DETERMINATION OF EMERGING DRUG RESISTANCE, HUMORAL IMMUNE RESPONSE AND ANTIGEN ANALYSIS OF *MYCOBACTERIA* Sp.

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BY

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This thesis is submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D) under the faculty of Post Graduate Medical Science & Research, University of Dhaka. This work has been carried out at the Department of Microbiology, BIRDEM, Dhaka. This is an original and innovative type of work and to the best of my knowledge it has not been done any where in Bangladesh.

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This thesis titled "Determination of emerging drug resistance, humoral immune response and antigen analysis of *Mycobacteria* Sp. "is submitted by Dr. Md. Mohiuddin, Regn No- 169/ 2006-07 (Re regn.) in the fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D) under the University of Dhaka. This is an original and innovative type of work and so far has not yet been done elsewhere in the country. This work is interesting to us and is being approved by:

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Abstract

Tuberculosis is a major public health problem in Bangladesh. It is estimated that 300,000 new cases occur and 70,000 people die every year. Drug resistance tuberculosis is wide spread and is now a threat to tuberculosis (TB) control program in many courtiers including Bangladesh. The resistance pattern of tuberculosis changes over time. Therefore, rapid detection of *M. tuberculosis* is important for case detection and effective control. The current methods of diagnosis are time-consuming. Therefore, new diagnostic methods are urgently needed. Serological assays remain attractive for use in resource-limited settings because these are simple, rapid and inexpensive and offer the possibility of detecting cases often missed by routine sputum smear microscopy.

In view of the above, the present study was undertaken to determine the drug resistance pattern of mycobacteria and the predictive efficacy of rifampicin as a surrogate marker for resistance to other anti-tubercular drugs. A molecular method was used to detect *M. tuberculosis* and *Mycobacterium* other than tuberculosis (MOTT) directly from clinical samples. Humoral immune response was also studied against specific mycobacterial antigens.

A total of 300 suspected cases of pulmonary and extra pulmonary tuberculosis cases were included in the study. Samples were subjected to culture in Lowenstein Jensen (L-J) medium and thermostable multiplex PCR for the detection and identification of mycobacteria. Drug resistance to first line anti-tubercular drugs namely isoniazid (INH), rifampicin (RIF), ethambutol (ETHAM) and streptomycin (SM) were determined by proportion method. IgM and IgG antibody response to purified antigens namely Ag 85 complex, culture filtrate protein (CFP), lipoarabinomannan (LAM) and cell wall fraction (CWF) was determined by enzyme linked immunosorbant assay (ELISA).

Out of 300 samples, there were 255 sputum and 45 lymph node (LN) aspirates from pulmonary and extra pulmonary tuberculosis cases respectively. *M. tuberculosis* was

isolated from 66.6% cases. Out of total isolates, MOTT was isolated from 4.0% cases (2.2% from sputum and 20.0% from lymph node aspirates).

A total of 45 culture positive samples were subjected to multiplex PCR. Out of 45, 42 (93.3%) was positive by PCR while 3 yielded negative results. Out of 42 PCR positive, 2 were identified as MOTT. These 2 isolates of MOTT were also identified as MOTT by culture and biochemical tests. The sensitivity of PCR was 93.7%, but the specificity could not be determined as two PCR positive but culture negative cases were not 'false positive'. Both responded to anti-tubercular treatment.

Overall drug resistance of *M. tuberculosis* and MOTT was 53.6% and 100% respectively to any first line drugs. Rate of multi drug resistant tuberculosis (MDR-TB) among new cases was 4.2% while it was 36.0% in treated cases. It was found that 83.3% rifampicin resistant *M. tuberculosis* was cross resistant to one or more other first line anti-tubercular drugs while cross resistance of INH, ETHAM and SM resistant isolates was much low.

The mean serum IgM and IgG antibodies against Ag 85 complex was significantly higher ($p<0.001$) in tubercular patients than that of healthy control subjects (OD value of IgM 1.176 versus 0.557 and IgG 1.5 versus 0.86). Similarly, the mean OD values of serum IgM and IgG antibodies against CFP were significantly higher in patients ($p<0.006$; $p<0.001$) than that of healthy control subjects. Antibody response (IgM and IgG) to LAM antigen in tuberculosis patients and healthy control subjects revealed that mean IgM antibody ($p<0.04$) to LAM was higher in the healthy control subjects than that of patients. But mean OD of IgG antibody to LAM was significantly higher ($p<0.0001$) in the serum of patients than that of healthy control subjects. Similar significantly higher ($p<0.001$) IgM antibody was observed in healthy subjects than that of patients against CWF. It was observed that out of 30 patients, Ag 85 complex IgM antibody was positive in 66.67% patients and it's sensitivity and specificity was 75.0% and 96.7% respectively. The anti-Ag 85 complex IgG was positive in 70.0% of patients and sensitivity and specificity was 76.9% and 93.75% respectively. The sensitivity and specificity of anti-

CFP IgM was 60.0% and 96.7% respectively while for IgG it was 69.8% and 93.75% respectively. But low level of sensitivity was observed for IgM and IgG antibodies against LAM and CWF.

The present study, therefore, revealed that high level of drug resistance exist to individual anti-tubercular drugs and MDR-TB is an emerging problem particularly in treated cases. Rifampicin resistance could be used as a marker for drug resistance to other drugs and could obviate the necessity of doing susceptibility test with other drugs in a resource constraint situation. Multiplex PCR in a thermostable format may be applicable for rapid detection of *M. tuberculosis* and MOTT. Serological tests utilizing specific antigen may be used as an adjunct test for diagnosis of tuberculosis where other diagnostic tools are lacking or ineffective. Lack of response to some antigens indicates underlying pathophysiology of tubercular infection.

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
AFB	Acid Fast Bacilli
BCG	Bacillus of Calmette and Guerin
BACTEC	Radiometric Mycobacterial Culture System
BIRDEM	Bangladesh Institute of Research in Diabetes, Endocrine & Metabolic Disorders
CWF	Cell Wall Fraction
CFP	Culture Filtrate Protein
DOTS	Directly Observed Treatment, Short course
DMCH	Dhaka Medical Hospital
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbant assay
EDTA	Ethylenediaminetetraacetic acid
EPTB	Extra Pulmonary Tuberculosis
ESAT	Early Secretory Antigen Target
ETHAM	Ethambutol
HIV	Human Immuno Deficiency
IgM	Immunoglobulin M
IgG	Immunoglobulin G
ICDDR,B	International Centre for Diarrhoeal Disease Research, Bangladesh
IGRA	Interferon Gamma Released Assays
INH	Isoniazid
IUATLD	International Union Against Tuberculosis and Lung Diseases

kDa	Kilo Dalton
L-J	Lowenstein Jensen
LAM	Lipo arabino mannan
LiPA	Line Probe Assay
MDR	Multi Drug Resistant
MODS	Microscopic Observation of Broth Culture
MGIT	Mycobacterial Growth Indicator Tube
MOTT	Mycobacterium Other Than Tuberculosis
NTM	Non-Tuberculous Mycobacteria
NTP	National Tuberculosis control Program
NaOH	Sodium Hydroxide
NALC	N-Acetyl-L-Cysteine-Sodium Hydroxide
NIDCH	National Institute Of Diseases of Chest and Hospital
PZA	Pyrazinamide
PAS	Para Aminosalicylic Acid
PNB	Para-Nitro Benzoic Acid
PBS	Phosphate Buffer Saline
PCR	Polymerese Chain Reaction
RIF	Rifampicin
SSCP	Single Strand conformation Polymorphism
SM	Streptomycin
TCATI	Tuberculosis Control and Training Institute
TMB	Tetramethylbenzidine
TDR	Totally Drug Resistant

TB	Tuberculosis
µl	Microliter
µg	Microgram
WHO	World Health Organization
XDR	Extensively Drug Resistant
XXDR	Extremely Drug Resistant
Z-N	Ziehl-Neelsen

CHAPTER 1

INTORDUCTION

1.1. Introduction

Tuberculosis caused by *M. tuberculosis*, is the largest single infectious cause of human mortality. The incidence of TB has remained high in most of the developing countries and the disease has recently reemerged as public health problem in the industrialized countries (Andersen, 1997). TB currently holds the seventh place in the global ranking of causes of death (Dye, 1999). In 2009 there were an estimated 9.4 million incident cases and 1.3 million deaths occurred among HIV negative TB cases (WHO, 2010). Most of the estimated number of cases in 2009 occurred in Asia (55%), Africa (30%), Eastern Mediterranean region (7%), Eastern Europe (4%) and the region of the Americas (3%). The 22 high burden countries account for 81% of all the estimated cases (WHO, 2010).

