 12-18 hours, so that cultures must be incubated for three to six weeks at 37ºC until proliferation becomes microscopically visible (Allen and Mitchison, 1992). Broth-based culture systems to improve the speed and sensitivity of detection have been developed (Hanna *et al*., 1995). In AFB smear-positive specimens, the BACTEC system can detect *M. tuberculosis* in approximately eight days (compared to approximately 14 days for smear-negative specimens (*Morgan et al*., 1983).

FIG 1.2 Colonies of *M. tuberculosis* on Lowenstein-Jensen medium

1.2.1.2 Pathophysiology

Inhalation of *M. tuberculosis* leads to one of four possible outcomes:

- Immediate clearance of the organism
- Latent infection
- The onset of active disease (primary disease)
- Active disease many years later (reactivation disease)

Among individuals with latent infection, and no underlying medical problems, reactivation disease occurs in 5 to 10 percent of cases (Comstock, 1982). The risk of reactivation is markedly increased in patients with HIV (National action plan to combat multidrug-resistant tuberculosis, 1992) .These outcomes are determined by the interplay of factors attributable to both the organism and the host.

Primary disease

Among the approximately 10% of infected individuals who develop active disease, about half will do so within the first two to three years and are described as developing rapidly progressive or primary disease.

The tubercle bacilli establish infection in the lungs after they are carried in droplets small enough (5 to 10 microns) to reach the alveolar spaces. If the defense system of the host fails to eliminate the infection, the bacilli proliferate inside alveolar macrophages and eventually kill the cells. The infected macrophages produce cytokines and chemokines that attract other phagocytic cells, including monocytes, other alveolar macrophages and neutrophils, which eventually form a nodular granulomatous structure called the tubercle. If the bacterial replication is not controlled, the tubercle enlarges and the bacilli enter local draining lymph nodes. This leads to lymphadenopathy, a characteristic clinical manifestation of primary tuberculosis (TB).

The bacilli continue to proliferate until an effective cell-mediated immune (CMI) response develops, usually two to six weeks after infection. Failure by the host to mount an effective CMI response and tissue repair leads to progressive destruction of the lung. Tumour necrosis factor (TNF)-alpha, reactive oxygen and nitrogen intermediates and the

contents of cytotoxic cells (granzymes, perforin) may all contribute to the development of caseating necrosis that characterize a tuberculous lesion. Unchecked bacterial growth may lead to haematogenous spread of bacilli to produce disseminated TB. Disseminated disease with lesions resembling millet seeds is termed miliary TB. Bacilli can also spread by erosion of the caseating lesions into the lung airways –and the host becomes infectious to others. In the absence of treatment, death ensues in 80% of cases (Barnes *et al*, 1928).The remaining patients develop chronic disease or recover. Chronic disease is characterized by repeated episodes of healing by fibrotic changes around the lesions and tissue breakdown. Complete spontaneous eradication of the bacilli is rare.

Reactivation disease

Reactivation TB results from proliferation of a previously dormant bacterium seeded at the time of the primary infection. Among individuals with latent infection and no underlying medical problems, reactivation disease occurs in 5 to 10% (Comstock, 1982). Immunosuppression is associated with reactivation TB, although it is not clear what specific host factors maintain the infection in a latent state and what triggers the latent infection to become overt.

The disease process in reactivation TB tends to be localized (in contrast to primary disease): there is little regional lymph node involvement and less caseation. The lesion typically occurs at the lung apices, and disseminated disease is unusual unless the host is severely immunosuppressed. It is generally believed that successfully contained latent TB confers protection against subsequent TB exposure (Heimbeck, 1930).

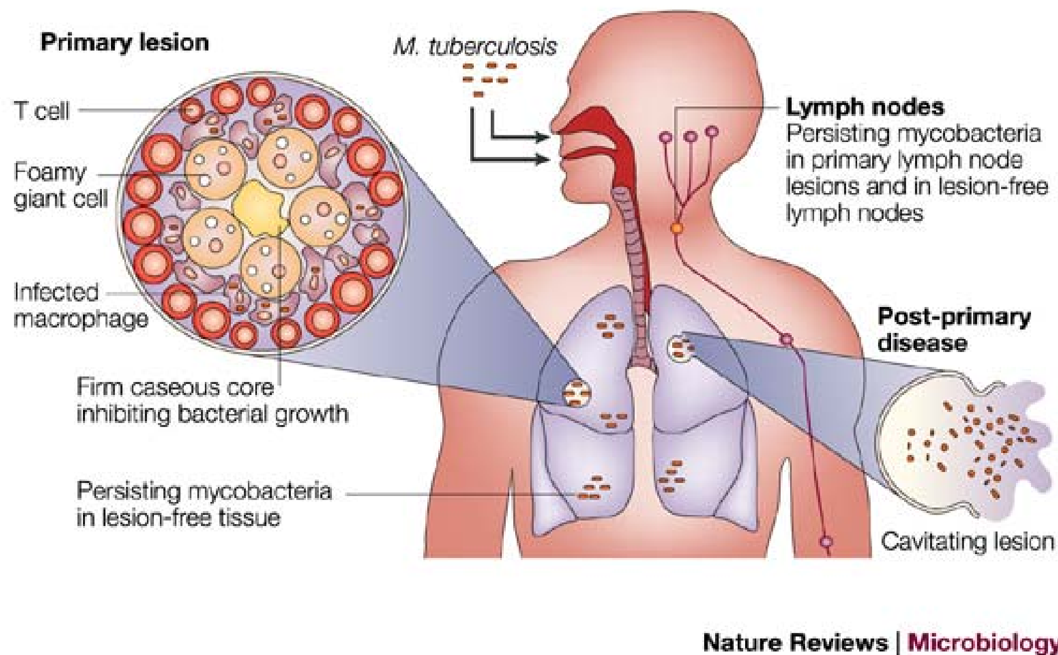

FIG 1.3 Pathophysiology of tuberculosis

1.2.1.3 Signs and symptoms

Tuberculosis may infect any part of the body, but most commonly occurs in the lungs (known as pulmonary tuberculosis). Extrapulmonary TB occurs when tuberculosis develops outside of the lungs, although extrapulmonary TB may coexist with pulmonary TB (Dolin *et al*., 2010).

General signs and symptoms include fever, chills, night sweats, loss of appetite, weight loss, and fatigue (Dolin *et al*., 2010). Significant finger clubbing may also occur (Peter *et al*., 2005).

Pulmonary Tuberculosis

Tuberculosis infection most commonly involves the lungs (in about 90% of cases) (Lawn *et al*., 2011; Behera, 2010). Symptoms may include chest pain and a prolonged cough producing sputum. About 25% of people may not have any symptoms (i.e. they remain "asymptomatic"). Occasionally, people may cough up blood in small amounts, and in very rare cases, the infection may erode into the pulmonary artery, resulting in

massive bleeding (Rasmussen's aneurysm). Tuberculosis may become a chronic illness and cause extensive scarring in the upper lobes of the lungs. The upper lung lobes are more frequently affected by tuberculosis than the lower ones (Dolin *et al*, 2010).

Extrapulmonary Tuberculosis

Extrapulmonary TB occurs more commonly in immunosuppressed persons and young children. In those with HIV, this occurs in more than 50% of cases (Golden and Vikram, 2005). Notable extrapulmonary infection sites include the pleura (in tuberculous pleurisy), the central nervous system (in tuberculous meningitis), the lymphatic system (in scrofula of the neck), the genitourinary system (in urogenital tuberculosis), and the bones and joints (in Pott's disease of the spine), among others. When it spreads to the bones, it is also known as "osseous tuberculosis" (Kabra *et al*., 2006), a form of osteomyelitis (Kumar *et al*., 2007). Sometimes, bursting of a tubercular abscess through skin results in tuberculous ulcer (Manual of Surgery, 2008). An ulcer originating from nearby infected lymph nodes is painless, slowly enlarging and has an appearance of "wash leather" (Burkitt and George, 2007).

A potentially more serious, widespread form of TB is called "disseminated" TB, commonly known as miliary tuberculosis (Dolin *et al*., 2010). Miliary TB makes up about 10% of extrapulmonary cases (Ghosh *et al*., 2008).

1.2.1.4 Transmission of tuberculosis

When people with active pulmonary TB cough, sneeze, speak, sing or spit, they expel infectious aerosol droplets 0.5 to 5.0 µm in diameter. A single sneeze can release up to 40,000 droplets (Cole and Cook, 1998). Each one of these droplets may transmit the disease, since the infectious dose of tuberculosis is very small (Nicas *et al*., 2005). The probability of transmission from one person to another depends upon several factors, including the number of infectious droplets expelled by the carrier, the effectiveness of ventilation, the duration of exposure, the virulence of the *M. tuberculosis* strain, the level of immunity in the uninfected person, and others (CDC, 2011). The cascade of person-to-person spread can be circumvented by effectively segregating those with active ("overt")

TB and putting them on anti-TB drug regimens. After about two weeks of effective treatment, subjects with nonresistant active infections generally do not remain contagious to others (Ahmed and Hasnain, 2011).

1.2.1.5 Risk factors

A number of factors make people more susceptible to TB infections. The most important risk factor globally is HIV; 13% of all people with TB are infected by the virus (WHO, 2011). This is a particular problem in sub-Saharan Africa, where rates of HIV are high (WHO, 2006; Chaisson *et al*., 2008). Of people without HIV who are infected with tuberculosis, about 5–10% develop active disease during their lifetimes; in contrast, 30% of those coinfected with HIV develop the active disease (Peter *et al*., 2005). Tuberculosis is closely linked to both overcrowding and malnutrition, making it one of the principal diseases of poverty (Lawn *et al*., 2011). Those at high risk thus include: people who inject illicit drugs, inhabitants and employees of locales where vulnerable people gather (e.g. prisons and homeless shelters), medically underprivileged and resource-poor communities, high-risk ethnic minorities, children in close contact with high-risk category patients, and health care providers serving these patients (Griffith and Kerr, 1996).

Those who smoke cigarettes have nearly twice the risk of TB than nonsmokers (Smit *et al*., 2010). Other disease states can also increase the risk of developing tuberculosis. These include alcoholism (Lawn *et al*., 2011) and diabetes mellitus (threefold increase) (Restrepo, 2007). Certain medications, such as corticosteroids and infliximab (an anti-αTNF monoclonal antibody) are becoming increasingly important risk factors, especially in the developed world (Lawn *et al*., 2011) .There is also a genetic susceptibility (Möller *et al*., 2010), for which overall importance remains undefined (Lawn *et al*., 2011).

1.2.1.6 Diagnosis of Tuberculosis

Tuberculosis is diagnosed by finding *M. tuberculosis* bacteria in a clinical specimen taken from the patient. While other investigations may strongly suggest tuberculosis as the diagnosis, they cannot confirm it. A complete medical evaluation for tuberculosis (TB) must include a medical history, a physical examination, a chest X-ray and microbiological examination (of sputum or some other appropriate sample). It may also include a tuberculin skin test, other scans and X-rays, surgical biopsy.

Medical history

The medical history includes obtaining the symptoms of pulmonary TB: productive, prolonged cough of three or more weeks, chest pain, and hemoptysis. Systemic symptoms include low grade remittent fever, chills, night sweats, appetite loss, weight loss, easy fatigability and production of sputum that starts out mucoid but changes to purulent (Kumar *et al*., 2007). Other parts of the medical history include prior TB exposure, infection or disease; past TB treatment; demographic risk factors for TB; and medical conditions that increase risk for TB disease such as HIV infection.

Physical examination

A physical examination is done to assess the patient's general health and find other factors which may affect the TB treatment plan. It cannot be used to confirm or rule out TB. However, certain findings are suggestive of TB. For example, blood in the sputum, significant weight loss and drenching night sweats may be due to TB.

A definitive diagnosis of tuberculosis can only be made by culturing *M. tuberculosis* organisms from a specimen taken from the patient (most often sputum, but may also include pus, CSF, biopsied tissue, etc). A diagnosis made other than by culture may only be classified as "probable" or "presumed". For a diagnosis negating the possibility of tuberculosis infection, most protocols require that two separate cultures both test negative (Kumar *et al*., 2007).

Sputum

Sputum smears and cultures should be done for acid-fast bacilli if the patient is producing sputum (Kumar *et al*., 2007). The preferred method for this is fluorescence microscopy (auramine-rhodamine staining), which is more sensitive than conventional Ziehl-Neelsen staining (Steingart *et al*., 2006).

In cases where there is no spontaneous sputum production, a sample can be induced, usually by nebulized inhalation of a saline or saline with bronchodilator solution. A comparative study found that inducing three sputum samples is more sensitive than three gastric washings (Brown *et al*., 2007).

Alternative sampling

In patients incapable of producing a sputum sample, common alternative sample sources for diagnosing pulmonary tuberculosis include gastric washings, laryngeal swab, bronchoscopy (with bronchoalveolar lavage, bronchial washings, and/or transbronchial biopsy), and fine needle aspiration (transtracheal or transbronchial). In some cases, a more invasive technique is necessary, including tissue biopsy during mediastinoscopy or thoracoscopy.

PCR

Other mycobacteria are also acid-fast. If the smear is positive, PCR or gene probe tests can distinguish *M. tuberculosis* from other mycobacteria. Even if sputum smear is negative, tuberculosis must be considered and is only excluded after negative cultures.

Others

Many types of cultures are available (Drobniewski *et al*., 2003). Traditionally, cultures have used the Löwenstein-Jensen (LJ), Kirchner, or Middlebrook media (7H9, 7H10, and 7H11). A culture of the AFB can distinguish the various forms of mycobacteria, although results from this may take four to eight weeks for a conclusive answer.

New automated systems that are faster include the MB/BacT, BACTEC 9000, VersaTREK, and the Mycobacterial Growth Indicator Tube (MGIT). The Microscopic Observation Drug Susceptibility assay culture may be a faster and more accurate method (Moore *et al*., 2006).

Immunological test

ALS Assay

Antibodies from lymphocyte secretion or antibody in lymphocyte supernatant or ALS Assay is an immunological assay to detect active diseases like tuberculosis, cholera, typhoid etc. Recently, ALS assay nods the scientific community as it is rapidly used for diagnosis of tuberculosis. The principle is based on the secretion of antibody from in vivo activated plasma B cells found in blood circulation for a short period of time in response to TB-antigens during active TB infection rather than latent TB infection.

Tuberculin skin test

Two tests are available: the Mantoux and Heaf tests.