Tuberculosis is a major public health problem in Bangladesh. The country ranks 6th out of 22 highest burden TB countries in the world. It is estimated that 300,000 new cases occur each year, of which about half of them are infectious TB. It is further estimated that about 70,000 people die every year (WHO, 2011) and hence, each hour eight persons die of the disease. The national tuberculosis prevalence survey was conducted from October 2007 to March 2009. This was a cross sectional community based multistage cluster survey in 40 clusters (20 urban and 20 rural). The survey included 52,098 persons of $\geq$ 15 years. The overall crude prevalence of new smear-positive tuberculosis was 63.3 per 100,000 among persons $\geq$ 15 years. The adjusted prevalence of smear positive tuberculosis was higher in rural (86.0 per 100,000) than urban areas (51.1 per 100,000) and among males (121.7 per 10,000) than females (40.3 per 100,000). The prevalence was much higher among the lowest socioeconomic groups (ICDDR, B, 2010).

Drug resistance TB is wide spread and is now a threat to TB control program in many courtiers (Johnson *et al.*, 2006). Shortly after the first introduction of anti-tubercular drugs like streptomycin (SM), isoniazid (INH), para-amino salicylic acid (PAS), resistance to these drugs was observed in clinical isolates of *M. tuberculosis* (Crofton and Mitchison, 1948). By the end of the 1960s, rifampicin (RIF) was introduced. There was decline in drug resistant and susceptible mycobacteria in developed countries with use of RIF in combination therapy. This led to a decline in funding and interest in TB control programs. As a result, no concrete monitoring of drug resistance was carried out for the following 20

years (Espinal, 2003). The arrival of HIV/AIDS in 1980s resulted in an increase in transmission of TB associated with out break of MDR-TB (Edlin *et al.*, 1992; Fischl *et al.*, 1992)

Globally the median prevalence of drug resistance to any drug in new cases was highest in South East Asia (19.8%) followed by Western Pacific (11.4%) and it was least in Europe (8.4%). The median prevalence of drug resistance to any drug in treated cases was highest in Eastern Mediterranean (63.3%) followed by South East Asia (39.9%) and it was least in Europe (15.9%). MDR-TB was highest in Eastern Mediterranean (48.3%) followed by South East Asia (20.4%) and it was least in Europe (4.7%) (WHO, 2006).

Data on 77 geographical sites collected from 1999 to 2002 showed that the highest prevalence of MDR-TB among previously treated cases was 58.3% in Oman and 56.4% in Kazakhstan. Multi-drug resistant TB is now thought to afflict 1 to 2 million patients annually (Mitnick *et al.*, 2008). Total number of MDR-TB cases in 2004 was 4.3% and among the newly infected cases and previously treated case were 2.7% and 18.5% respectively world wide (Zignol M *et al.*, 2006). In India, the rate of MDR-TB ranged from 0% to 14.2% in new cases and 0% to 58.3% in previously treated cases. The resistance to INH ranged from 10.0%-30.41%, to SM 8.0%-46.95%, to RIF 1.0%-58.55%, to ETHAM 3.67%-5.0% (Pereira *et al.*,2005; Deodhar *et al.*,1999 ; Shah *et al.*, 2002 ; Paramasivan *et al.,* 2004). In Pakistan, the rate of MDR-TB in new cases ranged from 11.9% to 12.2% and in treated cases it was 23%-27%. It was also showed that resistance to INH was 23.6%, to RIF 26.6%, to SM 19.6% and to ETHAM 11.8% (Iqbal *et al.*, 2003; Ikram *et al.*, 2008). In Turkey, for the period of 1982-1991 and 1992-1999 the resistance rates of *M. tuberculosis* to INH, SM, RIF and ETHAM was 14.2%, 14.4%, 10.5% and 2.7% and 14.4%, 21.1%, 10.6% and 2.4% respectively while MDR-TB was 7.3% and 6.6% respectively (Kart *et al.*, 2002).

The population weighted mean of MDR-TB in the South East Asian Region was 2.8% among new cases and 18.8% among previously treated cases with over 80% of these cases residing in Bangladesh, India, Indonesia, Myanmar and Thailand. XDR-TB has also been isolated in samples from India, Indonesia, Bangladesh and Thailand (WHO, 2011). There is no national data on drug resistance in Bangladesh. A few studies were conducted in sub national level. As per WHO report, estimated MDR-TB rate in Bangladesh was 1.8%

among new cases and 14% among previously treated TB cases (WHO, 2009a). A study conducted by the International Center for Diarrhoeal Disease and Research and Shyamoli Chest Disease Clinic on samples of 652 patients and found 3% and 15% MDR-TB among new and previously treated TB patients respectively (Zaman *et al.*, 2005). The Damien Foundation has also conducted two studies on drug resistance in 1995 and 2001 comprising 645 and 1041 patients respectively, where it was found that the overall MDR-TB has fallen from 0.7% to 0.4% in new cases and 6.8% to 3.0% in previously treated patients (Deun *et al.*, 1999; Deun *et al.*, 2004). However, this finding was not substantiated by further studies. In a study conducted in 2005-2006 by NTP-NIDCH on 96 patients of category II failures, 88% were found with MDR-TB (WHO, 2009a). NTP-NIDCH conducted another study with 1123 patient and found 87% MDR-TB in category II failure and 13% were in category I failure (Rahman *et al.*, 2009).

In Bangladesh, isolated surveys have indicated the rate of MDR-TB among newly diagnosed cases ranged from 0.4% to 3.0% and among treated cases it was 3.0% to 15.4%. In a published report, 63 isolates of sputum samples from different areas of Bangladesh were studied in NIDCH in 2005 and drug susceptibility testing was done on 42 of 63 isolates. Among 42 strains, 35 (83%) were MDR. Among these MDR strains, 40% were found resistant to any of the second line drugs (Deun *et al.*, 2008). In 1999, a study conducted in rural areas of Bangladesh reported initial resistant to INH as 5.4% and to RIF 0.5% while acquired resistance to INH and RIF as 25.9% and 7.4% respectively. MDR -TB was only one in new case and 5.5% in treated cases (Deun *et al.*, 1999). A study involving 106 TB cases reported that 29.7% of isolated *M. tuberculosis* was resistant to at least one drug, 4.95% to two drugs, 2.97% to three drugs. In this study, 15.8% was resistant to INH, 10.9% was resistant to RIF, 6.9% to SM and 3% to ETHAM and MDR was 4.95% (Miah *et al.*, 2000). Therefore, it appears that resistance to anti-tubercular agents is prevalent in both rural and urban areas of Bangladesh. MDR-TB is also present at a variable rate in new and treated cases.

Recently, it has been found that some of the multi drug resistant *M. tuberculosis* has become resistant to quinolones and at least to one second line injectable anti- tubercular drugs (namely amikacin, kanamycin and capreomycin). These *M. tuberculosis* has been termed as extensively drug resistant tuberculosis (XDR-TB). Globally 25,000 cases of

XDR-TB are estimated to emerge every year and 69 countries including Bangladesh have reported at least one case of XDR-TB by the end of 2010 (WHO, 2011). The pattern of drug resistance continuously changes over time in a given area and with the use of anti-tubercular drugs. Therefore, it is important to determine the rate of drug resistance at a certain interval. In Bangladesh, studies carried out so far were done with small number of isolates and no attempt was made to determine the presence of cross resistance among RIF resistant *M. tuberculosis*.

Tuberculosis may involve any organ or system in the body and is classified as pulmonary and extra pulmonary tuberculosis (EPTB). Common sites of EPTB include lymph nodes, pleura, abdominal organs and osteo-articular areas (Mariorie *et al.*, 2005). Lymph node involvement is the commonest form of EPTB. In developing countries where the incidence of TB is high, tubercular lymphadenitis (TBL) is one of the most frequent causes (30-52%) of lymphadenopathy (Gupta *et al.,* 1992). In Bangladesh, lymph node tuberculosis was found to be common (36.2%) among the EPTB (Karim *et al.*, 2006). Lymphadenitis by MOTT requires long term treatment for 12-18 months (Kanlikama *et al.*, 1997). Therefore, rapid and accurate diagnosis of TBL is of prime importance because delayed chemotherapeutic intervention is associated with poor prognosis (Clarridge *et al.*, 1993).