The Mantoux test for TB involves intradermally injecting PPD (Purified Protein Derivative) tuberculin and measuring the size of induration 48-72 hours later. If a person has had a history of a positive tuberculin skin test, another skin test is not needed. There is disagreement on the use of the Mantoux test on people who have been immunized with BCG. The Heaf test was used in the United Kingdom until 2005, and is graded on a four point scale. The Mantoux test is now used.

Nucleic acid amplification tests (NAAT)

This is a heterogeneous group of tests that use either the polymerase chain reaction (PCR) technique or transcription mediated amplification (TMA) or other forms of nucleic acid amplification methods to detect mycobacterial nucleic acid. These tests vary in which nucleic acid sequence they detect and vary in their accuracy. The two most common commercially available tests are the amplified *M. tuberculosis* direct test (MTD, Gen-Probe) and Amplicor (Roche Diagnostics).

Full blood count

Although a full blood count is never diagnostic, normocytic anemia and lymphopenia are common. Neutrophilia is rarely found [iron deficiency anemia may develop with isoniazid treatment]. Urea and electrolytes are usually normal, although hypocalcemia and hyponatremia are possible in tuberculous meningoencephalitis due to SIADH. In advanced disease, hypoalbuminemia, hyperproteinemia, and hyperglobulinemia may be present ("Tibione in the Treatment of Tuberculosis Activity, Dosage and Toxic Manifestations").

1.2.1.7 Treatment of tuberculosis

TB is highly contagious during the active stage of the disease and can infect an individual through inhalation of as few as 10 *M. tuberculosis* (MTB) bacteria. In the intensive phase of TB treatment, the antibiotics mainly kill rapidly growing bacteria, which causes rapid sputum conversion, and the eradication of clinical symptoms. However, in order to kill the persistent or slow growing strains of MTB, the continuation phase of the treatment is essential.

The waxy, hydrophobic cell wall of MTB gives it the ability to survive long exposure to substances such as acids, detergents, oxidative bursts, and antibiotics. In fact, the typical “short” treatment of MTB involves a four antibiotic treatment for two months and then a two antibiotic treatment for an additional four months. The antibiotics involved are isoniazid, rifampicin, pyrazinamide and ethambutol. The standard therapy for active TB is a six-month program with two months dedicated to isoniazid, rifampin, and pyrazinamide and four months of isoniazid, rifamate, and rimactane (Sharma, 2005; Swierzewski, 2007). Ethambutol or streptomycin is also added until the patient’s drug sensitivity is known (Swierzewski, 2007). This long period of treatment is a direct result of the slow reproductive time and resistant cell wall of MTB (Sharma, 2005; Todar, 2008).

FIG 1.4 First-line anti-TB agents from left to right: INH, RIF, PZA, and EMB form the core of initial treatment regimens

1.2.2 Multidrug-resistant tuberculosis

TB can be treated effectively by using first line drugs (FLD) isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (SM). However, this first-line therapy often fails to cure TB for several reasons. Relapse and the spread of the disease contribute to the emergence of drug resistant bacteria. Two types of drug resistant MTB strains are currently recognized.

The emergence of multidrug-resistant TB (MDR-TB), i.e. which is resistant to at least isoniazid (INH) and rifampicin (RIF), is of great concern, because it requires the use of second-line drugs that are difficult to procure and are much more toxic and expensive than FLDs (Espinal *et al*., 2001). Therefore, the detection and treatment of drug susceptible or single drug resistant TB is an important strategy for preventing the emergence of MDR-TB (Masjedi *et al*., 2006).

M. tuberculosis strains with extensively drug resistant-TB (XDR-TB), that is resistant to either isoniazid or rifampicin (like MDR tuberculosis), any fluoroquinolone, and at least one of three second-line antituberculosis injectable drugs—i.e., capreomycin, kanamycin, and amikacin have also been reported (Eker *et al*., 2008).

First- and second-line drugs and mechanisms of drug resistance are presented in table 1.1 (Almeida and Palomino, 2011).

Drug	Gene	Role of gene product
Isoniazid	katG	catalase/peroxidase
	inhA	enoyl reductase
	ahpC	alkyl hydroperoxide reductase
Rifampicin	rpoB	β-subunit of RNA polymerase
Pyrazinimide	pncA	PZase
Streptomycin	rpsL	S12 ribosomal protein
	rrs	16S rRNA
	gidB	7-methylguanosine methyltransferase
Ethambutol	embB	arabinosyl transferase
Fluoroquinolones	gyrA/gyrB	DNA gyrase
Kanamycin	rrs	16S rRNA
	eis	aminoglycoside acetyltransferase
Amikacin	rrs	16S rRNA
Capreomycin	rrs	16S rRNA
	tylA	rRNA methyltransferase
Ethionamide	inhA	enoyl reductase
p-aminosalicylic acid	thyA	thymidylate synthase A

TABLE 1.1 First- and second-line drugs and genes associated with drug resistance

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1.2.2.1 Molecular basis of antimicrobial resistance in tuberculosis

M. tuberculosis and other members of the *M. tuberculosis* complex use several strategies to resist the action of antimicrobial agents. First, the mycobacterial hydrophobic cell wall that results in decreased permeability to many compounds (Jarlier and Nikaido, 1994; Lee *et al*.,1996); active drug efflux systems, and degrading or inactivating enzymes, and the genes that are associated with these functions, have been found in *M. tuberculosis* (Kwon *et al*., 1995; Cole *et al*., 1998).

However, genetic studies have shown that resistance of *M. tuberculosis* to antimycobacterial drugs is the consequence of spontaneous mutations in genes that encode either the target of the drug, or enzymes that are involved in drug activation.

In addition, resistance to a single drug may involve multiple genetic alterations locating to different genes, as well as multiple genetic alterations within a single gene. Presumably, this accumulation of various resistance mutations, all associated with resistance to a single drug, will either affect (increase) phenotypic resistance or ameliorate the fitness cost associated with a defined resistance mutation (Buttger, 2011).

Resistance mechanisms for the first-line anti-tuberculous agents are summarized below

Isoniazid

Isoniazid (INH) is one of the most effective and specific antituberculosis drugs, which has been a key to treatment since its introduction in 1952 (Bernstein *et al*., 1952). INH is only active against growing tubercle bacilli, and is not active against non-replicating bacilli or under anaerobic conditions. INH enters the mycobacterial cell by passive diffusion (Bardou *et al*., 1998). The most significant adverse reactions associated with isoniazid administration are hepatotoxicity and neurotoxicity.

Mutations in several genes, including *katG*, *ahpC*, and *inhA*, have all been associated with isoniazid resistance. INH is a prodrug that is activated by the mycobacterial enzyme *katG* (Zhang *et al*., 1992). INH-resistant clinical isolates of *M. tuberculosis* often lose catalase and peroxidase enzyme encoded by *katG* (Middlebrook, 1954), especially in high-level resistant strains (MIC > 5 μg/ml) (Winder, 1982). Low-level resistant strains (MIC < 1 μg/ml) often still possess catalase activity (Winder, 1982). Although mutations in *katG* have been shown to be responsible for INH resistance (Zhang *et al*., 2000), it is not clear whether the regulation of *katG* expression plays a role in INH resistance. The *katG* gene encodes a bifunctional catalase-peroxidase that converts INH to its active form (Zhang *et al*., 1992). Activated INH inhibits the synthesis of essential mycolic acids by inactivating the NADH-dependent enoyl-acyl carrier protein reductase encoded by *inhA* (Banerjee *et al*., 1994).

Mutations in *inhA* or its promoter region are usually associated with low-level resistance and are less frequent than *katG* mutations (Zhang *et al*., 2000; Hazbón *et al*., 2006). INH-resistant *M. tuberculosis* harboring inhA mutations could have additional mutations in *katG*, conferring higher levels of INH resistance (Heym *et al*., 1995).

In *M. tuberculosis*, *ahpC* codes for an alkyl hydroperoxidase reductase that is implicated in resistance to reactive oxygen and reactive nitrogen intermediates. It was initially proposed that mutations in the promoter of *ahpC* could be used as surrogate markers for the detection of isoniazid resistance (Rinder *et al*., 1998).

Rifampicin

One of the main reasons for treatment failure and fatal clinical outcome in tuberculosis patients is resistance to rifampin (Wilson *et al*., 1998). In addition to a significant early bactericidal effect on metabolically active *M. tuberculosis*, rifampicin also exhibits excellent late sterilizing action on semidormant organisms undergoing short bursts of metabolic activity. The recognition of this late effect of rifampin, and the additional effectiveness of pyrazinamide, has allowed for the reduction of routine tuberculosis treatment from 1 year to 6 months (Mitchison and Nunn, 1986).

Whereas monoresistance to isoniazid is quite common, monoresistance to rifampicin is rare. Instead, rifampicin resistance occurs most often in strains that are also resistant to isoniazid; thus, rifampicin resistance can be used as a surrogate marker for MDR. The mechanism of action of rifampicin is to inhibit mycobacterial transcription by targeting DNA-dependent RNA polymerase. The development of resistance to rifampicin is due to mutations in a well-defined, 81 base pair (bp) (27 codons) central region of the gene that encodes the β-subunit of RNA polymerase (*rpoB*) (Telenti *et al*., 1993).

More than 96% of the rifampicin-resistant strains contain a mutation in this 81 bp region of *rpoB*, thus facilitating a straightforward approach to detect rifampicin resistance and/or MDR rapidly (Zhang and Telenti, 2000; Ramaswamy and Musser, 1998). The most common mutations (65–86%) alter either codon 526 or codon 531, and result in high-level resistance to rifampicin (minimal inhibitory concentration [MIC] >32 μg/ml). However, not all mutations within the 81 bp region exhibit the same level of resistance. For example, alterations in codons 511 and 516 result in organisms that have low-level resistance to rifampicin and another rifamycin derivative (rifapentin), but remain susceptible to two other rifamycins (rifabutin and rifalazyn) (Moghazeh *et al*.,1996; Ohno *et al*., 1996) . Rare mutations associated with rifampicin resistance have also been found in the amino-terminal region of *rpoB* (Espinal *et al*., 2000; Huyen *et al*., 2010).

Streptomycin (STM) is another first-line TB drug. Mutations associated with streptomycin resistance in tuberculosis have been identified in the 16S rRNA gene (rrs) and rpsL gene. In contrast to other bacteria that have multiple copies of rRNA genes, *M. tuberculosis* complex members have only one copy. Therefore, single nucleoside changes can potentially produce antibiotic resistance. Mutations in the rrs are clustered in two regions around nucleotides 530 and 951. The 530 loop 16S rRNA is highly conserved and is located adjacent to the 915 region in secondary structure models. The majority of mutations producing streptomycin resistance occur in rpsL. The most common mutation

is at the codon 43. Mutations also have occurred in codon 88. About 65-75% of streptomycin resistant isolates have resistance-associated changes in rpsL or rrs. Failure to identify resistance-associated variations in these genes in 25-35% of organisms indicates that other molecular mechanisms of streptomycin resistance exist (Mendez, 2001).

Pyrazinamide (PZA) is a structural analogue of nicotinamide that is used as a first-line TB drug. PZA kills semi-dormant tubercle bacilli under acidic conditions. It is believed that in the acidic environment of phagolysosomes the tubercle bacilli produce pyrazinamidase, an enzyme that converts PZA to pyrazinoic acid, the active derivative of this compound. To define the molecular mechanism of PZA resistance the pncA gene encoding pyrazinamidase has been sequenced. For clinical isolates identified mutations at codons 63, 138, 141, and 162. In contrast, susceptible organisms had wild type sequences. Lack of pncA mutations in 28% of PZA resistant isolates suggested the existence of at least one additional gene participating in resistance. It is presumed that these structural changes detrimentally change enzyme function, thereby altering conversion of PZA to its bioactive form (Mendez, 2001).

Ethambutol (EMB) is a bactericidal first-line TB drug. This agent is thought to act on the mycobacterial cell wall, with arabinan synthesis as the primary site of action. Drug susceptibility testing for EMB is particularly problematic (Buttger, 2011). To understand the mechanism of resistance of ethambutol a two gene locus (embAB) that encodes arabinosyl transfer has been established. Sequencing of these regions in clinical isolates from diverse geographical areas discovered that 69% of ethambutol resistance isolates had an amino acid substitution in EmbB that was not found in ethambutol susceptible strains. The great majority (98%) of strains had mutations in codon 306, however, mutations were also identified in 3 additional codon 285, 330 and 630. The cause of ethambutol resistance in many organisms lacking mutations in ERDR of EmbB is unknown (Mendez, 2001).

1.2.2.2 Epidemiology

Cases of MDR tuberculosis have been reported in every country surveyed (Farmer and Paul, 2001). MDR-TB most commonly develops in the course of TB treatment (Wood *et al*., 1993) and is most commonly due to doctors giving inappropriate treatment, or patients missing doses or failing to complete their treatment. Because MDR tuberculosis is an airborne pathogen, persons with active, pulmonary tuberculosis caused by a multidrug-resistant strain can transmit the disease if they are alive and coughing (Farmer & Paul, 2001). TB strains are often less fit and less transmissible, and outbreaks occur more readily in people with weakened immune systems (e.g., patients with HIV) (CDC, 1991; Edlin *et al*., 1992).

Outbreaks among non immunocompromised healthy people do occur (CDC, 1990), but are less common (Wood *et al*., 1993). As of 2013, 3.7% of new tuberculosis cases have MDR-TB. Levels are much higher in those previously treated for tuberculosis - about 20%. WHO estimates that there were about 0.5 million new MDR-TB cases in the world in 2011. About 60% of these cases occurred in Brazil, China, India, the Russian Federation and South Africa alone (WHO, 2013).

When patients have MDR-TB, they require longer periods of treatment—about two years of multidrug regimen. Several of the less powerful second-line drugs, which are required to treat MDR-TB, are also more toxic, with side effects such as nausea, abdominal pain, and even psychosis. The Partners in Health team had treated patients in Peru who were sick with strains that were resistant to ten and even twelve drugs. Most such patients require adjuvant surgery for any hope of a cure (Farmer, 2005).

1.2.2.3 Rapid Detection of Drug-resistant TB Strains

Early diagnosis of tuberculosis and drug resistance improves survival and by identifying infectious cases promotes contact tracing, implementation of institutional cross-infection procedures, and other public-health actions. There have been many advances in methodology for tuberculosis diagnosis (Dinnes *et al*., 2007; Lange and Mori, 2010; Wallis *et al*., 2010).

For every stages of diagnosis, there are new approaches. New tests are available by level of laboratory and phase of application.