Microscopic examination of sputum is the only rapid, relatively simple, and inexpensive test for diagnosis of active pulmonary and extra pulmonary tuberculosis. But, the reported sensitivity of Z-N staining of unprocessed sputum smears from adults is only 40 to 70% because 5×10^3 to 5×10^4 organism/ml specimen is needed for the detection of AFB (Pfyffer and Vincent, 2005). Culture of mycobacterium is also done for isolation and identification of MTB but it is a cumbersome, bio-hazardous and needs bio-safety cabinet. It also needs an average time of 23.6 days on L-J media (Moore *et al.*, 2004). Sensitivity and specificity of this method is 48.9% and 100% respectively (Negi *et al.*, 2005). In newer liquid culture method like Microscopic Observation of Drug Susceptibility Assay (MODS), about nine days is required for culture and drug susceptibility (Caviedes *et al.*, 2000) and its sensitivity is 92% and specificity 94.4% (Caviedes *et al.*, 2000; Ha *et al*,. 2009). But in this method chance of contamination is more and skilled laboratory personnel are required and it is bio-hazardous also. The average turnaround time for other liquid based culture methods like

MGIT (Mycobacterium growth Indicator Tube) and automated systems like BACTEC is around 6.5 to 9 days with specificity between 80-100% (Li-li *et al.*, 2011).

Therefore, many investigators have used molecular techniques like polymerase chain reaction (PCR) or hybridization for the diagnosis of pulmonary and extra pulmonary tuberculosis by comparing the results with those of acid fast stained smears and culture. Advantage of PCR is that the mycobacteria specific DNA can be amplified and can be detected within a few hours directly from the clinical samples with sensitivity and specificity ranging from 65-98% and 70-100% respectively (Abe *et al.,* 1993 ; Claridge *et al.*, 1993; Miller *et al.*, 1994; Notle *et al.*, 1993; Shawar *et al.*, 1993; Yuen *et al.*, 1993; Cohen *et al.,* 1998; Querol *et al.*, 1995; Gopinath *et al.,* 2009; Bennedsen *et al.,* 1996; Hajia *et al.*, 2009; Jafarian *et al.,* 2008; Beige *et al.*, 1995; Ginesu *et al.*, 1998; Mustafa *et al.,* 1999; Rodriguez *et al.*, 1997). The researchers concluded that PCR might be a useful tool for the diagnosis of tuberculosis within a day. So far, there is only one reported study from Bangladesh which showed the sensitivity of PCR as 92% with specificity of 70% (Parvez et *al.,* 2003). But no study has yet been done on the diagnosis of EPTB by PCR in Bangladesh.

The current understanding of humoral immune response to tuberculosis is from the serological studies that aimed in developing serological assays to diagnose tuberculosis. Genome analysis has revealed that there are about 113 secreted and 49 excreted proteins of *M.* tuberculosis. The proteins of mycobacteria induce variable degree of humoral immune responses in infected people. The most studied secreted proteins of *M. tuberculosis* are ESAT-6 (early secretory antigen target), CFP-10, 38kDa, 16kDa and Ag85 complex. The ability of these proteins to elicit serological response makes them to be utilized as the candidates for sero-diagnosis. The other proteins eliciting humoral immune response are cell wall fraction (CWF) and lipoarabinomannan. Serological methods have been regarded as attractive tools for rapid diagnosis of tuberculosis due to their simplicity, rapidity and low cost. Serodiagnosis also does not require safety measures associated with handling of live bacilli as in culture and offer the possibility of detecting cases often missed by routine sputum smear microscopy. It is estimated that a rapid and widely available diagnostic assay with 85% sensitivity and 95% specificity would result in 400,000 fewer deaths each year and would greatly reduce the global health cost of TB (Ireton *et al.*, 2010). Some

serological tests are available with variable degree of sensitivity and specificity. The reactive antigens demonstrated various levels of sensitivities, ranging from 12% to 78% and specificities of 79 to 100% (Ireton *et al.*, 2010). Antigen 85 complex, CFP and LAM of *M. tuberculosis* elicits detectable IgG response in tubercular patients. Sensitivity and specificity of assays utilizing Ag 85 complex ranged from 82-84.1% and 85.2-86% respectively for LAM it was 80.3-93% and 99-100% respectively. Serological tests detecting IgG antibody against CFP showed sensitivity and specificity of 60.4 to 66% and 73.8- 85.2% respectively (Kumar *et al.*, 2010; Kashyap *et al.*, 2007; Boechme *et al.*, 2005; Brown *et al.*, 2003; Chan *et al.*, 2000; Sada *et al.*, 1992; Xueqiongwu *et al* 2010). Serological assays using sonicate supernatant, purified protein derivative (PPD), A60, 38kDa, cell wall and cytosolic antigens showed sensitivity of 53-88% and specificity of 52.5-96% (Agarwal *et al.*, 1989; Kalantri *et al.*, 2005; Raheman *et al.*, 1998; Senol *et al.*, 2007). Recently introduced interferon gamma released assay (IGRA), using ESAT-6 and CFP-10, is a promising test for the diagnosis of TB infection. Several studies have been conducted in various clinical settings on the accuracy and utility of IGRA. Most of these studies have reported that the sensitivity of IGRA is modest to detect active TB disease (Jafari *et al.,* 2006; Kobashi *et al.*, 2006).

Therefore, a sensitive and specific point of care serological test for the rapid diagnosis of patients with active TB would facilitate early treatment and reduce *M. tuberculosis* transmission. Understanding of humoral response to particular antigens would facilitate effective prevention, better management and development of serological assays for rapid diagnosis of tuberculosis. This is particularly important for pediatric and extra pulmonary tuberculosis and in those unable to produce sputum. No studies have yet been carried out among Bangladeshi population regarding humoral immune response to specific mycobcaterial antigens. There is no reported study on the role of serological diagnosis of tuberculosis in Bangladeshi patients.

In view of the above, the present study was undertaken for the rapid detection of *Mycobacterium* by PCR. Also, drug susceptibility pattern of mycobacteria in different groups of patients was determined to understand the emerging drug resistance problem. Attempt was also made to determine the humoral immune response to specific mycobacterial antigens and their application in sero-diagnosis of active infection.

1.2. Objectives

The objectives of the study were to:

a. isolate and determine the distribution of *Mycobacterium tuberculosis* and *Mycobacterium* other than tuberculosis in pulmonary and extra pulmonary tuberculosis cases.

b. determine role of PCR in rapid detection of mycobacteria in clinical samples.

c. identify different *Mycobacterium* species directly in clinical samples by PCR using species specific primers.

d. determine the drug resistant pattern of *Mycobacterium tuberculosis* to first line anti-tubercular drugs.

e. determine the humoral immune response to different antigens of *Mycobacterium tuberculosis* in active tuberculosis and healthy individuals.

f. assess specific antibody response as a sero-diagnostic tool for detection of active tuberculosis

CHAPTER 2

REVIEW OF LITERATURE

2. Reveiew of Literature

2.1 History of human tuberculosis

Tuberculosis is a chronic infectious disease caused by a bacteria known as *Mycobacterium tuberculosis*. It is a contagious and air born disease. Tuberculosis predominantly affects lungs but central nervous system (CNS), circulatory system, joints and bones can also be affected. The name 'tuberculosis' was used for the first time in the 19th century. It is believed to have originated from the word "tubercle" meaning a 'protuberance, swelling or nodule'- such nodules are found in infected lungs and bones (Madhura, 2010).

Tuberculosis was present before the beginning of the prerecorded history and has left its mark on human creativity, music, art and literature. Its causative agent *'Mycobacterium tuberculosis'* has killed more persons than any other microbial pathogens (Daniel,2006)

The first reference of a disease similar to tuberculosis in humans dates back to ancient Egypt (Madhura, 2010). Examination of mummies and tomb painting reveal that Tuberculosis was present around 5000 BC. Skeletal remains from a Neolithic Settlement in the Eastern Mediterranean shows that prehistoric humans (700BC) had Tuberculosis and tubercular decay has been found in the spines of mummies from 3000 to 2400 BC (Hart *et al.,* 1996). Consumption, king's evil, lupus vulgaris, and phthisis are some of the more colorful names for tuberculosis (Mathema, 2006).

Robert Koch, a German physician in 1882, first isolated *Mycobacterium tuberculosis (M .tuberculosis),* the causative agent of tuberculosis. He received Nobel prize in 1905 for the discovery. French scientists Calmette and Guerin in 1908 cultured a bovine strain of *M. bovis* on a unique medium that was found to weaken the organism. They discovered that the weakened organisms were incapable of causing tuberculosis, even in guinea pigs. . Later, they used the strain as a vaccine candidate and tried the vaccine on human successfully. Hence, in 1921 the vaccine was named BCG (Bacillus Calmette Guerin) after the name of two inventors. Even today, it is the most widely used vaccine given to children after birth to prevent tuberculosis (Hart *et al.*, 1996).

After the world war I in 1920, a conference on tuberculosis held in Paris with participation of delegates from 31 countries and thus International Union Against Tuberculosis and Lungs Disease (IUATLD) was established (Leao and Portaels, 2007).

The concept of sanatorium cure provided the first widely practiced approach to anti tuberculosis treatment. In the 19th century it provided duel function throughout Europe and the United states-firstly it protected the general population by isolating the sick persons who were the source of infection, secondly they offered tuberculosis patients bed rest exercise fresh air and good nutrition, all of which assisted the healing process.

Prior to the introduction of the anti tubercular drugs, the only treatment was care at sanatorium and besides that was the surgical intervention including pneumothorax or plombage technique i.e. collapsing an infected lung to "rest" it and allow the lesions to heal but this was discontinued by 1950s (Leao and Portaels, 2007.). The history of tuberculosis changed dramatically after the introduction of anti-mycobacterial agents. Anti-tuberculosis drug treatment started in 1944, when streptomycin (SM) and paraminosalicylic acid (PAS) were discovered. In 1950, the first trial was performed comparing the efficacy of SM and PAS both as monotherapy or combined. The study demonstrated that combined therapy was more effective and resulted in the first multidrug antituberculosis treatment that consisted of a long course of both drugs. In 1952, a third drug, isoniazid (INH),was added to the previous combination, greatly improving the efficacy of treatment, but which still had to be administered for 18-25 months. In 1960, ethambutol (ETHAM) substituted PAS, and the treatment course was reduced to 18 months. In the '70s' with the introduction of rifampicin (RIF) into the combination and treatment was shortened to just nine months. Finally, in 1980, pyrazinaide (PZA) was introduced into the antituberculosis treatment, which could be reduced further to only six months (Silva and Anisa , 2007).

Two biological features explain why combined drug therapy is more effective at curing tuberculosis than monotherapy. Treatment of active Tuberculosis with a single drug results in the selection of drug resistant bacilli and the other is that different populations of tubercle bacilli- each of them showing a distinct pattern of susceptibility for antitubercular drugs-may co-exist (Shamputa, 2006). Tuberculosis program activities was reinforced by

successful chemotherapy and resulted in a pronounced reduction of infection and death rates. The disease became greatly controlled but it never quite disappeared. Then, in around 1985, cases of tuberculosis began to rise again in industrialized countries. Several interrelated forces drove this resurgence including increase in Prison populations, homelessness, injection drug use, crowded housing and increased immigration from countries where tuberculosis continued to be endemic.

Above all, the decline in tuberculosis control activities and the human immunodeficiency virus (HIV)/acquired immodeficiency syndrome (AIDS) epidemic were two major factors fueling each other in the resurgence of tuberculosis (Leao and Portaels, 2007). In light of the resurgence of tuberculosis worldwide, directly observed treatment short course (DOTS) strategy was promoted as the official policy of the World Health Organization (WHO) in 1991 to contain the disease. In 1998, IUATLD joined with the WHO and other international partners to form the stop TB initiative, a defining moment in the restructuring of global effort to control Tuberculosis. In spite of these global efforts, TB continues to pose a dreadful threat. A notorious example is the sudden emergence of a deadly form of TB associated with HIV/ AIDS in a rural hospital located in Kwa-zulu-natal, a South African province in 2005. This deadly form of TB is called extensively drug resistance TB (XDR-TB) (Gandhi, 2006). XDR-TB is defined as MDR-Tuberculosis caused by *M. tuberculosis* which is further resistant to second line anti tubercular drugs (Raviglione, 2007).

2.2. Microbiology of mycobacteria

The genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae* orders *Actinomycetales* and related to other mycolic acid-containing genera. The minimal characteristics for including a bacterium in this genus are:

- Acid fastness
- The presence of mycolic acids containing 60-90 carbon atoms,
- G+C content of the DNA is 61-71 mol% (exception being *M. leprae*, <57%; Good and Shinnick, 1998)

Mycobacteria are nonmotile, nonspore-forming, weakly gram-positive, aerobic or microaerophilic, straight or slightly curved rod-shaped bacteria (0.2-0.6 × 1.0 -10μm). (Wayne and Kubica, 1986).

At the beginning of the twentieth century, *M. tuberculosis* was the only species of *Mycobacterium* routinely isolated from, and associated with, human disease. As time went on, more and more species of environmental mycobacterium, called nontuberculous mycobacteria (NTM) (formerly atypical mycobacteria or mycobacteria other than tubercle bacilli), were recognized as cause of human disease. Currently, there are about 100 mycobacterial species that are usually grouped into two major divisions – 'slowly growing ' and 'rapidly growing' mycobacteria. The slow growers require more than 7 days to produce visible colonies on solid media under ideal culture conditions.

Mycobacteria have been classified as *M. tuberculosis* complex and non-tuberculous mycobacteria (NTM). *M. tuberculosis* complex consists of *M. tuberculosis, M. bovis, M. bovis, M..africanum, M. microti, and M..canetti.* NTM include photochromogens, scotochromogens, non-photochromogens and rapid growers (Kazda 1983).

Photochromogens are defined as mycobacteria that are non pigmented when grown in the dark but acquired a lemon-yellow colour after exposure to daylight or artificial light. All species in this group are potentially pathogen. This group includes *M. kansassi*, *M. marinum*, *M. simiae* and *M asiaticum*. Scotochromogens are those Mycobacteria which produce pigment (yellow to orange even red) when grown in the dark and the light namely *M. scrofulaceum*, M. *szulgai*, *M. xenopi* and *M. flavescens*. Nonphotochromogens do not develop pigment in light or darkness, eg. *M.avium M. intracellularae*, *M. gastri*, *M. malmoense*, *M.terrae* & *M. trival*. Rapid growers produce visible growth in less than 7 days eg. *M. fortuitum*, *M. chelonai*, *M. smegmatis*. (Pfyffer and Vincent, 2005).

2.3. Epidemology of tuberculosis

The consequences of tuberculosis on society are immense. The WHO estimated that one third of the global community is infected with *Mycobacterium tuberculosis* (*M.tuberculosis*) and two billion people in total. Tuberculosis accounts for 2.5% of global burden of disease and holds the seven places in the global ranking of causes of death (Dye, 1999). Effective drugs to treat and cure the disease have been available for more than 50 years, yet every 15 seconds, someone in the world dies from tuberculosis. If left untreated, a person with active tuberculosis will infect an average of 10 to 15 other people year (Dye, 2005).

Tuberculosis hinders socioeconomic development because 75% of people with tuberculosis are within the economically productive age group of 15-54 years. Ninety five percent all cases and 99% of deaths occur in developing countries, with the greatest burden in sub-Saharan Africa and South East Asia (Dye, 2006; WHO, 2006a) . The WHO estimated 8.9 million new cases of tuberculosis in 2004 (140/100,000 population) and 1.7 million people (27/100,000) died from tuberculosis in 2004, including those co-infected with HIV (248,000), but the majority of patients with tuberculosis live in the most populous countries of Asia. Bangladesh, China, India, Indonesia and Pakistan together account for half (48%) of new cases that arise every year. In terms of total estimated number of new tuberculosis cases arising annually, about 80% of new cases occur in the 22 top-ranking countries (Dye 2006, WHO 2006). In 2004, the estimated tuberculosis incidence per capita was stable or falling in five out of six WHO regions, although it was still growing at 0.6% per year globally. The exception is the African region where the incidence of tuberculosis is still rising in line with the spread of HIV. (Dye, 2006; WHO, 2006)

Tuberculosis death rates in high-burden countries varied dramatically from 9 per 100,000 populations in Brazil to 139 per 100,000 in South Africa. In these two countries, the overall case fatality rates for tuberculosis were 13% and 27% respectively, and the difference was due largely to the difference in HIV infection rates (Dye, 2006; WHO, 2006).

Tuberculosis is a major cause of morbidity and mortality globally with an estimated 9.4 million incident cases and 11.1 million prevalent cases. In 2008 there were 1.3 million deaths from tuberculosis in HIV negative persons and 0.52 million deaths in HIV positive persons (ICDDR,B, 2010).

In 2009, Asia and Africa accounted for 55% and 30% of total cases of tuberculosis respectively. Smaller portion of cases occurred in Eastern Mediterranean region (7%), European region (4%) and in Americas (3%). The 22 high burden countries that have received particular attention at the global level since 2000 account for 81% of all estimated cases worldwide. The five countries with the largest number of incidence cases in 2009 were India (1.6-2.4 million), China (1.1-1.5 million), South Africa (0.4-059 million), Nigeria (0.7-055 million) and Indonesia (0.35-5.2 million). India alone accounts for one fifth (21%) of all tuberculosis cases worldwide and China and India combined account for 35% of total cases (WHO, 2010). The estimated global tuberculosis incidence, prevalence and mortality in 2009 in different WHO regions of the world is shown in Table 2.1. The estimated tuberculosis cases in different countries of the world are shown in Fig-2.1.