1.2.2.3.1 Microscopy

Microscopy has been a diagnostic tool for TB for over a century, and still currently the most rapid diagnostic method. Standard light microscopy (LM) and fluorescent microscopy (FM) are common methods. The recent development of light emitting diodes (LED), with the appropriate fluorescent light output for FM and low power consumption, has led to the development of simple, robust LED FM microscopes, requiring minimal mains or battery power and no dark room requirement. The WHO has recommended rolling it out as an alternative to LMs in resource-limited settings, based on studies that have shown comparable performance of LM and standard FM systems (Minion *et al*., 2011; Trusov *et al*., 2009).

1.2.2.3.2 Drug resistance testing

1.2.2.3.2.1 Phenotypic methods

Significant effort has been invested into further development of simple, alternative phenotypic methods such as the nitrate reductase assay (NRA), thin-layer agar (TLA), colour test (Color Test), the microscopic observation drug susceptibility assay (MODS), the colorimetric redox indicator (CRI) method and phage-based assays, most of which can be set up directly on specimens (Bwanga *et al*., 2009; Balabanova *et al*, 2009; WHO, 2011).

These methods can detect MTB and resistance to INH and RIF. While MODS, NRA and CRI have been endorsed by the WHO, current evidence was considered to be insufficient for recommending the use of TLA or phage-based assays (WHO, 2011).

Drug susceptibility tests (DSTs) should be done on initial isolates from all patients to identify an effective anti-TB regimen. These tests should be repeated if patients continue to produce culture-positive sputum after 3 months of treatment or if cultures become positive after a period of negative cultures. Results of DSTs may take up to 8 weeks if

conventional bacteriologic methods are used, but several new molecular DSTs can detect drug resistance to rifampin or to rifampin and isoniazid in a sputum sample within hours. MODS is an extensively validated method that has almost perfect agreement with conventional DST for INH, RIF and MDR-TB (100%, 97% and 99%). The results are available within a median of 7 days; the method is cheap, non-commercial and works well on all types of primary specimens as well as on isolates. However, it requires relatively long, detailed staff training (Bwanga *et al*., 2009; Balabanova *et al*, 2009; Leung *et al*., 2012).

TLA recently demonstrated a good performance of the MDR-/XDR-TB colour test for the identification of MTB complex and detection of resistance to INH, RIF and ciprofloxacin in cultures (Toit *et al*., 2012).

1.2.2.3.2.2 Genotypic methods

Genotypic methods are not routinely used in the mycobacterium laboratory; they are essentially for research purposes (Somoskovi *et al*., 2003; Lin *et al*., 2004).

The molecular techniques also aimed detecting resistance genes. Example includes; DNA probe and DNA sequencing of MTB gene such as catalase (*katG*) or RNA polymerase (*rpoB*). Mutations in these genes have been associated with resistance to isoniazid and rifampicin respectively.

Among the many techniques used to identify drug resistance-associated mutations, automated DNA sequencing of PCR products has been the most widely applied. One important advantage of sequence-based approaches is that the resulting data are virtually unambiguous because the resistance- associated mutation is either present or absent. Until recently, the considerable cost required to purchase a sequencing instrument was a major disadvantage.

Conventional DNA sequencing remains the gold-standard of DNA sequencing, is highly accurate, and offers the advantage of being able to read larger amounts of DNA. It is the

foundation on which many of the rapid molecular assays, (i.e., line probe assays, molecular beacon-based real time-polymerase chain reactions (RT-PCR), and pyrosequencing) were developed. These assays have the capacity to both identify *M. tuberculosis* isolates and evaluate for drug resistance to isoniazid and rifampicin.

The using of molecular primers in real- time PCR reaction can differentiate between the presence of the wild- type sequence and mutated sequence associated with drug resistance. Molecular tests are rapid (within few hours), highly sensitive and specific, but expensive, requires expertise and may not differentiate active infection as DNA from a dead organism during antibiotic treatment can be detected and amplified by PCR (Dinnes *et al*., 2007).

1.2.2.4 Treatment of drug-resistant TB

The emergence of MDR- and XDR-TB has shattered the initial optimism that DOTS based programmes would progressively eliminate TB. In order to control the spread of drug resistant TB, the WHO extended the DOTS programme in 1998 to include the treatment of MDR-TB (called "DOTS-Plus") (Iseman, 1998).

Implementation of DOTS-Plus requires the capacity to perform drug-susceptibility testing and the availability of second-line agents, in addition to all the requirements for DOTS. Clinical pilot experiences from the past few years showed that high cure rates of drug resistant TB are achieved in settings where DOTS-Plus has been established (Tupasi *et al*., 2003, 2006; Riekstina *et al*., 2007).

Resistance to INH is the most common form of TB drug resistance reported, either in isolation or in combination with other drugs (Zhang and Yew, 2009). INH monoresistant TB is relatively easy to treat with SCC treatment. Up to 98% cure and less than 5% relapse can be achieved when all four drugs INH, RIF, PZA and EMB are used during the 6-month treatment period (Five-year follow-up of a controlled trial of five 6-month regimens of chemotherapy for pulmonary tuberculosis, 1987).

RIF-resistant TB often carries a much more ominous prognosis, as the outcome of SCC treatment is poor in terms of both disease status at the end of the treatment and relapse (Zhang and Yew, 2009). Moreover, RIF monoresistance in *M. tuberculosis* is rare and usually reflects resistance to INH as well, i.e., MDR-TB (O'Riordan, 2008). In fact, SCC cures less than 60% of MDR-TB, with a recurrence rate of about 28% among patient with apparent success (Espinal *et al*., 2000; Becerra *et al*., 2000).

The current recommendation for individualized treatment regimens is a combination of at least four drugs to which the *M. tuberculosis* isolate is likely to be susceptible. Drugs are chosen with a stepwise selection process through 5 groups of TB drugs on the basis of efficacy and safety (Caminero *et al*., 2010).

1.2.2.5 Prevention of MDR-TB

There are several ways that drug resistance to TB, and drug resistance in general, can be prevented (Gao *et al*., 2010; Lobue and Philip, 2009).

Rapid diagnosis and treatment of TB: One of the greatest risk factors for drug resistant TB is problems in treatment and diagnosis, especially in developing countries. If TB is identified and treated soon, drug resistance can be avoided.

1. Completion of treatment: Previous treatment of TB is an indicator of MDR TB. If the patient does not complete his/her antibiotic treatment, or if the physician does not prescribe the proper antibiotic regimen, resistance can develop. Also, drugs that are of poor quality or less in quantity, especially in developing countries, contribute to MDR TB.
2. Patients with HIV/AIDS should be identified and diagnosed as soon as possible. They lack the immunity to fight the TB infection and are at great risk of developing drug resistance.
3. Identify contacts who could have contracted TB: i.e. family members, people in close contact, etc.
4. Research: Much research and funding is needed in the diagnosis, prevention and treatment of TB and MDR TB.

1.3 Aims and objectives

Since the incidence of mycobacterium infection is common in our country, several institutions and research organizations have already undertaken several diagnostic programmes to isolate *M. tuberculosis* from infected patients, mostly by using conventional methods. We have evaluated the molecular techniques for rapid detection of multidrug resistant strains from MTB strains and sputum. We analyzed 55 samples by PCR amplification followed by DNA sequencing. Rapid detection of drug resistance could not only optimize the treatment and improve the outcome of patients with drug resistant TB, but is especially important in the prevention of transmission of drug resistant TB. It can also be used in drug surveillance studies. To be cost-effective in resource poor countries where most MDR-TB patients reside, it will be crucial that molecular genetic tests fulfill the criteria of accuracy, speed and simplicity.

The present study is undertaken to achieve the following objectives:

(i) amplify the genes (*rpoB* and *katG*) responsible for rifampicin and isoniazid resistance

(ii) sequence analysis of amplicon to find out the position of mutation

(iii) comparing sequence analysis with the results of DST

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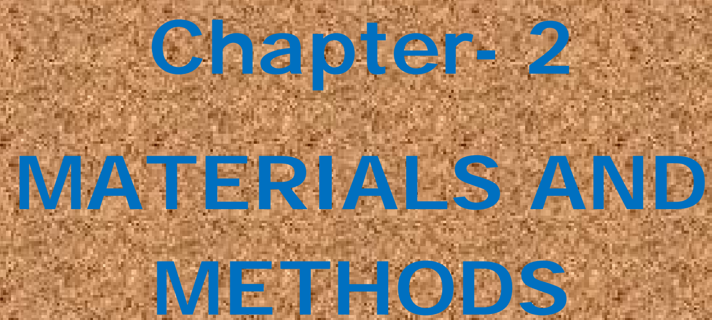

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2.0 MATERIALS AND METHODS

2.1 Selection of Patients and Samples

Clinically diagnosed patients with pulmonary tuberculosis were selected based on their signs and symptoms. A brief study questionnaire was used to select TB suspected patients. Fifty five sputum samples were collected from fifty five patients. Samples were collected from the National Tuberculosis Reference Laboratory (NTRL), Mohakhali, Dhaka- 1212.

The tests were carried out in National Tuberculosis Reference Laboratory (NTRL), NIDCH, Mohakhali, Dhaka- 1212 and in the Department of Microbiology, University of Dhaka, Dhaka-1000.

2.2 Sample Collection and Transportation

The samples were collected from patients with suspected pulmonary tuberculosis. As sputum samples from clinically suspected patients are potentially infectious and hazardous, samples were transported to the laboratory with utmost care as early as possible. Before collection of samples, the patients were suggested to rinse their mouth with water to remove any food particles, oral drugs or mouthwash that might contaminate the samples or inhibit the growth of acid-fast bacilli. Early morning sputum, which is an exudative material after a deep and productive cough, was collected in robust, leak-proof, clean, sterile and disposable container and was labeled with patient name and laboratory identification number. The minimum volume of each collected sputum sample was 2 mL (optimum volume 5 mL). The sputum samples were transported to the laboratory as early as possible to avoid leakage and breakage of the sealed and packaged container.

2.3 Microscopy - Acid Fast Staining

For direct microscopic examination of mycobacteria in sputum samples, Auramine O fluorochrome acid-fast staining was performed. Smear were prepared, stained and examined properly.

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2.3.1 Smear preparation

Sputum sample was directly used to make smear of approximately 2-3 cm^2 in diameter. A sterile toothpick was used to make smears and allowed to air dry and then heat-fixed by passing the slide over a bunsen burner flame.

2.3.2 Auramine O Fluorochrome Acid-Fast Staining Procedure

Auramine O staining procedure involves primary stain (0.1% Auramine O), decolorizer (5.0% Acid-alcohol) and counterstain (0.3% Methylene blue). The prepared smear was flooded with 0.1% auramine O solution to allow staining for minimum of 20 minutes, making sure that the staining solution remains on the smear. Then each slide was rinsed with distilled water. After that, 5.0% acid-alcohol was added to each slide and let stand for 1-2 minutes. After rinsing the slide with water, excess water was poured off. The smear was then flooded with 0.3% methylene blue solution to allow counter-staining for 30 seconds to 1 minute and then rinsed with water and kept for air-drying away from direct sunlight. The smear was examined using a LED fluorescent microscope (Primo Star iLED) (examination at 20× to 40×). In Auramine O staining method, mycobacteria appear bright yellow fluorescent color against dark background.

To determine degree of positivity, a quantitative report on number of AFB seen on a smear was made according to IUATLD/WHO scale (Table 2.1).

TABLE 2.1 IUATLD/WHO scale for a quantitative report on number of AFB seen on a smear

IUATLD/WHO scale	**Magnification 200×** **1 length = 30 fields** **1 length = 300 HPF (High power fields)**
Negative	0 AFB/ 1 length
Scanty	1-29 AFB/ 1 length
1+	30-299 AFB/ 1 length
2+	10-100 AFB/ 1 field on average
3+	Greater than 100 AFB/ 1 field on average

2.4 Gene Xpert MTB/RIF

Among the most important diagnostic techniques one is the Gene Xpert which detects gene mutation (*rpoB*) associated with RIF resistance. Gene Xpert test can be performed on sputum or bronchial washing samples. Results become available in less than 2 hours. Gene Xpert test is a semi-quantitative nested real-time PCR *in-vitro* diagnostic test for: (i) The detection of *M. tuberculosis* complex DNA in sputum samples or concentrated sediments prepared from induced or expectorated sputa that are either acid-fast bacilli (AFB) smear positive or negative; and (ii) The detection of rifampin resistance associated mutations of the *rpoB* gene in samples from patients of rifampin resistance (Ashok *et al*., 2008; Curry, 2008).

2.4.1 Procedure

A 200-µl portion of the deposit was resuspended in phosphate-buffered saline to a 500-µl volume. The sample reagent supplied with the test (1.5 ml) was then added. The mixture was then shaken by hand according to test instructions. The mixture was vortexed for 30 s to ensure all bacteria were resuspended. The sample was left to stand for 15 min, as per the manufacturer's instructions, with intermittent manual shaking. The solution was then transferred to the Xpert cartridge using a Pasteur pipette, and the cartridge was loaded onto the Xpert machine for analysis. Results are reported as positive or negative for *M. tuberculosis*. Positive results were placed in one of four categories; very low, low, medium, or high. Rifampin resistance results were reported as susceptible or resistant.

2.5 Processing of sputum sample for culture techniques

The sputum samples were transferred into the sterile 50 mL falcon centrifuge tubes. Aseptically collected samples were subjected to digestion-decontamination for liquefaction of the organic debris and elimination of unwanted normal flora microorganisms. The digestants used in this study for processing of clinical samples were N-acetyl-L-cysteine (NALC) and 4% sodium hydroxide (NaOH).

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2.5.1 Decontamination of sputum sample

Decontamination was performed according to N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method. It is the preferred method for the decontamination step because it is the least toxic to the mycobacteria, and therefore provides the highest yield of positives. NALC perform the liquefaction step, and permits the use of a lower concentration of NaOH than that required when NALC is omitted. Sodium citrate is also included to bind heavy metal ions, which, if present in the specimen, can inactivate the N-acetyl-L-cysteine. NALC loses activity rapidly in solution, so the digestant was prepared fresh before use. Fresh digestant solution was prepared by mixing an equal volume of 4% NaOH and 2.9% sodium citrate. Then 0.5g NALC powder was added per 100 mL of NaOH- Na citrate solution.

Equal volume of NALC-NaOH solution was added into the 50 mL flacon centrifuge tube containing sputum samples. After addition of digestant, the cap of the tube was tightened and the tube was kept standing at room temperature for 15 minutes with occasional shaking. Equal volume of NALC-NaOH solution was then added into the 50 mL falcon centrifuge tube containing sputum samples. Sterile phosphate buffer solution of pH 6.8 (Appendix) was added to make up to approximately 50 mL. Then the 50 mL falcon centrifuge tube was centrifuged at 3,000 × g for 15 minutes. Supernatant was discarded into container containing disinfectant (3% glutaraldehyde) and pellet was resuspended with 1 mL phosphate buffer solution. Then this resuspended pellet was used for smear preparation, inoculation into liquid and solid culture media and molecular analysis.

2.6 Detection of Mycobacteria by Rapid Culture Technique

As rapid culture method BACTEC™ MGIT™ 960 TB System was used, all sputum samples (irrespective of their microscopic result) were tested for presence of mycobacteria using this automated liquid culture system.

2.6.1 Inoculation of MGIT medium

The MGIT 960 tube contains 7.0 mL of modified 7H9 broth base. The caps of the tubes were kept closed until any addition was made to the medium. Each MGIT tube was

labeled with specimen number. MGIT PANTA was reconstituted with 15 mL MGIT growth supplement. Then, 0.8 mL of MGIT growth supplement/PANTA was aseptically added to each MGIT tube. Using a sterile pipette, up to 0.5 mL of well mixed digested-decontaminated sputum sample was added to the appropriately labeled MGIT tube. The tube was immediately recapped tightly and was mixed by inverting the tube several times. All the tubes and caps were wiped with mycobacterial disinfectants. Then inoculated tubes were kept at room temperature for 30 minutes. The whole work of specimen inoculation was carried out in a class-II biologic safety cabinet.

2.6.2 Incubation

All inoculated MGIT (7 mL) tubes were placed in the BACTEC MGIT 960 instrument after scanning each tube. The caps were kept tightly closed and incubated at 37ºC ± 1ºC.

MGIT tubes were kept incubated until the instrument flagged them as positive. After a maximum of six weeks, the instrument usually flags the tubes as negative if there is no growth.

2.6.3 Indication of positive growth

When the instrument signaled a tube positive for growth, an indicator green light showed the exact location of the positive tube in the drawer of the instrument. At this point, the tube was removed and scanned outside the instrument. The tube was observed visually. Mycobacterial growth usually appears granular and not very turbid while contaminating bacterial growth appears very turbid. Growth, especially of *M. tuberculosis* complex, settles at the bottom of the tube.

In some instances, especially if mycobacterial growth is extremely slow or there is less oxygen consumption during mycobacterial growth, there may be growth in the MGIT broth without the presence of fluorescence. For this reason, at the termination of incubation protocol, all negative tubes were observed visually for turbidity and growth before discarding. If there was any suspicion of growth, an AFB smear and subculture were performed.

2.7 Isolation of *M. tuberculosis* by Conventional Culture Technique

Isolation of *M. tuberculosis* by conventional culture technique is considered to be the gold standard for detection of this organism in clinical specimens. In this study, Lowenstein-Jensen slant was used as solid culture medium.

2.7.1 Preparation of homogenized whole egg

Fresh eggs, not more than one week old, were cleaned by hand brush with soap solution and submerged in soap solution for 15 minutes and then rinsed thoroughly in running tap water. The cleansed eggs were soaked in 70% ethanol for 15 minutes. The eggs were then broken into a sterile flask containing sterile glass beads. The eggs were homogenized and filtered through four layers of sterile gauze into a sterile, graduated cylinder.

2.7.2 Preparation of egg based Lowenstein-Jensen Medium

After making homogenized egg solution, salt solution and 2% malachite green solution were prepared and autoclaved (Appendix-I). The egg based L-J modified medium was prepared via mixing of sterile salt solution, 2% malachite green and homogenized egg in a particular ratio (Appendix-I). Approximately 6.7 mL of medium was dispensed into McCortney bottle. The tubes were slanted and coagulated by inspissations at 90°C for 50 minutes. The tubes were incubated at 35-37°C for 48 hours to check the sterility of the media. Then the prepared L-J tubes were stored in the refrigerator by keeping the caps tighten enough to prevent evaporation (when not immediately used).

2.7.3 Inoculation of media

An aliquot of 100 μL of the digested sputum sample was transferred on to tube containing egg-based Lowenstein-Jensen medium and spread evenly over the entire surface of the medium using a bacteriological loop. Tubes of inoculated media were kept at room temperature for half an hour until fluid inoculums were absorbed.

2.7.4 Incubation and observation

The inoculated tubes were incubated at 37°C in slanted position. The caps of the tubes were kept loose for the first 48 hours of incubation to let the inoculums dry. After 48 hours, the caps of the tubes were tightened to avoid dehydration of the medium. Within 5 to 7 days, the tubes were examined to permit the early detection of rapidly growing mycobacteria and enable prompt removal of contaminated cultures. All cultures were incubated for 8 weeks with weekly examination of growth.

Presence or absence of *M. tuberculosis* in a sputum sample was preliminarily determined by observing colony morphology on L-J slant where colonies appear as bread crumb or cauliflower. Colonies are buff colored (never pigmented), rough and waxy. On the other hand, colonies produced by *M. bovis* in L-J medium are white, small, round, wrinkled surface and thin margins.

Presence or absence of contamination on solid culture media was carefully observed. Usually if media color changes and liquefaction happens then it can be assumed that contamination of culture media has happened. The usual contaminants are bacteria, fungi and yeasts.

Quantification of growth on solid culture was also performed. For scoring amount of growth on single solid culture medium, WHO scoring guideline was followed (Table 2.2).

TABLE 2.2 WHO guideline for scoring amount of growth on single solid culture medium

No growth	0
Fewer than 10 colonies	Number of colonies
10-100 colonies	1+
More than 10 colonies	2+
Innumerable colonies or confluent growth	3+

2.7.5 Ziehl-Neelsen acid-fast staining for confirmation of positive cultures

Ziehl-Neelsen acid-fast staining was performed to confirm growth of acid-fast bacilli in Lowenstein-Jensen medium. Smear was prepared on a microscopic slide from positive cultures using a loop. After air drying and heat fixing the smear was flooded with carbol fuchsin stain. The slide was heated gently and stain was being added as the slide was getting dry during heating. After 5-10 minutes of staining cooling was performed and then the stain was washed using water. Decolorization was performed with acid-alcohol for 2 minutes. After washing with water, methylene blue (counter stain) was poured on the smear and staining was allowed for 2 minutes. Air drying was performed after washing counter stain with water and the slide was observed under oil-immersion objective lens of the microscope. Under microscope acid-fast bacteria appeared red as they retained carbol fuchsin dye. Non acid-fast bacteria appeared blue as they picked up methylene blue.

2.8 Drug Susceptibility Testing of *M. tuberculosis*

2.8.1 Preparation of stock solution

Each drug was dissolved in 20 mL distilled water and subsequently homogenized by magnetic stirrer. The homogenized solutions were let stand for one hour to scrutinize any undissolved constituent's. After that the solutions were centrifuged at 6000 rpm for 5 minutes. The supernatants were collected and transferred to fresh tubes and centrifuged again. The supernatants were collected and stored at 4°C as stock solutions.

2.8.2 Procedure

Drug susceptibility of the isolates to isoniazid (INH), rifampicin (RIF), streptomycin (SM) and ethambutol (EMB) was performed by standard proportion method (Strong and Kubica, 1981). Briefly, LJ media with drug incorporated in various concentrations (0.2 ug/mL isoniazid, 40 ug/mL rifampicin, 4 ug/mL streptomycin and 2 ug/mL ethambutol) and plain LJ medium for control were prepared. The growth from a 3-4 week old culture was scraped with a loop and bacterial suspension was made in sterile distilled water, vortexed and matched with McFarland opacity tube No.1. Dilutions of 10^{-2} and 10^{-3} were

made and inoculated on both the control and drug containing media and incubated at 37°C. The first reading was taken on 28th day of incubation and the second on 40th day. The percentage resistance (R) was calculated as the ratio of the number of colonies on the drug containing media to those on the control medium.

2.9 Amplification of *M. tuberculosis* genes

2.9.1 DNA extraction from *M. tuberculosis* strains

DNA was extracted from *M. tuberculosis* strains grown on solid culture media by using InstaGene matrix kit (Bio-Rad, USA). The growth from a culture was scraped with a loop and bacterial suspension was made in 1 mL of sterile distilled water in a microcentrifuge tube. The bacterial cell suspension was centrifuged for 1 minute at 10,000–12,000 rpm and supernatant was removed. 200 µL of InstaGene matrix (InstaGene matrix was being mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension) was added to the pellet and incubated at 56°C for 30 minutes. After vortexing the tube at high speed for 10 seconds, it was placed in a 100°C heat block or boiling waterbath for 8 minutes. Then the microcentrifuge tube was again vortexed at high speed for 10 seconds and then centrifuged at 13,000 rpm for 2-3 minutes. The resulting supernatant was transferred to another microcentrifuge tube and stored at -20°C.

2.9.2 DNA extraction directly from sputum samples

DNA was extracted directly from heat killed sputum samples using RTP® Mycobacteria Kit (STRATEC molecular, Germany) according to the provided instructions. In brief, 200 µL of decontaminated and heat killed sputum was transferred into a 1.5 mL reaction tube and 200 µL of NAC buffer was added. After short vortexing, the sample was incubated at room temperature under continuous shaking for 20 minutes. The sample was centrifuged at 10,000 rpm for 15 minutes and supernatant was removed completely. The resulting pellet was resuspended in 400 µL resuspension buffer R and then the entire sample was transferred into the extraction tube L. Extraction tube L was vortexed for few seconds and placed into a thermomixer for 15 minutes at 95°C under continuous shaking. Then

extraction tube L was placed on ice for 1 minute and 20 µL proteinase K was added to it. Then the extraction tube L was incubated in a thermomixer at 68°C for 10 minutes under continuous shaking. Four hundred microliter of binding solution was added to the sample and vortexed shortly. The sample was then loaded onto the RTA spin filter set and incubated for 2 minutes. The RTA spin filter set was centrifuged at 11,000 rpm for 1 minute. After discarding the filtrate the RTA spin filter was placed back into the RTA receiver tube. Then, 500 µL of wash buffer I was added to the RTA spin filter and centrifuged at 11,000 rpm for 1 min. The filtrate was discarded and RTA spin filter reassembled with the RTA receiver tube. Then, 800 µL of wash buffer II was added to the RTA spin filter and centrifuged at 11,000 rpm for 1 minute. The filtrate was discarded and the RTA spin filter was placed back into the RTA receiver tube. Finally, The RTA spin filter set was centrifuged for 4 minutes at 13,000 rpm to remove the ethanol completely. After that, the RTA spin filter was placed into a new 1.5 mL receiver tube and 100 µL of prewarmed (at 65°C) Elution Buffer was added. After 3-5 minutes of incubation at room temperature, centrifugation was done at 11,000 rpm for 1 minute. The RTA Spin Filter was discarded and elute was stored at -20°C.

2.9.3 PCR amplification of *M. tuberculosis* IS*6110* sequence

IS*6110* sequence is present in almost all *M. tuberculosis* strains. To confirm the presence of *M. tuberculosis* in all samples, the IS*6110* sequence was amplified by polymerase chain reaction (PCR).

2.9.3.1 Preparation of reaction mixture

Firstly, a master mix, including all the reagents for PCR reaction except the template was prepared (Table 2.4) and aliquoted into PCR tubes before adding different extracted DNA templates. After a brief spin, the PCR tubes were placed in a thermal cycler (Bio-Rad, USA). The primers used for PCR are given in the table 2.3.

TABLE 2.3 Primers used for PCR amplification of IS*6110, rpoB* and *katG* gene sequence

Target	Primer Name	Sequence	Amplicon length
IS*6110* sequence	IS1	5'-CCT GCG AGC GTA GGC GTC GG-3'	123 bp
	IS2	5'-CTC GTC CAG CGC CGC TTC GG-3'	
rpoB gene	PR1	5'-CCG CGA TCA AGG AGT TCT TC-3'	315 bp
	PR2	5'-ACA CGA TCT CGT CGC TAA CC -3'	
*kat*G gene	RTB 59	5'-TGC CCGCGGCGGTCGACATT-3'	807 bp
	RTB 36	5' -TCGGGGTCGTTGACCTCCCA-3'	

(Reference: Eisenach *et al*., 1990; Sekiguchi *et al*., 2007, Chia *et al*., 2012)

TABLE 2.4 Components of PCR reaction mixture for amplification of IS*6110* sequence

Reagents	Component Volume in 25 µL Reaction Mixture	Final Concentration
PCR grade water	10.575 µL	
10× PCR reaction buffer	2.5 µL	1×
$MgCl_2$ (25 mM)	3.8 µL	3.8 mM
dNTP mixture (2.5 mM each)	2.0 µL	200 µM
IS1 (25 pmol/µL)	0.5 µL	0.5 pmol/µL
IS2 (25 pmol/µL)	0.5 µL	0.5 pmol/µL
Taq polymerase (5 U/µL)	0.125 µL	0.025 U/µL
Template DNA	5 µL	

2.9.3.2 Thermocycling parameters

The PCR reaction was performed according to the following program:

95 °C…………………10 minutes …………..initial denaturation

95 °C……………….... 30 seconds
68 °C………………… 30 seconds   30 cycles
72 °C.........................30 seconds

72°C………………......7 minutes……………final extension

After this, PCR tubes were stored at -20°C until further analysis. The cycling profile for each primer: target combination was optimized accordingly.

2.9.3.3 Post - PCR detection of amplified IS*6110* sequence by electrophoretic analysis

The PCR products were analyzed by 2% agarose gel electrophoresis to detect specific band for IS*6110* sequence.

2.9.3.3.1 Preparation of agarose gel

A 2% agarose gel was prepared by melting 2.0 gm agarose (Sigma, USA) in 100 mL 1× TBE buffer (Appendix-II). The melted agarose was allowed to cool to about 50°C and poured into gel electrophoresis unit (Sigma, USA) with spacers and comb. After solidification of the gel, the comb was removed and wells were formed. Then the gel was submerged in 1× TBE buffer in a gel running tank.

2.9.3.3.2 Loading and running the sample

Five microliter of PCR product was mixed with 1μL of 6× gel loading dye (Appendix-II). The mixture was slowly loaded into the well using disposable micro-pipette tips. Marker DNA of known size (Invitrogen, USA) was loaded in one well to determine the size of the PCR products. Electrophoresis was carried out at 90 volts for approximately 45 minutes.

2.9.3.3.3 Staining and visualization of the gel

After electrophoresis, the gel was submerged in staining solution containing ethidium bromide (Et-Br) (Appendix-II) for 15 minutes. Destaining was performed by submerging the gel in distilled water for about 15 minutes. The EtBr stained DNA bands were observed on a UV transilluminator (Vilber Lourmat, France). Photographs were taken using a gel documentation system (Vilber Lourmat, France) and bands were analyzed.