Of the 9.4 million incident cases in 2009, and estimated 1.0 -1.2 million (11-13%) were HIV positive. Of these HIV positive tuberculosis cases, 80%were approximately in African region (WHO, 2010).

Tuberculosis incidence rate peaked in 2004. Since then, the incidence rates are falling in five of WHOs six regions (exception is the South East Asia Region, where the incidence rate is stable). All WHO regions are on track to achieve the 50% mortality and prevalence reduction target, except for the Africa region even though rates of mortality are falling there (WHO, 2010). The overall trend of tuberculosis in terms of incidence, prevalence and mortality in SEA region is shown in Fig 2.2.

2.4. Tuberculosis in Bangladesh

Tuberculosis (TB) remains a major public health problem from a single infectious agent in Bangladesh. Over 3, 00,000 new tuberculosis cases and 70,000 deaths are estimated to occur per year in Bangladesh and the country ranks 6th on the global list of high burden countries. The estimated prevalence and incidence rates of all forms of tuberculosis were 387 and 223 per 1,00,000 population in 2007 and the estimated death rate per 1,00,000 was 45. Two previous national tuberculosis prevalence surveys were conducted in Bangladesh in 1964-66 and in 1987-88.

Table 2.1: Estimated global Tuberculosis incidence, prevalence and mortality, 2009

	Incidence[1]			Prevalence[2]		Mortality	
WHO region	Number x1000	% of global total	Rate per 100,000 pop[3]	Number x1000	Rate per 100,000 pop3	Number x1000	Rate per 100,000 pop3
Africa	2800	30%	340	3900	450	430	50
Americas	270	2.9%	29	350	37	20	2.1
Eastern Mediterranan	660	7.1%	110	1000	180	99	18
Europe	420	4.5%	47	560	63	62	7
South East Asia	3300	35%	180	4900	280	480	27
Western Pacific	1900	21%	110	2900	160	240	13
Global Total	9400	100%	140	14000	164	1300	19

Source: WHO fact sheet, November 2010; [3]*Pop indicates population; Mortality excluding HIV*

Fig 2.1: Estimated new tuberculosis cases in different countries of the world in 2009 (WHO 2010)

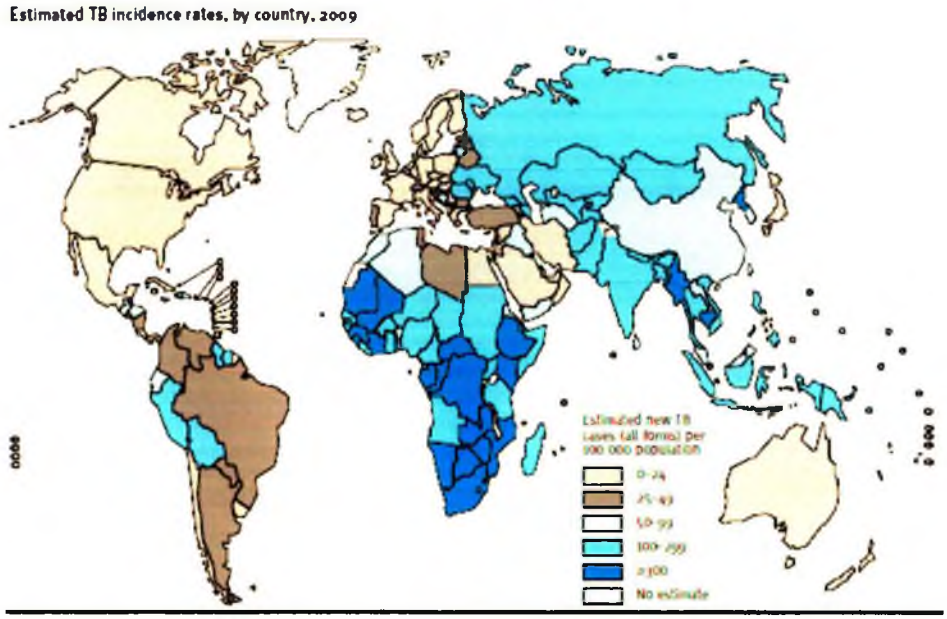
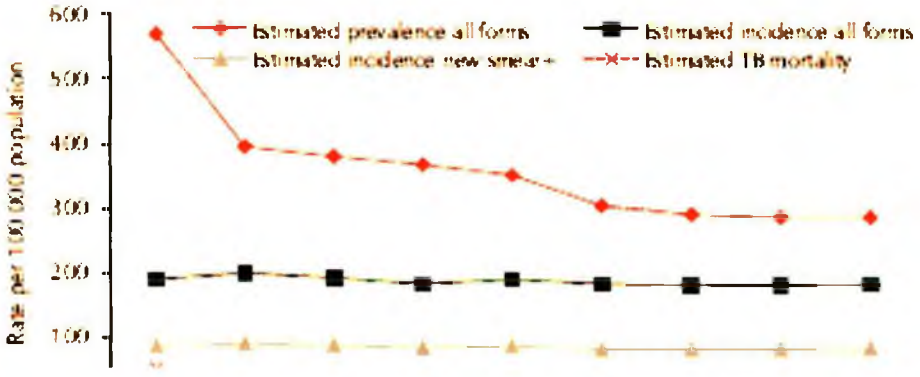


Fig 2.2: The overall trends in the estimated tuberculosis prevalence, incidence and mortality rates per 100 000 population in South- East Asian Region from 1990 to 2007



Source: Global Tuberculosis Control, WHO Reports 2001-2008

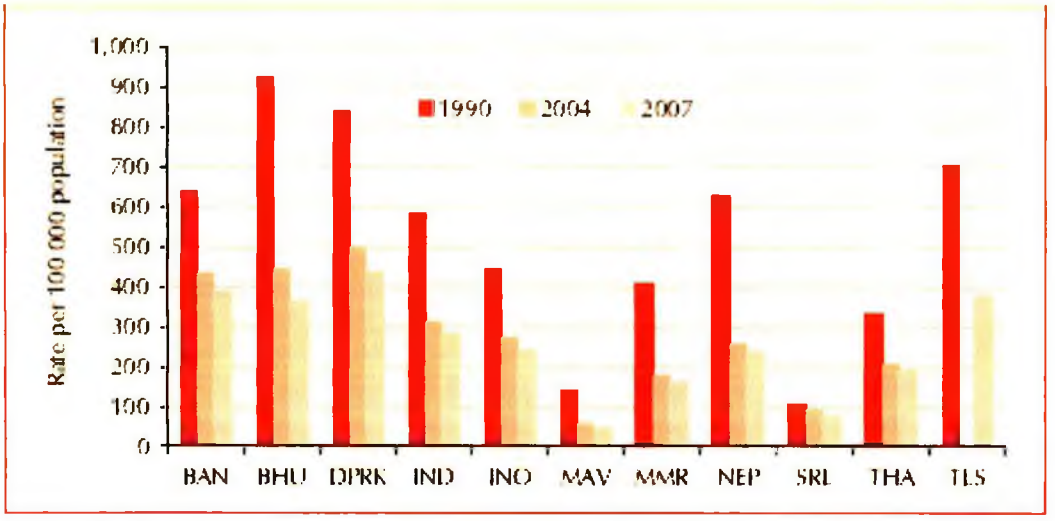
The 1964-66 surveys estimated a prevalence of 318/1,00,000 persons ≥15 Years, while the 1987-88 survey reported a much higher prevalence of 870/1,00,000 persons ≥ 15 Years (WHO, 2009; ICDDR,B 2010). A new assessment was undertaken by ICDDR,B in collaboration with the National Tuberculosis Control Program, BRAC, Damien Foundation, the WHO, Netherlands Tuberculosis foundation (KNCV) and other partners from October 2007 to March 2009. The over all crude prevalence of new smear positive tuberculosis was 79.4 per 1,00,000 among persons ≥15 years. The adjusted prevalence of smear positive tuberculosis was higher in rural (86 per 1,00,000) than Urban areas (51.1 per 1,00,000) and it was higher among males than females. The prevalence was much higher among the lowest socioeconomic quintile (Zaman *et al.*, 2009). This survey was the first nation wide representative survey to determine the prevalence of smear positive tuberculosis since the introduction of the DOTS Program in Bangladesh in 1993. The estimated incidence of tuberculosis in Bangladesh in 2008 was 223/100 000 population. The estimated trends of prevalence and mortality of tuberculosis in Bangladesh and other neighboring countries of the region are shown in Fig 2.2 and Fig 2.3. Rates of HIV infection in patients with tuberculosis have so far remained below 1% in Bangladesh, China, Indonesia and Pakistan (Asamoah-Odaei, 2004; Dye, 2005; Dye, 2006). The summary of the tuberculosis situation in Bangladesh is given in Table 2.2.