2.9.4 PCR amplification of *rpoB* gene of *M. tuberculosis*

In more than 95% cases, the reason for resistance to rifampicin is mutation in the *rpoB* gene, the gene encoding the β- subunit of RNA polymerase. So the *rpoB* gene of the *M. tuberculosis* was amplified by polymerase chain reaction (PCR).

Master mix, including all the reagents was prepared as mentioned before (Section 2.9.3.1). Only the primer pair and $MgCl_2$ concentration were different. The primers used for this PCR protocol are PR1 and PR2 (Table 2.3). In a 25 µL reaction mixture, final concentration of each primer and $MgCl_2$ were 0.2 pmol/µL and 4 mM respectively.

The master mix and template DNA containing PCR tubes were placed in thermal cycler (Bio-Rad, USA). The PCR reaction was performed according to the following program:

95°C…………………10 minutes …………..initial denaturation
95°C………………... 30 seconds
63°C…………………30 seconds } 30 cycles
72°C.........................50 seconds
72°C…………………...7 minutes...…………final extension

After this, PCR tubes were stored at -20° C until further analysis. The cycling profile for each primer: target combination was optimized accordingly.

The PCR products were analyzed by 2% agarose gel electrophoresis according to the protocol described in section 2.9.3.3.

2.9.5 PCR amplification of *katG* gene of *M. tuberculosis*

In *M. tuberculosis* isolates mutation in *katG* gene encoding catalase-peroxidase enzyme is most frequently associated with INH resistance as demonstrated by several studies. So the *katG* gene of the *M. tuberculosis* was amplified by polymerase chain reaction (PCR).

The amplification mixture used in the PCR was the same as described in the Table 2.4, except the primers. RTB59 (forward) and RTB36 (backward) (Table 2.3) were the primers used in this PCR.

Amplification was carried out according to the following directions:

94°C…………………10minutes …………..initial denaturation

94°C………………........ 1 minute
64°C……………………. 1 minute
72°C………………...... 1.30 minutes
} 35 cycle

72°C……………………10minutes ……………final extension

The reaction was stopped by cooling at 4°C. To detect the specific band (807bp) corresponding to *kat*G gene, agarose gel (0.8-1.0%) electrophoresis was done as described previously.

2.10 DNA sequencing

There are three basic steps in DNA sequencing:

1. Purification of PCR products

2. Cycle sequencing

3. Purification of cycle sequence product and detection of nucleotide sequence

2.10.1 Purification of PCR products

The *rpoB* gene PCR products of 315 bp which were produced by primer pair PR1and PR2 and the *kat*G gene PCR products of 807bp which were produced by primer pair RTB 59 and RTB 36 were purified with the AccuPrep® PCR Purification Kit (Bioneer, Korea) according to the provided instructions. Before starting the procedure, 48 mL of absolute ethanol was added to 12 mL of buffer-2 to make working solution of buffer-2. In the beginning, 5 volumes of buffer-1 (PCR product binding buffer) were mixed with 1 volume of PCR product (e.g. 35 µL of PCR product and 175 µL of buffer-1) by vortexing. The mixture was transferred to a DNA binding filter column tube which was placed inside a 2 mL collection tube and centrifuged for 1 minute at 13,000 rpm. The flow through was poured off and the DNA binding filter column was reassembled with the 2 mL collection tube. Then 500 µL of working solution of buffer-2 (washing buffer) was added to the DNA binding filter column tube and the tube was again centrifuged for 1 minute at 13,000 rpm. The washing procedure was repeated using 500 µL of buffer-2 (wash buffer). In order to remove residual ethanol, the DNA binding filter column tube was centrifuged for an additional 1 minute at 13,000 rpm for drying. Binding column tube was then placed in a clean 1.5 mL microcentrifuge tube. After air drying for 3-5 minutes, 30 µL of buffer-3 (elution buffer) was added to the center of the binding column filter, and was stood for 5 minutes. Purified PCR product was eluted by centrifugation at 13,000 rpm for 1 minute. The purified PCR products were stored at -20°C until further use.

2.10.1.1 Measurement of DNA concentration

Concentration of DNA was measured using nanodrop spectrophotometer. 1 µL of DNA sample was applied to the nanodrop machine and concentration of DNA per micro liter was measured. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as “pure” for DNA.

2.10.2 Cycle sequencing

In this step only one stand of the desired DNA was amplified and the DNA fragments of different length that had been terminated with different ddNTPs were produced. The reaction mixture composition for cycle sequencing is shown in table 2.5.

TABLE 2.5 The reaction mixture composition for cycle sequencing

Reagents	Volume (µl)
Terminator ready reaction mixture (Big dye)	2
5X buffer	2
Template	1
Primer (forward or reverse 3.2 Pico-mol)	1
De-ionized water	14
Total	20

The big dye terminator contains the following reagents:

- Big dye terminator
- Deoxynucleotide triphosphates
- AmpliTaq DNA polymerase
- $MgC1_2$
- Buffer reaction mixture was mixed well and vortexed briefly

The tubes were then placed in a thermal cycler under the following conditions:

2.10.2.1 Thermal cycling

96°C………………………..1 minute …………..initial denaturation

96°C……………….......... 10seconds

50°C……………………… 5 seconds } 25 cycle

60°C……………….......... 4 minutes

60°C………………………..1minute ……………final extension

2.10.3 Purification of cycle sequence product and detection of nucleotide sequence

2.10.3.1 Ethanol Precipitation of Cycle Sequence Product

Cycle sequence products were purified by ethanol precipitation method to remove the unincorporated dye.

1. Each cycle sequencing product was transferred to a 1.5 ml micro-centrifuge tube containing 2µl of 3 M Na-acetate (pH 4.8) and 50µl of 100% ethanol
2. The tubes were then kept on ice for 15 minutes for precipitating the extraction products
3. Centrifuged at 13000 rpm for 30 minutes
4. The supernatant was carefully aspired and discarded
5. After brief vortex the pellet was rinsed with 20µl of 70% ethanol
6. Centrifuged at 13000 rpm for 30 minutes
7. Repeated the step 5 and 6
8. The supernatant was carefully aspired and discarded
9. The pellet was carefully dried in the drier for 20 minutes
10. Added template suppressor reagent (Hi-formamide) and stored at -20°C for overnight

11. Thawed the whole sample and placed in a sequence tube which was placed in a thermal cycler at 95°C for 3 minutes for denaturation
12. Immediately the tube was kept on ice and left there until sequencing

2.10.3.2 Detection of the nucleotide sequence

The purified cycle sequenced product was analyzed by electrophoresis in the ABI-Prism 3130 genetic analyzer ABI Prism, USA). DNA was separated through the POP-7 contained in a capillary and detected by laser beam. When the nucleotides reached a detector window in capillary electrophoresis, the laser beam excited the fluorescent-labeled fragments. The laser exits the fluorescent dye labels and emitted fluorescence was detected by CCD camera and the data was measured by specific software.

2.11 Sequence Analysis and Detection of Mutations Associated with Rifampicin and Isoniazid Resistance in *M. tuberculosis*

2.11.1 Sequence editing

Raw sequences (available in .abi chromatogram file format from automated DNA sequencer) were edited using Molecular Evolutionary Genetics Analysis (MEGA)-6 software (Tamura *et al*., 2013). The sequences were aligned with reference strain sequence (H37Rv) and compared with those sequences and edited. As the product size were 315bp (*rpoB*) and 807bp (*katG*), sequencing was done from both reverse and forward end. As a result reverse and forward sequences were subjected to blast searches with their respective sequences.

2.11.2 Similarity searches on sequence databases

Database searches for matching sequences are frequently carried out. The commonest type of search is to look for sequences that are similar to a query sequence. Two quicker methods for searching have been developed. These are: FASTA (Fast-A11) and BLAST (Basic Local Alignment Search Tool).

2.11.2.1 BLAST

To check whether *rpoB* and *katG* genes of the multi-drug resistant *M. tuberculosis* strains under study had the similar sequences with those from the reference resistant strain *M. tuberculosis* H_{37}RV, sequence similarity search was performed using BLAST (www.ncbi.nlm.nih.gov/blastf) algorithm.

2.11.3 Comparison of Sequence

Using Molecular Evolutionary Genetics Analysis (MEGA)-6 software (Tamura *et al*., 2013), we compared nucleotide and amino acid sequence of potential MDR strain with reference sensitive strain-H37Rv (GenBank accession no. NC_000962.3).

2.11.3.1 Sequence alignment for detection of RIF-associated mutations

DNA sequences, obtained by using forward (PR1) and reverse (PR2) primers, were aligned with the *rpoB* gene sequence of a wild type (rifampicin sensitive) *M. tuberculosis* strain- H37Rv). Presence or absence of specific mutations at *rpoB* gene codon D516, H526 and S531 were identified by comparing DNA sequence of an isolate with wild type DNA sequence. Mutations at any of those 3 positions indicate high possibility of resistance to rifampicin.

2.11.3.2 Sequence alignment for detection of INH-associated mutations

DNA sequences, obtained by using forward (RTB59) and reverse (RTB36) primers, were aligned with the *katG* gene sequence of a wild type (isoniazid sensitive) *M. tuberculosis* strain- H37Rv. Presence or absence of mutation at the Ser315 codon of *katG* gene was identified by comparing DNA sequence of an isolate with wild type DNA sequence. Mutation at codon 315 of the *katG* gene indicates high-level resistance to isoniazid.

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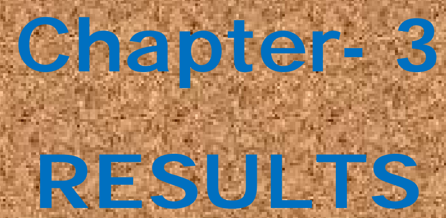


3.0 RESULTS

3.1 Patients' Samples

The whole dissertation includes samples from MDR-TB patients in National Tuberculosis Reference laboratory (NTRL). As the target of this study was establishment of a MDR-TB detection method in patients with pulmonary tuberculosis, we only included sputum samples from patients. For this purpose, 55 sputum samples from 55 patients were enrolled in this study. These samples were from previously treated patients (drug relapse or failure, chronic cases) and did not respond to anti-TB drugs after a sufficient duration of treatment.

All sputum samples were tested for microscopic examination, conventional culture method, drug susceptibility testing (DST) and GeneXpert assay. Then, 55 samples (36 from MTB strain, 19 from direct sputum) were subjected to *ropB* gene mutation analysis and 21 samples out of 55 (12 from MTB strain, 9 from direct sputum) for *katG* gene mutation analysis by polymerase chain reaction and DNA sequencing to detect multidrug- resistant tuberculosis (MDR-TB).

The table 3.1 shows the demographic data of the collected samples of MDR-TB patients. The age of the patients included in this study ranges from 11-80 years with a prevalence within the age group 21-30 years. On the other hand, number of male patients was little bit higher than the female counterparts.

TABLE 3.1 Number of sputum samples collected with demographic data

Age Group	Sex		Total
	Male	**Female**	
11-20	2	7	9
21-30	13	9	22
31-40	6	3	9
41-50	4	4	8
51-60	1	3	4
61-80	3	0	3
Total	**29**	**26**	**55**

3.2 Identification of *Mycobacterium tuberculosis* Complex

3.2.1 Microscopic identification

Acid-fast staining was performed by Auramine O staining method. The presence of acid-fast bacilli (AFB) in the sputum samples was confirmed as bright yellow fluorescent colored rod-shaped organisms were observed. The appearance of acid-fast bacilli after Auramine O fluorochrome staining is shown in figure 3.1. Forty six out of 55 samples were AFB positive. Degree of positivity of each specimen was determined according to IUATLD/WHO scale (Table 2.1). The microscopic identification results of each sample are given in table 3.3.

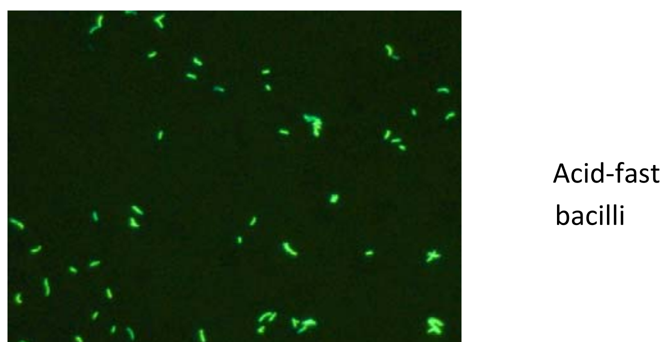


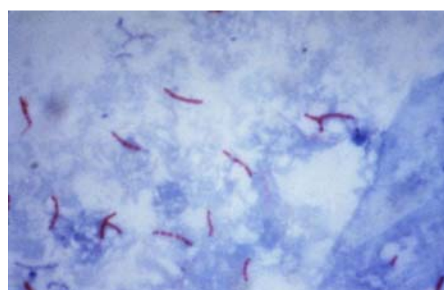
FIG 3.1 Acid-fast bacilli revealed under LED microscope after Auramine O fluorochrome staining

3.2.2 Identification by Rapid culture

All sputum samples (irrespective of their microscopic results) were tested for the presence of mycobacteria using BACTEC™ MGIT™ 960 TB automated liquid culture system. When the instrument signaled an inoculated MGIT tube positive for growth after incubation at 37°C within six weeks, the tube was removed and scanned visually outside the instrument. Forty eight samples showed growth of mycobacteria. Rapid culture results of individual samples are listed in table 3.3. All negative tubes were observed visually for turbidity and growth before discarding. If there was any suspicion of growth, an AFB smear and subculture were performed.

3.2.3 Identification by conventional solid culture technique

All sputum samples were tested for the presence of *M. tuberculosis* complex by conventional culture technique using Lowenstein-Jensen medium. An aliquot of 100μl of digested and decontaminated sputum sample was cultured into two tubes containing Lowenstein Jensen (LJ) media for primary isolation. All cultures were incubated for 8 weeks with weekly examination of growth. Preliminary identification of *M. tuberculosis* was made by observing colony morphology (Fig 3.3B). Presence or absence of contamination in culture media was initially suspected by observing indications like change in media color (Fig 3.3C) and liquefaction of media. Moreover, growth of acid-fast bacilli on L-J medium was confirmed by performing Ziehl-Neelsen acid-fast staining from colonies (Fig 3.2). All 55 samples were positive for *M. tuberculosis* complex with varying degree of growth. Degree of growth for each sample was determined according to WHO scoring guideline mentioned in table 2.2. The conventional solid culture results of these samples are given in table 3.3.