Table 2.2: Tuberculosis situation in Bangladesh as of 2010

Country ranking among the 22 high burden countries	6th
Estimated incidence of all forms of tuberculosis per 100,000 population per year	223
New smear-positive cases per 100,000 population per year	100
Estimated mortality from all forms of tuberculosis per 100,000 population per year	45
Estimated prevalence of all forms of tuberculosis cases per 100,000 population	387
Proportion of MDR-TB among new cases	3.5%
Proportion of MDR-TB among re-treatment cases	20%
DOTS population coverage	100%
Cases detection rate- New smear-positive cases	74%
Treatment success rate for new smear-positive cases	92%

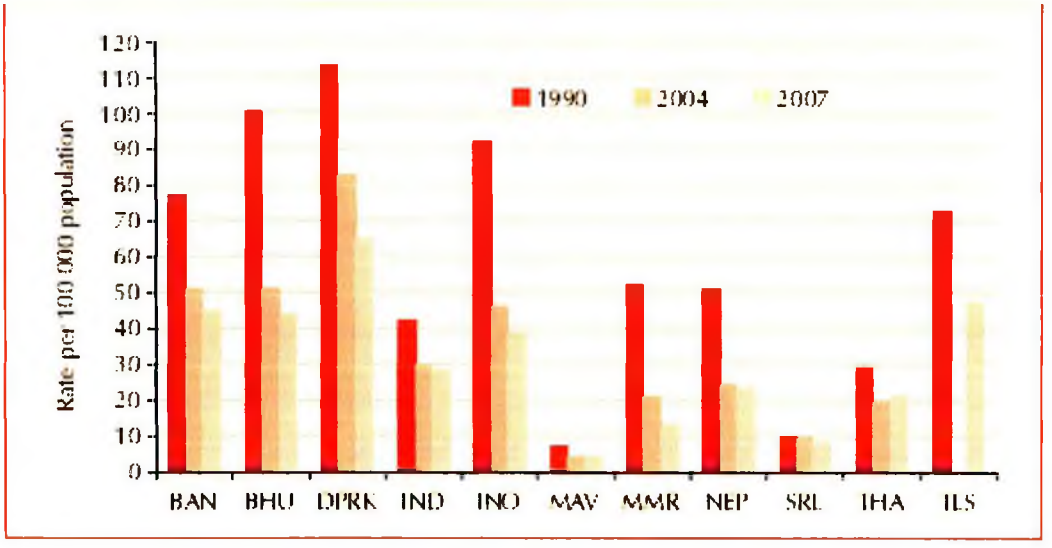
Source: Bangladesh Bureau of Statistics and Global Tuberculosis Control WHO Report 2009

Fig 2.3: The estimated prevalence of tuberculosis in 1990, 2004 and 2007 in Bangladesh and neighboring countries



Source: Global Tuberculosis Control, WHO Reports 2001-2008

Figure 2.4: The estimated tuberculosis mortality rates for all forms of tuberculosis per 100 000 populations, in 1990, 2004 and 2007 in Bangladesh and neighboring countries



Source: Global Tuberculosis Control, WHO Reports 2001-2008

2.5 Drug resistant tuberculosis

Drugs used to treat tuberculosis are classified as first line and second line anti tubercular agents. The first line essential antitubercular agents are the most effective and necessary component of any short- course therapeutic regimen. The two drugs in this category are isoniazid and rifampicin. First line supplemental agents either can shorten chemotherapy (e.g., Pyrazinamide) or are highly effective and infrequently toxic (ethambutol and streptomycin). Second line antitubercular drugs are clinically much less effective than first line agents and much more frequently elicit severe reaction. They include para-aminosalicylic acid (PAS), ethionamide, cycloserine, vincomycin, kanamycin, amicacin and thiacetazone. Newer antituebercular drugs, which have not yet been placed in the above categories, include rifabutin and the quinolones, especially ciprofloxacin, ofloxacin, and sparfloxacin.

Soon after the introduction of the first anti-mycobacterial drugs, drug resistant bacilli started to emerge, but the launch of both combination therapy and new and more effective drugs seemed to be enough to control disease. In fact, it was thought that tuberculosis could be eradicated by the end of 20th century. However, Tuberculosis unexpectedly re-emerged in the '80s' and in the following years there was an important increase in the incidence of poly, multiple, and extensively drug resistant strains.

Contributing factors for development of drug resistance include population growth, the human immunodeficiency virus epidemic, and poorly implemented public health, measures (Raviglione *et al.*,1995). In the developing countries, efforts to curb tuberculosis are further hindered by overcrowding, poverty, poor infection control in hospitals, widespread AIDS, and sever lack of financial and technical resources for effective control programs. The number of tuberculosis patients infected with multiple-drug resistant strains is also on the rise, particularly in developing countries; this is due in large part to the failure of control programs to provide adequate treatment and to the marked increase in high risk groups, such as patients with AIDS. The main cause of drug resistance is the failure to ensure correct treatment of each patent in tuberculosis. programs are often at fault due to:

a. Improper prescription of treatment regimens

b. Inadequate drug supply

c. Poor case holding,

d. Poor quality of drugs

The most frequent drugs involved in mono-resistance are isoniazid (INH) and streptomycin (SM). Nowadays, SM is not regularly used in the standard therapeutic schemes, and resistances to INH has limited clinical or epidemiological relevance (Nardell and Rubin, 2005). Likewise poly-resistance is relatively easy to overcome as long as susceptibility to RIF is preserved. In contrast, the standard anti-tuberculosis chemotherapy often fails in patients infected with RIF resistant M tuberculosis which are therefore at an increased risk of developing added INH resistance, that is, to become MDR. In many settings, resistance to RIF is a strong predictor of MDR-TB (Traore *et al.*, 2000). Furthermore, poor outcome and death are associated with the resistance to RIF alone or in combination with resistance to other drugs (Espinal *et al.,* 2000). Mono-resistance to RIF is rather unusual and occurs mainly in association with HIV/AIDS.

Drug resistant tuberculosis can be classified as:

- Monoresistant tuberculosis: It is caused by *M. tuberculosis* resistant to a single drug.
- Polyresistant tuberculosis: It is caused by *M. tuberculosis* resistant to at least two drugs but not involving isoniazid (INH) and rifampicin (RIF) simultaneously.
- Multidrug resistant tuberculosis (MDR -TB): It is caused by *M. tuberculosis* resistant to at least two drugs, always involving INH and RIF.
- Extensivly drug resistant tuberculosis (XDR -TB): It is defined as MDR-TB with additional resistance to any fluoroquinolone, and to at least one of the three following injectable drugs used in anti-tuberculosis treatment: capreomycin, kanamycin, and amikacin (Raviglione, 2007).
- Extremely drug-resistant tuberculosis(XXDR-TB):It is defined as tuberculosis caused by strains of *M. tuberculosis* which are resistant to all first line (INH, RIF, ETHAM, SM and pyrazinamide) and second line (ofloxacin,ciprofloxacin, cycloserine,prothionamide,amikacin,kenamycin,capreomycin and ethionamide) anti-tubercular drugs. It is also known as totally drug resistant (TDR) tuberculosis.

As for the epidemiological mode of development, drug resistant tuberculosis is classified in two groups:

- Primary or initial drug resistant tuberculosis – It is defined as a resistance to anti-tubercular drug(s) detected in a tuberculosis patients who has never received

tuberculosis chemotherapy. Primary drug resistance is believed to be occurred by infection with drug resistant bacilli from another patient who had secondary drug resistances.

- Secondary or acquired drug resistant tuberculosis – It is defined that as a resistance to any anti-tubercular drugs in a tuberculosis patients occurred during the course of therapy or after irregular drug treatment.

Global surveillance has shown that drug resistance tuberculosis is wide spread and is now a threat to tuberculosis control program in many countries. Shortly after the first introduction of anti-tuberculosis drugs like SM, INH, PAS, resistance to these drugs was observed in clinical isolates of *M. tuberculosis* (Crofton and Mitchison, 1948). By the end of the 1960s, RIF was introduced and with the use of combination therapy, there was a decline in drug resistant and drug susceptible Tuberculosis in developed countries. This led to a decline in funding and interest in tuberculosis control programs. As a result, no concrete monitoring of drug resistance was carried out for the following 20 years (Espinal, 2003). The arrival of HIV/AIDS in 1980s resulted in an increase in transmission of tuberculosis associated with breakout of multidrug resistant tuberculosis (MDR-TB) (Edlin *et al.*, 1992; Fischl *et al.*, 1992).