Acid-fast bacilli

FIG 3.2 Acid-fast bacilli (red) revealed under bright field microscope after Ziehl-Neelsen acid-fast staining

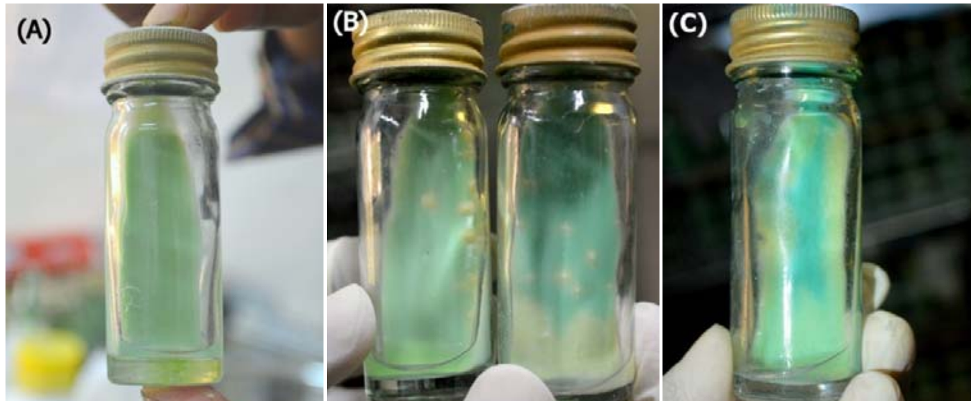


FIG 3.3 Identification by conventional solid culture method (A) Negative control tube (B) Colonies of *M. tuberculosis* grown on Lowenstein-Jensen (L-J) medium (C) A contaminated culture tube showing change in media color

3.2.4 Molecular identification by amplification of IS*6110* insertion sequence

IS*6110* sequence is present in multiple copies in almost all *M. tuberculosis* strains. All 55 samples were tested for the presence of IS*6110* sequence by PCR. For 36 out of 55 samples, DNA was extracted from *M. tuberculosis* strains grown on solid culture. All of these 36 isolates were found to be positive for IS*6110* sequence. For remaining 19 samples, DNA was extracted from sputum samples directly. All of these 19 sputum samples were positive for IS*6110* sequence.

Upon amplification, the 123-bp fragment was detected by agarose gel electrophoresis and visualized by gel documentation system. The IS*6110* specific bands are shown in figure 3.4

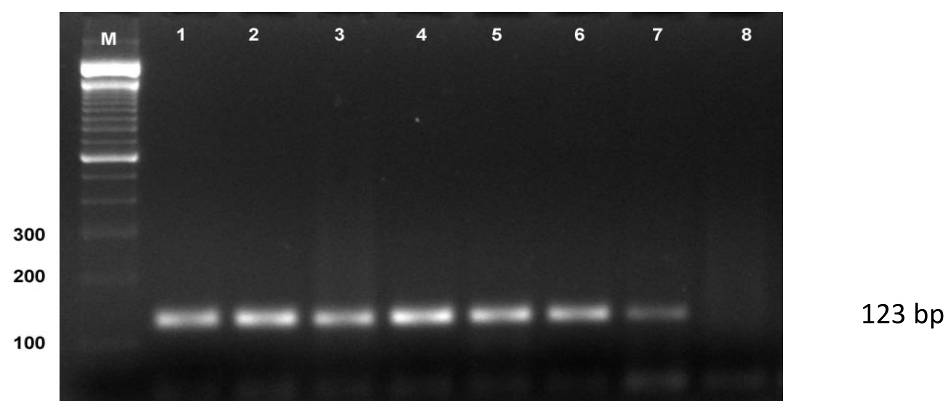


FIG 3.4 Analysis of PCR products by agarose gel electrophoresis showing 123 bp band corresponding to IS*6110* sequence in *M. tuberculosis*. Lane M: 100 bp DNA ladder; Lane 1: Positive control (MTB positive); Lane 2-7: Sample ID- 5, 9, 22, 27, 36, 37; Lane 8: Negative control

3.3 Detection of Multidrug-Resistant Tuberculosis (MDR-TB)

3.3.1 Detection of MDR-TB by drug susceptibility testing (DST)

All 55 samples were tested for drug sensitivity to four drugs such as rifampicin, isoniazid, streptomycin and ethambutol. All isolates resistant to isoniazid and rifampicin were considered as multidrug- resistant (MDR). The result shows that 4 isolates are rifampicin mono-resistant, 12 are resistant to both rifampicin and isoniazid; 10 isolates are resistant to rifampicin, isoniazid and one more drug (streptomycin or ethambutol); 24 isolates were found to be resistant to all four drugs, 5 were sensitive to all 4 drugs. The results of drug susceptibility test are given in table 3.3. This result shows that detection of rifampicin resistance alone can be used for detection of multidrug resistant tuberculosis (MDR-TB). Overall result of DST is summarized in table 3.2.

TABLE 3.2 Drug sensitivity test results of MTB positive strain of all four drugs in individual form

Name of resistant drug	Rifampicin only	Rifampicin and Isoniazid	Rifampicin, Isoniazid and Streptomycin ⁄Ethambutol	All four drugs	None
Number of resistant isolates	4	12	10	24	5

3.3.2 Detection of MDR-TB by Gene xpert

The Xpert MTB/Rif test is a cartridge-based fully automated NAAT (nucleic acid amplification test) for TB case detection and rifampicin resistance testing. The Gene Xpert can detect *M. tuberculosis* in sputum sample.

Results are reported as positive or negative for *M. tuberculosis*. The samples were simultaneously analyzed by GeneXpert MTB/RIF assay and only those which were positive for *M. tuberculosis* were included in this study. Positive results were placed in one of four categories; very low, low, medium, or high. Rifampicin resistance results were reported as susceptible or resistant. GeneXpert assay shows that 45 samples are refampicin resistant and 10 are rifampicin sensitive.

When the Xpert assay results for rifampicin resistance were compared against phenotypic DST results, 8 results were discordant. Eight samples had discordant DST results showing resistance to rifampicin but negative Xpert assay results for such resistance (false-negative Xpert assay result for rifampicin resistance). Using phenotypic DST as the reference standard, sensitivity and specificity of GeneXpert assay for the detection of Rif- resistance were 86.21% and 62.5%. The results are given in the table 3.3.

TABLE 3.3 Microscopic identification, GeneXpert, rapid culture, conventional solid culture and drug susceptibility test (DST) results of sputum samples along with patient ID, age and sex

Sample ID.	Age	Sex	Microscopic Identification Result	Gene Xpert result	Rapid Culture Result	Solid Culture Result	Drug Susceptibility Test (DST) Result			
							RIF	**INH**	**SM**	**EMB**
1	40	F	Negative	RR	Negative	2+	R	R	S	S
2	75	M	2+	RR	Positive	1+	R	R	R	R
3	30	M	2+	RR	Positive	3+	R	R	S	S
4	21	M	3+	RR	Positive	3+	R	R	R	R
5	22	M	3+	RR	Positive	3+	R	R	R	R
6	25	M	2+	RR	Positive	3+	R	R	R	R
7	25	M	1+	RR	Positive	3+	R	R	R	R
8	49	M	2+	RS	Positive	3+	R	R	R	S
9	19	F	2+	RR	Positive	3+	R	R	R	R
10	42	F	2+	RR	Positive	3+	R	R	R	S
11	60	F	Negative	RR	Positive	1+	R	R	R	R
12	21	F	2+	RR	Positive	3+	R	R	R	R
13	18	M	3+	RR	Positive	2+	R	R	R	S
14	23	M	Negative	RS	Positive	3+	R	R	R	R
15	25	F	Negative	RR	Positive	2+	R	R	R	S
16	35	M	Negative	RR	Positive	2+	R	R	R	R
17	40	M	1+	RR	Positive	3+	R	R	S	S
18	24	F	2+	RS	Positive	3+	R	R	R	R
19	13	M	Negative	RR	Negative	1+	R	R	S	S
20	26	M	3+	RS	Positive	3+	S	S	S	S
21	25	M	1+	RR	Positive	3+	R	R	S	R
22	28	F	3+	RR	Positive	1+	R	R	R	R
23	40	F	3+	RR	Positive	3+	R	R	R	S
24	27	F	2+	RR	Positive	3+	R	R	R	R
25	18	F	Negative	RR	Negative	2+	R	R	R	R
26	50	M	Negative	RR	Negative	2+	R	R	R	R
27	47	M	1+	RR	Positive	2+	R	R	S	S
28	25	M	1+	RS	Positive	2+	R	R	S	S
29	43	F	2+	RR	Positive	2+	R	R	R	R
30	37	M	1+	RR	Positive	1+	R	R	R	R
31	21	F	2+	RR	Positive	2+	R	R	R	S
32	34	M	1+	RS	Positive	2+	R	R	S	S
33	75	M	2+	RR	Positive	1+	R	R	R	R
34	21	M	3+	RR	Positive	3+	R	R	R	R

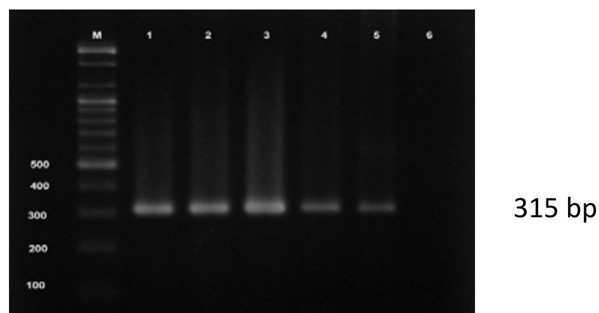
35	25	M	2+	RR	Positive	2+	R	R	R	R
36	42	F	2+	RR	Positive	3+	R	R	R	S
37	16	F	Negative	RR	Negative	1+	R	S	S	S
38	11	F	Scanty	RR	Negative	3+	S	S	S	S
39	45	F	3+	RS	Positive	3+	R	R	S	S
40	30	F	3+	RR	Positive	3+	R	S	S	S
41	40	M	2+	RR	Positive	2+	R	S	S	S
42	30	F	2+	RR	Positive	2+	R	R	R	R
43	18	F	2+	RR	Positive	2+	R	R	R	R
44	22	M	2+	RR	Positive	2+	R	R	R	R
45	22	F	2+	RR	Positive	2+	R	R	S	S
46	55	F	2+	RR	Positive	2+	R	S	S	S
47	60	F	3+	RR	Positive	3+	R	R	S	S
48	26	M	3+	RS	Positive	3+	R	S	S	S
49	45	M	2+	RR	positive	2+	R	R	S	R
50	38	M	3+	RR	positive	3+	R	R	R	R
51	35	F	3+	RS	positive	3+	S	S	S	S
52	20	F	2+	RR	positive	3+	R	R	S	S
53	13	F	scanty	RR	negative	3+	S	S	S	S
54	65	M	3+	RR	positive	3+	S	S	S	S
55	55	M	3+	RS	positive	3+	R	R	S	S

M = Male**, F** = Female**, R** = Resistant**, S** = Sensitive, **RR** = Rifampicin resistant, **RS** = Rifampicin sensitive

3.3.3 Detection of MDR-TB by molecular techniques

3.3.3.1 PCR amplification of *rpoB* gene of *M. tuberculosis* grown on culture and sputum directly

For 36 (sample ID: 1-36) of 55 samples, DNA was extracted from *M. tuberculosis* strains grown on solid culture with rifampicin / DST. For remaining 19 samples (sample ID: 37-55), DNA was extracted from sputum samples directly. All 55 samples were subjected to *rpoB* gene amplification by PCR and showed positive results. The primer pair (PR1 and PR2) was used for amplification of *rpoB* gene of all samples. The amplified PCR product of 315 bp was detected by agarose gel electrophoresis (Fig.3.5).



315 bp

Fig 3.5 Analysis of PCR products by agarose gel electrophoresis showing 315 bp band corresponding to *rpoB* gene in *M. tuberculosis*. Lane M: 100 bp DNA ladder, Lane 1: Positive control (MTB positive); Lane 2-5: Sample ID: 37-40; Lane 6: Negative control

3.3.3.2 PCR amplification of *katG* gene of *M. tuberculosis* grown on culture and sputum directly

RTB 59 and RTB 36 were the two primers used to amplify the *katG* gene of all samples. For 12 (sample ID: 1-3, 5-7, 9-13, 26) of 21 samples, DNA was extracted from *M. tuberculosis* strains grown on solid culture with isoniazid / DST. For remaining 9 samples (sample ID-47-55), DNA was extracted from sputum samples directly. All 21 samples were subjected to *katG* gene amplification by PCR and showed positive results. After PCR amplification, bands corresponding to 807-bp were observed for all samples (Fig 3.6).

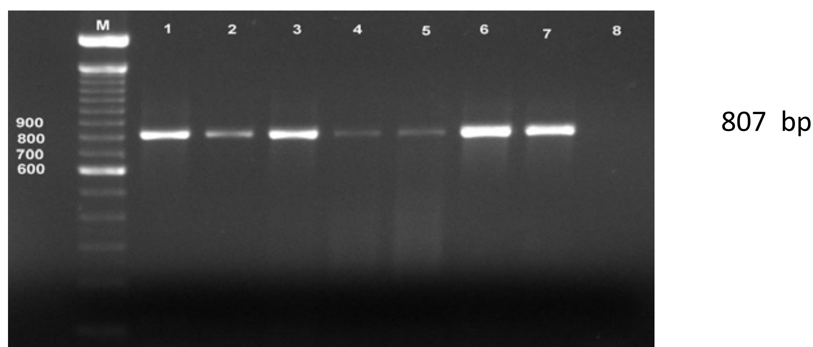


FIG 3.6 Analysis of PCR products by agarose gel electrophoresis showing 807 bp band corresponding to *katG* gene in *M. tuberculosis*. Lane M: 100 bp DNA ladder, Lane 1: Positive control (MTB positive); Lane2-7: Sample ID: 50-55; Lane 8: Negative control

3.3.3.3 Detection of *rpoB* and *katG* gene mutations by DNA sequencing

Resistance to rifampicin is associated with presence of mutation in 81bp hotspot region in *rpoB* gene. Inside this 81 bp region, mutations in codon H526 and S531 are responsible for high degree of resistance against rifampicin. Mutations in codon 511 and 516 are associated with development of low level of resistance. Presence or absence of these RIF associated mutations were detected by sequencing segment of *rpoB* gene containing the 81 bp hotspot region.

Analysis of mutations in the *katG* gene in *M. tuberculosis* may contribute to the development of reliable and rapid tests for detection of INH resistance. Mutations in codon 315 of the *katG* gene are associated with high-level resistance to isoniazid.