In 1996, the WHO and IUATLD launched the global project on drug resistance surveillance based on data collected and reported by an international network of laboratories acting as Supranational Reference Laboratories. The network includes 26 Supranational Reference Laboratories distribution in the five WHO regions and is coordinated by the Prince Leopold Institute of Tropical Medicine in Antwerp Belgium (Martin and Portaels, 2007).

2.5.1 Global situation of drug resistant tuberculosis

According to WHO/IUAT LD Global project on anti-tuberculosis drug resistance reports, the prevalence of resistance to at least one drug (any resistance) ranged from 0% in sum Western European countries to 57.1% in Kazakhstan (median=10.2%). Median prevalence of resistance to individual drugs was: SM 6.3%, INH 5.9%, RIF 1.4% and ETHAM 0.8%. Prevalence of MDR- TB ranged from 0% in eight countries to 14.2% in Kazakhstan and Israel (median=1.1%). The highest prevalence of MDR-TB was observed in Tomsk Oblast (Russian Federation) (13.7%), Uzbekistan (13.2%), Estonia (12.2%), China (7.8%-10.4%),

Lithuania (9.4%), Latvia (9.3%), and Ecuador (6.6%). Significant increase in prevalence of any drug resistance was found in Botswana, New Zealand, Poland, and some provinces of Russian Federation. Decreasing trends in MDR-Tuberculosis were observed in Cuba, Hong Kong, Thailand and the USA (Martin and Portaels, 2007). A total of 3718 pulmonary tuberculosis cases were studied in Turkey for the period of 1982-1991, and 1992-1999 and it was found that resistance rates of *M. tuberculosis* to INH, SM, RIF and ETHAM for the period of 1982-1991 were 14.2%, 14.4% 10.5% and 2.7% respectively and resistance rate for the period of 1992-1999 was 14.4%, 21.1%, 10.6% and 2.4% respectively. For the above period MDR-Tuberculosis was 7.3% and 6.6% respectively (Kart *et al.,* 2002). Another study from Turkey reported resistance to SM as11.3% followed by INH 5(8%) and rifampicin 3(4.8%). MDR-TB was 4.8% (Ozturk *et al.*, 2005).

During Nov. 2004 to Nov. 2005 the CDC and WHO surveyed the Supranational Tuberculosis reference laboratory (SRL) network and found that the new MDR cases had been emerging every year all over the world and it is now estimated that more than 4,00,000 new cases occur annually. The SRL network identified that XDR-TB was the most common in South Korea showing 15% of all the MDR-TB cases. It has been estimated worldwide that XDR- TB increased from 5% of MDR isolates in 2000 to 7% of MDR isolates in 2004 (CDC, 2000; CDC, 2004).

WHO/IUAT LD Global project on anti-tuberculosis drug resistance reports also evaluated RIF resistance as a predictor of MDR-TB in order to explore the significance of rapid testing for RIF resistance to identify cases likely to have MDR-TB. The report also confirmed that, globally, more isolates were resistant to INH than to any other drug (range 0-42%). INH and SM resistance were more prevalent than RIF or ETHAM resistance. Resistance to INH, SM, RIF and ETHAM was the most prevalent pattern among previously treated cases and the proportions of isolates resistance to three or four drugs were significantly greater than among new cases, suggesting and amplification of resistance. It appeared that monoresistance to either INH or SM is the main gateway to the acquisition of additional resistance ((Martin and Portaels, 2007). The distribution of drug resistant *M. tuberculosis* in different regions of the world among new and treated cases is shown in Table 2.3 and Table 2.4

The WHO/ IUATLD global survey in 2000 showed that in Myanmar, 33.3% of the isolates from new pulmonary tuberculosis patients were resistant to at least one anti-tuberculosis drugs (INH, RIF, ETHAM and SM). Among the new cases resistance ranged from 1.7%-36.9% and MDR- TB was 1.25% (Phyu *et al.*, 2003).

Pereira *et al.*,(2005) reported INH, SM, RIF and ETHAM resistance as 30%, 23.3%,10% and 10% respectively in HIV infected tuberculosis cases in India. Another study carried out by Deodhar *et al.*,(1999) in India, reported that out of 707 culture positive *M. tuberculosis*, resistance to ethionamide was 60.67% followed by RIF 58.55%, INH 30.41%, SM 46.95% and ETHAM 3.67%. MDR-TB was 25.25%. Shah *et al.*, (2002) carried out a study in Gujrat, India on 822 previously treated culture positive tuberculosis patients (373 treatment failure and 449 were relapse cases). Among them 58.6% were resistant to one or more drugs and 35.2% were MDR.

Table 2.3: Median prevalence of drug resistance among new tuberculosis cases by region

Region	Resistance to any drug (%)	Polyresistance (%)	MDR-TB (%)
Africa	7.1	1.3	1.4
Americas	9.7	2.1	1.1
Eastern Mediterranean	9.9	2.5	0.4
Europe	8.4	1.1	0.9
South-East-Asia	19.8	4.0	1.3
Western Pacific	11.4	2.5	0.9
Overall median	10.2	1.9	1.1

Source: Adapted from Reference WHO, 2006

Table 2.4: Median prevalence of drug resistance among previously treated tuberculosis cases by region

Region	Resistance to any drug (%)	Polyresistance (%)	MDR-TB (%)
Africa	16.7	1.8	5.9
Americas	24.6	3.7	7
Eastern Mediterranean	63.3	5.8	48.3
Europe	15.9	2.6	4.7
South-East-Asia	39.9	7.3	20.4
Western Pacific	32.8	6.1	15.5
Overall median	18.4	3.2	7

Adapted from Reference WHO, 2006

2.5.2 Situation of drug resistant tuberculosis in neighboring countries

Paramasivan and Venkataraman (2004) of Chennai, India found that globally more strains were resistant to INH than to any other drugs (range 0-42%). In general, INH and SM resistance was more prevalent than that of RIF or ETHAM. In the previously treated cases, the proportion of strains resistant to 3 or 4 drugs was significantly greater than that of new cases. This relationship was found globally as well as regionally. It also appeared that INH and SM monoresistance are the main gateway to acquisition of additional resistance. The mediun prevalence of MDR- TB in new cases was 1.1% (range 0-14.2%) and that among treated cases it was 7% (range 0-58.3%).

Iqbal et al (2003) of Lahore, Pakistan, conducted a study with the total of 678 positive cases of mycobacterium tuberculosis and showed that resistance to pyrazinamide was the highest (29%) followed by RIF and INH 28% and 26% respectively. Resistance to SM was

24% and to ETHAM was 15% in this study. MDR-TB in primary tuberculosis cases was 11.9% and in secondary cases it was 23%. Drug resistance pattern for 2 drugs was 14.6%, for 3 drugs was 8.11% for 4 drugs 5% and for 5 drugs it was 4.71%. Another study from Pakistan with 1247 samples reported overall anti-TB drug resistance as 44.83% and MDR - TB as 30.69%. Resistance to single drug was 7.9%, majority of which were against INH and SM, resistance to 2 drugs (INH, RIF) were 15.15% and 15.17% isolates showed resistance to 4 drugs (Ikram *et al.,* 2008). In 2008 a report from Pakistan on 582 confirmed isolates of *M. tuberculosis* stated the rate of MDR-TB as 12.8% and 27.0% in new and treated cases respectively. Overall resistance to RIF was 26.6%, to INH 23.5%, to SM 19.5%, to ETHAM 11.8%. It was also shown that resistance to 1 drug was 20.1%, 2 drugs 14.6%, 3 drugs 9.6%, 4 drugs 5.8% and 5 drugs 4.8% (Iqbal et *al.*, 2008).

2.5.3 Drug resistant tuberculosis in Bangladesh

Tuberculosis (TB) remains a major public health problem in Bangladesh. Over 3,00,000 new cases and 70,000 deaths are estimated to occur in Bangladesh and the country ranks 6th on the global list of high burden countries and 9th among 25 high priority multidrug resistant (MDR) and extensively drug resistant (XDR) tuberculosis countries (WHO, 2008).