55 samples (sample ID: 1-55) were subjected to DNA sequencing for *rpoB* gene and 21 samples (sample ID: 1-3, 5-7, 9-13, 26, 47-55) for *katG* gene mutation analysis. After sequencing, the individual sequences were aligned and compared with the sequence of wild type reference (H37Rv) using MEGA 6 software. The pattern of mutation and change in amino acid level identified by DNA sequencing are given in table 3.4 and 3.5

3.3.3.3.1 Detection of RIF-Associated Mutations

RIF-associated mutations were found in (44/55) samples (80%). These samples showed commonly occurring mutations at codon 531, 526 and 516. Out of 55, thirty (54.54%) had mutation at codon 531; while seven (12.73%) had mutation at codon 526, five samples (9.09%) showed mutation at codon 516 and two (3.64%) had mutation at codon 511. The most frequent mutation was Ser → Leu (T**C**G→ T**T**G) occurred at codon 531. Changes of His → Asp (**C**AC → **G**AC) and His → Tyr (**C**AC → **T**AC) occurred at codon 526. Mutations at codon 516 occurred less frequently, mostly changing aspartate to valine (G**A**C → G**T**C) or tyrosine (**G**AC → **T**AC). Other resistance associated mutation occurred at codon 511, changing Leu → Pro (C**T**G → C**C**G). Nine samples showed no mutation in comparison to the standard sequence (H37Rv strain) in the targeted locations investigated, suggesting other mutation sites within *rpoB* gene. Moreover, 2 samples were found to contain both mutated and wild type sequences (Sample ID. 24 and 28). DST result of these two samples showed that both were multidrug- resistant *M. tuberculosis.*

TABLE 3.4 *rpoB* gene mutation pattern determined by DNA sequencing

Sample ID	**DNA Sequencing Result**		
	Position of mutation	**Change in nucleotide level**	**Change in amino acid level**
01	531	TCG → TTG	Ser → Leu
02	526	CAC → GAC	His → Asp
03	531	TCG → TTG	Ser → Leu
04	531	TCG → TTG	Ser → Leu
05	531	TCG → TTG	Ser → Leu
06	531	TCG → TTG	Ser → Leu
07	531	TCG → TTG	Ser → Leu

08	No mutation	No Change	No Change
09	531	TCG → TTG	Ser → Leu
10	516	GAC → GTC	Asp → Val
11	531	TCG → TTG	Ser → Leu
12	531	TCG → TTG	Ser → Leu
13	531	TCG → TTG	Ser → Leu
14	526	CAC → TAC	His → Tyr
15	531	TCG → TTG	Ser → Leu
16	531	TCG → TTG	Ser → Leu
17	526	CAC → TAC	His → Tyr
18	531	TCG → TTG	Ser → Leu
19	526	CAC → GAC	His → Asp
20	No mutation	No Change	No Change
21	531	TCG → TTG	Ser → Leu
22	531	TCG → TTG	Ser → Leu
23	531	TCG → TTG	Ser → Leu
24	526 + wild type	CAC → AAC and no mutation	His → Asn and no mutation
25	531	TCG → TTG	Ser → Leu
26	516	GAC → GTC	Asp → Val
27	516	GAC → TAC	Asp → Tyr
28	516 + wild type	GAC → GTC and no mutation	Asp → Val and no mutation

29	531	TCG → TTG	Ser → Leu
30	516	GAC → GTC	Asp → Val
31	531	TCG → TTG	Ser → Leu
32	No mutation	No change	No change
33	526	CAC → GAC	His → Asp
34	531	TCG → TTG	Ser → Leu
35	531	TCG → TTG	Ser → Leu
36	516	GAC → GTC	Asp → Val
37	531	TCG → TTG	Ser → Leu
38	511	CTG → CCG	Leu → Pro
39	No mutation	No Change	No Change
40	No mutation	No Change	No Change
41	526	CAC → GAC	His → Asp
42	511	CTG → CCG	Leu → Pro
43	No mutation	No Change	No Change
44	531	TCG → TTG	Ser → Leu
45	No mutation	No Change	No Change
46	526	CAC → TAC	His → Tyr
47	531	TCG → TTG	Ser → Leu
48	No mutation	No Change	No Change
49	531	TCG → TTG	Ser → Leu
50	No mutation	No Change	No Change
51	531	TCG → TTG	Ser → Leu
52	531	TCG → TTG	Ser → Leu

53	531	TCG → TTG	Ser → Leu
54	531	TCG → TTG	Ser → Leu
55	531	TCG → TTG	Ser → Leu

3.3.3.3.2 Detection of INH-Associated Mutations

A total of 21 samples were analyzed for INH-associated mutations. Out of 21, sixteen samples (76.19%) showed mutations at codon 315. The most commonly occurring mutation was A**G**C→A**C**C, changing serine to threonine (Ser315Thr) in the *katG* gene of *M. tuberculosis*. Five samples did not have any mutation in codon 315 when compared with the reference strain (H37Rv).

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TABLE 3.5 *katG* gene mutation pattern determined by DNA sequencing

Sample ID	DNA Sequencing Result		
	Position of mutation	**Change in nucleotide level**	**Change in amino acid level**
1	315	AGC→ACC	Ser→Thr
2	315	AGC→ACC	Ser→Thr
3	315	AGC→ACC	Ser→Thr
5	315	AGC→ACC	Ser→Thr
6	315	AGC→ACC	Ser→Thr
7	315	AGC→ACC	Ser→Thr
9	315	AGC→ACC	Ser→Thr
10	315	AGC→ACC	Ser→Thr
11	315	AGC→ACC	Ser→Thr
12	315	AGC→ACC	Ser→Thr
13	315	AGC→ACC	Ser→Thr
26	315	AGC→ACC	Ser→Thr
47	No mutation	No change	No change
48	No mutation	No change	No change
49	315	AGC→ACC	Ser→Thr
50	315	AGC→ACC	Ser→Thr
51	No mutation	No change	No change
52	No mutation	No change	No change
53	315	AGC→ACC	Ser→Thr
54	315	AGC→ACC	Ser→Thr
55	No mutation	No change	No change

3.4 Comparison of the results of drug susceptibility testing (DST) and DNA sequencing

In case of 36 isolates which were grown on DST media, 35 were resistant to RIF as found by DST, while 31 of them showed RIF-associated mutation by DNA sequencing. For 19 sputum samples, which were subjected directly to PCR amplification and sequencing, 15 of them were rifampicin resistant (RR) by DST, DNA sequencing results of these 19 samples showed that 13 samples contained RIF-associated mutations in *rpoB* gene of *M. tuberculosis*. Comparison of DST and sequencing results for *rpoB* gene are presented in table 3.6.

TABLE 3.6 Number of samples showing rifampicin resistance (RR) detected by two different techniques

Type of Sample	Number of Samples	No. of Samples Showing Rifampicin Resistance (RR) on DST Result	No. of Samples Showing Rifampicin Resistance (RR) on DNA Sequencing Result
Isolates grown on culture	36	35	31
Sputum Samples	19	15	13
TOTAL	**55**	**50**	**44**

Sensitivity and specificity of DNA sequencing for detecting of Rif-resistance: Compared with the DST results, sensitivity and specificity of sequencing for the detection of Rif- resistance were 98% and 100%.

We analyzed 21 samples out of 55 for *katG* gene. 12 isolates which were grown on DST media had INH-associated mutation by DST and DNA sequencing. For 9 sputum samples, 5 samples showed INH resistance on DST, whereas 4 samples showed INH associated mutation on DNA sequencing result. Comparison of DST and sequencing results for *katG* gene are presented in table 3.7.

TABLE 3.7 Number of samples showing isoniazid resistance detected by two different techniques

Type of Sample	Number of Samples	No. of Samples Showing Isoniazid Resistance on DST Result	No. of Samples Showing Isoniazid Resistance on DNA Sequencing Result
Isolates grown on culture	12	12	12
Sputum Samples	9	5	4
Total	**21**	**17**	**16**

Sensitivity and specificity of DNA sequencing for detecting isoniazid resistance: Compared with the DST results, sensitivity and specificity of sequencing for the detection of isoniazid resistance were 94% and 100%.

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CHAPTER- 4

DISCUSSION

AND

CONCLUDING REMARKS

4.0 DISCUSSION AND CONCLUDING REMARKS

4.1 Discussion

Tuberculosis (TB) is one of the ancient and deadliest disease of mankind, still posing a major health, social and economic burden at a global level and primarily in low and middle income countries (WHO, 2012). In the last decade, tuberculosis has reemerged as one of the leading causes of death (nearly 3 million deaths annually) (Bloom and Murray, 1992). The emergence of AIDS and decline of socioeconomic standards contribute to the disease's resurgence in industrialized countries (Barnes *et al*., 1991). The emergence and spread of drug-resistant strains of *M. tuberculosis*, especially multidrug-resistant (MDR) strains, are serious threats to the control of tuberculosis and comprise an increasing public health problem (Zumia and Grange, 2001).

MDR-TB is a potentially untreatable, transmissible disease associated with a high mortality (Shi *et al*., 2006). Patients infected with MDR strains, which are defined as strains resistant to both rifampin (RIF) and isoniazid (INH), are difficult to cure and are more likely to remain sources of infection for a longer period of time than are patients with drug-susceptible. Multidrug-resistant TB (MDRTB), associated with high death rates of 50% to 80%, spans a relatively short time (4 to 16 weeks) from diagnosis to death (Dooley *et al*., 1992). Delayed recognition of drug resistance, which results in delayed initiation of effective therapy, is one of the major factors contributing to MDRTB outbreaks, especially in health-care facilities (Edlin *et al*., 1992). The ratio of multidrug resistance (MDR) in new cases ranged from 0 to 22.3%. The highest proportion of MDR-TB reported was 60% among previously treated cases. It has been estimated that 489,139 cases of MDR-TB emerged in 2006 and the global proportion of such resistance among all cases amounted to 4.8% (WHO, 2008). In 2009, 250,000 TB patients were diagnosed and of these, 30,000 (12%) were notified as MDR TB (WHO, 2011).

In most countries, MDR-TB has increased in incidence and interferes with TB control programs, particularly in developing countries, where prevalence rates are as high as 48% (Iseman and Sbarbaro, 1992; Cohn *et al*., 1997). In Eastern Europe and central Asia, levels of MDR-TB remain high, reaching 32% of new cases and over 50% of previously treated cases in 2011 (WHO, 2012). MDR TB is an emerging threat in

Bangladesh. In 2008, the World Health Organization (WHO) ranked Bangladesh sixth among the world's 22 high-burden TB countries and 9th among 25 high priority MDR and extensively drug resistant (XDR) TB countries. In Bangladesh there is an estimated 1.4% of new TB and 29% of re-treatment TB cases with MDR (WHO, 2013).

The high infection and death rates pose an urgent challenge to rapidly detect cases (Iseman and Sbarbaro, 1992; Cohn *et al*., 1997). Expeditious identification of antimicrobial susceptibility patterns of MTB is essential for the control of MDR-TB. Slow diagnosis and insufficient testing for MDR-TB contribute to its dissemination (Victor *et al*., 2002). New molecular biology techniques are being introduced for rapid diagnosis and detection of multi-resistant strains. This is possible due to a better understanding of molecular mechanisms responsible for drug resistance in those strains (Palomino, 2009). This study assessed the usefulness of the PCR sequencing approach for *rpoB* and *katG* genes for rapid detection of MDR TB strains directly from sputum and culture to guide treatment regimens for MDR-TB. Some regions of *katG* and *rpoB* gene which had the highest percentage of mutations were investigated which will optimize the treatment and can be used in drug surveillance studies. Further, this study would be of great interest when evaluating the efficiency with which the GeneXpert can identify MDR TB. For this purpose, we collected 55 sputum samples from 55 MDR-TB patients from National Tuberculosis Reference laboratory (NTRL), NIDCH, Dhaka. By using PCR and subsequent sequencing, we were able to detect the mutations related to drug resistance to RIF and INH. Based on the obtained results, we found out that PCR amplification followed by DNA sequencing is a preferred method for detecting drug resistance in MTB in shorter time compared with conventional drug susceptibility testing.

Conventional drug susceptibility testing is time-consuming, costly and raises technical problems due to difficulties to control the inoculums size and the stability of the compounds in different culture media. Culture-based drug susceptibility testing is widely recognized as a reference method but requires several weeks and multiple methodologies to complete (Reller *et al*., 2000). Drug susceptibility testing by the conventional solid medium culture method is highly sensitive and specific but extremely slow due to the slow growth of *M. tuberculosis*. Liquid culture methods can

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reduce the turnaround time but require specialized instrumentation and reagents and are not feasible in most resource-limited settings. It is widely accepted that rapid drug susceptibility testing is critical in preventing emergence and expansion of drug resistance. Hence, there is an urgent need for new, rapid and effective diagnostics to prevent the emergence and spread of MDR.

During the past few years, molecular methods have been developed to identify drug resistance causing gene mutations (Neonakis *et al*., 2008; Viedma, 2003). One of latest techniques is the GeneXpert MTB/RIF, which can detect mutations in the *rpoB* gene only; due to close association of rifampicin resistance and MDR TB, this technique has been used to detect MDR TB cases (WHO; 2011). In our study, out of 55 samples, 45 (81.82%) had RIF-resistant *M. tuberculosis* and 10 (18.18%) were found to have RIF-sensitive *M. tuberculosis.* It is widely accepted that genotypic drug susceptibility testing has a high sensitivity and specificity as compared with conventional culture-based drug susceptibility testing, but is still unable to detect all drug-resistant strains (Müller *et al*., 2011; Barnard *et al*., 2008). Using phenotypic DST as the reference standard, sensitivity and specificity of GeneXpert assay for the detection of Rif- resistance were 86.21% and 62.5%. The sensitivity of the Xpert assay for detecting rifampin resistance has been reported to be between 60% to almost 100%, depending on the characteristics of the population being tested and the bacterial loads in their samples (Helb *et al*., 2010; Boehme *et al*., 2011; Lawn *et al*., 2011). Inconsistent results between the Xpert assay and phenotypic DST have been recognized in this study. We identified eight cases with negative Xpert assay and positive phenotypic DST results for rifampicin resistance; four of them were also found to be negative by DNA sequencing. Discrepancies between Xpert MTB/RIF and DST results regarding rifampin resistance (false resistance or false sensitivity) have also been reported previously (Boehme *et al*., 2010; Hillemann *et al*., 2011; Marlowe *et al*., 2011). Since drug susceptibility could only be diagnosed from the growth of *M. tuberculosis* in culture which can take as long as six weeks, selection of resistant strains during this prolonged incubation period might lead to an apparent switch from a susceptible to a resistant phenotype.

The polymerase chain reaction (PCR) sequencing-based strategy, designed to detect mutations associated with drug resistance rapidly, is able to provide a "same-day" diagnosis from culture and even clinical samples with high sensitivity and specificity. This gold standard method can also detect new mutations that could be associated with drug resistance. Automated sequencing has been used by several groups in the clinical setting for detection of the most frequent mutations and has been found to confer excellent benefit for patient care (Kourout *et al*., 2009; Abdelaal *et al*., 2009; Choi *et al*., 2010). In this study, for preliminary detection of MTB, the PCR assay was performed using the primers based on the *IS6110* gene. Based on PCR and sequencing, the most common mutations related to drug resistance were demonstrated as RIF (80%) and INH (76.19%). In comparison to DST, sensitivity and specificity for the detection of Rif- resistance were 98% and 100%; sensitivity and specificity for the detection of isoniazid resistance were 94% and 100%.

Resistance to RIF can be used as a marker for MDR-TB, because it is usually preceded by that to INH, isolated resistance to RIF being extremely rare (Mokrousov *et al*., 2003; Gillespie, 2002). In approximately 98% of cases, RIF-resistance is caused by mutations in the *rpoB* gene encoding the RNA polymerase- subunit (Victor *et al*., 2002; Ramaswamy and Musser, 1998; Mokrousov *et al*., 2003; Telenti *et al*., 1993). Most mutations occur in the short 81-bp region in *rpoB* gene (codons 507 to 533) (Ramaswamy and Musser, 1998; Telenti *et al*., 1993; Cavusoglu *et al*., 2002), and according to Mokrousov *et al*., up to ninety five per cent of RIF-resistant strains have mutations in codons 531, 526 and 516 (Mokrousov *et al*., 2003). Ninety five per cent of rifampicin resistances of *M. tuberculosis* strains have mutations from 511 to 533 codons and the rest of them have mutations in 481, 505, 508 and 509 codons (Simon *et al*., 1998). DNA sequencing of segment of *rpoB* gene was performed for all 55 samples. In this assay, some mutations had been identified in 516, 526 and 531 codons of *rpoB* gene.

The most frequent mutation found among the samples was located at codon 531 (54.54%). This is in accordance with reports from other countries: India-54.5% (Mani *et al*., 2001), Brazil -54% (Andre *et al*., 2000), Greece- 53% (Bernard *et al*.,1998), Iran-53.3% (Khosravia *et al*., 2012), Turkey - 56.1% (Cavusoglu *et al*., 2002), Pakistan- 56.45% (Yasmin *et al*., 2014), Italy-59.5% (Pozzi *et al*., 1999), Italy - 60.4% (Miotto *et al*., 2006), China -63.2% (Huang *et al*., 2009), Romania -63.82%

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(Cătălina *et al*., 2013) and Bulgaria - 65% (Valcheva *et al*., 2008). However, our study showed great differences from some countries, Hungary with a lower frequency -31% (Bártfai *et al*., 2001) and Republic of Moldova with a higher frequency -86.8% (Crudu *et al*., 2012); Germany - 75.7% (Hillemann *et al*., 2005), Ukraine -73% (Dymova *et al*., 2011).

Mutation in codon 526 occurred at a rate of 12.73%. This is in accordance with reports from other countries: Ukraine -12.5% (Miotto *et al*., 2006), Germany - 13.6% (Dymova *et al*., 2011), Turkey-17.1% (Hillemann *et al*., 2005), Taiwan-18.9% (Hwang *et al*., 2003), China -10% (Barnard *et al*., 2008), Romania-10.63% (Cătălina *et al*., 2013). Other studies also mentioned low frequencies for this type of mutation in countries like: Republic of Moldova - 0.9% (Crudu *et al*., 2012), Hungary - 6.9% (Bártfai *et al*., 2001), Italy - 6.5% (Cavusoglu *et al*., 2002), South Africa - 8.6% (Huang *et al*., 2009).

The less common mutation was found in codon 516 (9.09%) which has a similarity with the mutation rate 9.6% in South Africa (Hillemann *et al*., 2005) and China-10.5% (Ling *et al*., 2010), but higher than in some countries: China - 2% (Huang *et al*., 2009), Germany- 2.9% (Barnard *et al*., 2008), Republic of Moldova - 4.4% (Crudu *et al*., 2012), Pakistan- 4.84% (Yasmin *et al*., 2014), Brazil -7% (Andre *et al*., 2000) and Ukraine - 8% (Dymova *et al*., 2011) and much smaller than in Hungary - 37.9% (Bártfai *et al*., 2001).

Lower resistance is attributed by the mutations at codon 511 (Moghazeh *et al*., 1996; Bodmer *et al*., 1995, Yasmin *et al*., 2014, Madania *et al*., 2012). In this study, 2 samples had mutation in codon L511 (3.64%). Moreover, DNA sequencing of 2 samples were found to contain both mutated and wild type sequences. DST result of these two samples showed that both were multidrug resistant. It is possible that these samples comprised a heterogeneous population of organisms with both wild-type and mutated alleles in the *rpoB* gene, leading to amplification of the corresponding wild-type PCR product.

However, Isoniazid (INH) is one of the most effective and specific agents for the treatment of infection with *M. tuberculosis*. Recent increases in INH-resistant (INHr) and multidrug-resistant (MDR) tuberculosis are jeopardizing the continued utility of

this drug (Espinal *et al*., 2001; WHO, 2000). Furthermore, the development of INH resistance is a common first step in the evolution to MDR (Dye and Espinal, 2001). Between 40 and 95% of INHr, clinical *M. tuberculosis* isolates have mutations in *katG*, 75 to 90% of which are located in codon 315, with 10 to 25% of mutations located in other *katG* loci (Escalante *et al*, 1998; Mokrousov *et al*., 2004; Ramaswamy *et al*, 1998, 2004, 2003; Soolingen *et al*., 2000). The catalase-peroxidase coding *katG* gene is the most commonly targeted, with the majority of mutations occurring at codon 315 (Musser *et al*., 1995). Mutations in *katG*315 may be favored because mutations at this location appear to decrease INH activation without abolishing catalase-peroxidase activity, a potential virulence factor (Kapetanaki *et al*, 2005). In this study, 21 samples were subjected to DNA sequencing for *katG* gene. Our findings showed that all of the resistance mutations to INH occurred in codon 315 (76.19%) and this was in agreement with other mentioned studies showing the major involvement of this codon in INH resistance all over the world. The percentage of efficient mutations in isoniazid resistance has been reported by researchers, such as 91.7% in Petersburg, 62.2% in New York, 59.2% in South Korea, 34.6% in Madrid, 32% in Barcelona and 0% in Akvatvryal Gvynh which they had mutations in 315 codon region (Aktas *et al*.2005; Herrera *et al*., 2004).

4.2 Concluding Remarks

In conclusion, the results of this study suggested that molecular techniques can be used as a potentially rapid and sensitive alternative to conventional diagnostics for the identification of drug resistance *M. tuberculosis* from culture and sputum directly. The present study focused on the use of PCR based sequencing as a potential screening method for MDR-TB in our country. Our sequencing-based method can rapidly and efficiently assess MDR *M. tuberculosis*. We described PCR-based method for simultaneous detection of mutations in *rpoB* and *katG* genes responsible for resistance to isonizid and rifampicin. The technique allowed the detection of MDR *M. tuberculosis* circulating in our country, by identifying the prevalent mutations which appear in codons 531 (54.54%), 526 (12.73%) and 516 (9.09%) for *rpoB* gene and in codon 315 (76.19%) for *katG* gene. Thus, the technique could represent a useful diagnostic tool, offering the advantage of rapidity and the possibility for optimization

of antibiotic therapy. This strategy does have a few disadvantages. The molecular assays only detect known mutations, which is the most important limitation in detection of drug resistance by such techniques. Moreover, the DNA sequencer and sequencing are costly, and the procedure is somewhat complicated. However, this issue may be addressed if DNA sequencing costs are reduced by new sequencing methods and equipment.

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APPENDIX-I

Media composition

1. Lowenstein-Jensen Media Preparation

A. Preparation of Salt solution

Ingredients	For 1000 mL
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	1.5 gm
Magnesium sulfate	0.15 gm
Magnesium citrate	0.375 gm
Asparagine	2.25 gm
Glycerol	7.5 mL
Distilled water (Up to)	375 mL

B. Preparation of 2% Malachite Green

Distilled Water	50 mL
Malachite Green	1 gm

C. Preparation of complete L-J media

Ingredients	For 1000 mL
Salt solution	375 mL
Malachite Green	12.5 mL
Homogenized Egg	625 mL

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APPENDIX-II

Buffers and Reagents

A. Lysozyme

Lysozyme (10mg/ml) was made by dissolving 10 mg of Chicken egg white lysozyme (sigma grade-4) in 1 ml of Tris/EDTA/NaCl (TEN) buffer.

B. 10%SDS

10gm SDS was dissolved in 100ml distilled H_2O.

C. Proteinase-K (10mg/ml)

50 mg of proteinase-K was dissolved in 5 ml TE-buffer.

D. 5M NaCl

292 NaCl was added to distilled water and made the volume upto 1L.

E. 70% ice-cold ethanol

70ml ethanol was added to 30ml dist.H2O and stored at -20°C.

F. 10x-TBE (Tris-borate –EDTA pH 8.0)

108 gm of Tris-base, 55 gm of boric acid and 40m1 of 0.5 M EDTA (pH 8.0) were taken and distilled water was added to the mixture to make 1000 ml. The buffer was stored at room temperature.

G. 0.5 M EDTA

18.61 gm of $Na_2EDTA.2H_2O$ (disodium ethylene diamine tetra-acetic acid) was dissolved in 80 ml of distilled water and the pH was adjusted to 8.0 with pellets of NaOH. The final volume was made up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

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H. 1x-TE buffer (Tris-EDTA)

TE buffer (10 mM Tris-Cl/1mM EDTA, pH 8.0) was prepared was prepared by diluting concentrated stocks of 1 M Tris-Cl and 0.5 M EDTA in distilled water. For making 1000ml or 1L of TE buffer, 10 ml of 1 M Tris-HCl (pH 8.0) and 2 ml EDTA (0.5 M) and distilled water added up to 1000ml. The buffer was autoclaved and was stored at room temperature.

I. 6X-Gel loading buffer (Glycerol & bromophenol blue)

3ml glycerol (30%)

25mg bromophenol blue (0.25%)

Distilled H_2O to 10mL

J. EtBr (0.5 µg/ml)

10 µl of 10 mg/ml Ethidium bromide solution was added to 200 ml distilled water.
This solution was stored at room temperature and covered with aluminum foil.

K. 0.85% NaCl

0.85 g of NaCl, upto 100 ml by distilled water.

L. Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 8.0 gm of NaCl, 0.2 gm of KCl, 1.44 gm of Na_2HPO_4 and 2.0 gm of KH_2PO_4 in 800 ml distilled water. pH was adjusted to 7.4 with HCL. The final volume was adjusted to 1 L by distilled water.

APPENDIX-III

A. The amount of template needed to sequence a PCR product

Template Size(per reaction)	Concentration (ng/µl)	Amount needed (ng)
100-200 bp	5	25-30
200-500 bp	5-16	25-100
500-1000 bp	16-42	100-250
1000-2000 bp	42-83	250-500
> 2000 bp	83-100	500-600

B. Short name and single letter to identify amino acid

Alanine ALA (A)

Asparagine ASN (N)

Cysteine CYS (C)

Glutamine GLN (Q)

Histidine HIS (H)

Leucine LEU (L)

Methionine MET (M)

Proline PRO (P)

Threonine THR (T)

Tyrosine TYR (Y)

STOP=TGA, TAA, TAG (Termination signal)

Arginine ARG (R)

Aspartic acid ASP (D)

Glutamic acid GLU (E)

Glyeine GLY (G)

Isoleucine ILE (I)

Lysine LYS (K)

Phenylalanine PHE (F)

Serine SER (S)

Tryptophan TRP (W)

Valine VAL (V)

START= Start codon (ATG)

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C. The most commonly used symbols in molecular sequence are

R	A or G	PURINE
Y	C or T	PYRIMIDINE
M	A or C	AMINO
K	G or T	KETO
N	A, T,G C	ANY

D. Table of standard genetic code of amino acid

	T	C	A	G
T	TTT Phe (F) TTC Phe (F) TTA Leu (L) TTG Leu (L)	TCT Ser (S) TCC Ser (S) TCA Ser (S) TCG Ser (S)	TAT Tyr (Y) TAC Tyr (Y) TAA Stop TAG Stop	TGT Cys (C) TGC Cys (C) TGA Stop TGG Trp (W)
C	CTT Leu (L) CTC Leu (L) CTA Leu (L) CTG Leu (L)	CCT Pro (P) CCC Pro (P) CCA Pro (P) CCG Pro (P)	CAT His (H) CAC His (H) CAA Gln (Q) CAG Gln (Q)	CGT Arg (R) CGC Arg (R) CGA Arg (R) CGG Arg (R)
A	ATT Ile (I) ATC Ile (I) ATA Ile (I) ATG Met (M) Start	ACT Thr (T) ACC Thr (T) ACA Thr (T) ACG Thr (T)	AAT Asn (N) AAC Asn (N) AAA Lys (K) AAG Lys (K)	AGT Ser (S) AGC Ser (S) AGA Arg (R) AGG Arg (R)
G	GTT Val (V) GTC Val (V) GTA Val (V) GTG Val (V)	GCT Ala (A) GCC Ala (A) GCA Ala (A) GCG Ala (A)	GAT Asp (D) GAC Asp (D) GAA Glu (E) GAG Glu (E)	GGT Gly (G) GGC Gly (G) GGA Gly (G) GGG Gly (G)

APPENDIX-IV

Instruments

The important equipments used through the study are listed below:

Autoclave, Model no: HL-42AE	Hirayama corp, Japan
Sterilizer, Model no: NDS-600D	Japan
Class II Microbiological safety cabinet	Labcaire, USA
Room temperature horizontal shaker	Gerhardt, Germany
Water bath, Model:SUM	England
Electric balance, Scout, SC4010	USA
Freezer (-30°C)	Liebherr, Germany
Refrigerator (4°C)	Vest frost
Incubator	Japan
Gel documentation	Sigma, USA
Horizontal gel elctrophoresis apparatus Hl-SET	UK
Microcentrifuge, Mikro20	Germany
Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
Automated thermocycler, Model: 12137	Bio-Rad, USA
Power pack	Toledo, Germany
pH meter, Model no: MP220	Eppendorf, Germany
Centrifuge, Mode1:5804	Eppendorf, Germany