In Bangladesh, no nationwide study has been conducted to evaluate the status of over all drug resistant tuberculosis and MDR-TB. However, isolated studies have indicated the presence of single and poly resistant tuberculosis and MDR-tuberculosis. In a published report, 63 isolates of sputum samples from different areas of Bangladesh were studied in NIDCH, a referral center for tuberculosis in Dhaka, in 2005. Among 42 strains tested, 35 (83%) were MDR. Among these MDR strains 40% were found resistant to any of the second line drugs. Study conducted in a rural area of Bangladesh demonstrated that the prevalence of MDR-TB was 3% among new and 15.4% in previously treated patients (Deun *et al.*, 2008). Another study conducted by (Deun *et al.*, 1999) found that initial resistant to INH was 5.4% and to RIF was 0.5% and acquired resistance to INH and RIF was 25.9% and 7.4% respectively. MDR-TB was one in new case and 5.5% in previously treated cases. Miah *et al.,* (2000) investigated 106 isolates of which 101 (95.3%) were identified as *M. tuberculosis* and 5 (4.7%) were MOTT. Among the 101 *M. tuberculosis* strains, 29.7% were resistant to at least one drug, 4.95% were resistant to two drugs, 2.97% to three drugs .In this study 16 (15.8%) were resistant to INH, 11 (10.9%) were resistant to

RIF, 7 (6.9%) to SM and 3 (2.9%) to ETHAM and MDR was 4.95%. In 2007, Rahim *et al.*, (2007) found that out of 95 isolates 31% were resistant to any drug, and resistant to SM was 18%, to INH 23%, to RIF 2%, to ETHAM it was 10% and MDR was 2%.

The MDR-TB rates among newly diagnosed cases ranged from 0.4% to 3.0% and among previously treated cases were 3.0% to 15.4% while the rate was 88% among patients failing category Π regimen (WHO, 2011). Study conducted in 1998 at BSMMU, MDR-TB in new cases was 4.9%. In 1995 it was 0.7% in new cases and 6.8% in previously treated cases (WHO, 2007). It was reported that MDR-TB as 3% in new cases and 15.4% in previously treated cases, (WHO, 2011). Zaman *et al.*, (2005) reported 48.8% as drug resistant of all cases and 5.5% as MDR-TB, where as WHO reported only 1.4% of new cases were MDR-TB in Bangladesh (WHO, 2004 a). In 2009, Rahaman *et al.*, (2009) reported that drug resistance rates for INH, RIF, ETHAM and SM as 76.03%, 71.63%, 27.55% and 55.65% respectively. Of the resistant cases 87% were MDR-TB category II and 13%were MDR-TB category 1. Table 2.5 describes the rate of reported drug resistance in Bangladesh by different studies in urban and rural population. The estimated MDR TB in Bangladesh and the other countries with high MDR-TB is shown in Table 2.6.

Table 2.5 Estimated resistances to any drug and MDR-TB in Bangladesh

Reference and country	Overall resistance (%)	MDR-TB in new cases (%)	MDR-TB in treated cases (%)
Deun *et al.*, 1999 (urban)	-	-	5.5%
Miah *et al.*, 2004 (urban)	29.7	4.9	-
Zaman *et al.*, 2005 (urban)	48.8	5.5	-
Rahim *et al.*, 2007 (rural)	31.0	2.0	-
Deun *et al.*, 2008 (urban)	-	3.0	15.4
Rahman *et al.*, 2009 (urban)	-	-	87.0 (Cat II), 13.0 (Cat I)

Table 2.6: Estimated MDR-TB cases in high MDR-TB burden countries

Countries	**Estimated % of all new MDR-TB**	**Estimated % of all retreated MDR-TB**	**Total number of estimated MDR-TB (thousands)**
Bangladesh	2.2	15	9.8
Myanmar	4.2	10	9.3
Pakistan	2.9	35	15
India	2.3	17	99
China	5.7	26	100
South Africa	1.8	6.7	13
Indonesia	2	15	9.3
Russian Federation	16	42	38

Source: WHO, Global Tuberculosis Control 2010

2.6 Extensively drug resistant tuberculosis (XDR-TB)

It is defined as MDR-tuberculosis with additional resistance to any fluoroquinolone and to at least one of the three following injectable drugs like capreomycin, kenamycin and amikacin (Raviglione, 2007). CDC and the WHO surveyed an international network of tuberculosis laboratories during 2000-2004 and found that out of 17,690 tuberculosis isolates, 20% were MDR-TB and 2% XDR-TB. In addition, population based data on drug susceptibility of tuberculosis isolates were obtained from United States (1993-2004), Latvia (2000-02) and South Korea (2004), where 4%, 19%, 15% of MDR-TB cases respectively, were XDR-TB. XDR-TB has emerged world wide as a threat to public health and tuberculosis control, raising concerns of a future epidemic of virtually untreatable tuberculosis. Globally, 69 countries including Bangladesh have reported at least one case of XDR- TB by the end of 2010. There are an estimated 25,000 cases of XDR-TB every year (WHO, 2011).

According to Centres for Disease Control (CDC) and the WHO, a survey was conducted based on an international network of TB laboratories for year 2000-2004. The result showed that 20% and 2% of *M. tuberculosis* isolates were MDR and XDR respectively. It was also reported that XDR-TB isolates increased from 5% of MDR isolates in 2000 to 7% of MDR isolates in 2004 (CDC, 2006).

2.7. Pathogenesis and immunity of tuberculosis

M. tueberculosis is an obligatory aerobic, intracellular pathogen which has a predilection for lung tissue rich in oxygen supply. The tubercle bacilli enter the body via respiratory route. The bacilli spread from the site of initial infection in the lungs through the lymphatics or blood to the other parts of the body. The lungs and the regional lymph nodes are the favored site. Extra pulmonary tuberculosis of the pleura, lymphatic, bone, genitourinary system, meninges, peritoneum or skin occurs in about 15% of tuberculosis patients. (Raja, 2004) Humans most commonly acquire tuberculosis infection by inhaling aerosolized *M.tuberculosis*, where by droplet nuclei containing 1-3 viable bacteria (particle size, <5μg) are considered sufficient as infectious inoculum (Andersen, 1997). These small particles are carried via the air stream and distributed to all regions of the lungs. The

mycobacteria are then taken up by the alveolar macrophages mediated by complement receptors (CR1, CR2, CR3, CR4), mannose receptors (MR) (Raja, 2004). The interaction between MR on phagocytic cells and mycobacteria seems to be mediated through the mycobacterial surface glycoprotein and lipoarabinomannan. Prostaglandin E2 (PGE2) and interleukin 4 (IL-4) upregulate CR and MR receptor expression and function and interferon-γ (INF γ) decrease the receptor expression, resulting in diminished ability of mycobacteria to adhere to the macrophages (Raja, 20004).

Recent studies showed that heparin or fibronectin-binding proteins, present on the bacterial surface, play a role to facilitate their binding to the epithelial cells or macrophage (Pethe *et al.*, 2001; Pasula *et al.*, 2002). Because *M. tuberculosis* survives and grows within the macrophages, the bacterial cell wall acts as both an interface and a barrier between the parasite and the host cell. It is involved in many complex interactions which result in both stimulation and inhibition of host cell responses. It acts as a permeability barrier which may determine susceptibility to antibacterial drugs, and contributes both to the stimulation of, and protection against, the immune response of the host. *Mycobacterium* can survive in the hostile habitat for a long period of time even for years. An important mechanism by which mycobacteria use to escape from host killing system is the avoidance of activation of macrophages which will subsequently inhibit non-specific host response. Lipoarabinomannan, a major lipopolysaccharide in mycobacterial cell wall, plays a key role in the persistence mechanism of *M. tuberculosis* (Britton *et al.*, 1994).

Phagocytosis of *M .tuberculosis* by alveolar macrophage is the first event in the host – pathogen relationship that decides the out come of the infection. Inside the macrophage the intracellular mycobacterium employ a variety of survival strategies, which include:

i) prevention of an oxidative burst in phagocytosing cells and inhibition of phagosome lysosome fusion .

ii) resistance to lysosomal enzymes and reactive oxygen intermediates by means of cell wall lipids, LAM and secretion of superoxide dismutase.

iii) escape of mycobacterium from the phagosome into the cytoplasm (Horwitz, 1989; Sathish and Shinnick, 1994; McDonough *et at.*,1993).

The out come of infection by the tubercle bacilli depends critically on whether the host response with a protective or tissue necrotizing reaction. If the bacilli are not destroyed, they replicate and kill the cell. A local area of inflammation is thus established and more phagocytes are attracted to the site. Some bacilli are transported, probably within phagocytes, to the regional lymph nodes, where they are engulfed by antigen-presenting cells (APC). Other bacilli are transported further afield and may cause one of the extrapulmonary forms of primary disease such as tuberculosis meningitis (Pfyffer and Vincent, 2005).

In the initial stage of host-bacterium interaction, either the macrophages contain bacillary multiplication by producing proteolytic enzymes or cytokines or the bacilli begin to multiply. If the bacilli multiply, their growth quickly kills the macrophages, which lyses. Non-activated monocytes attracted from the bloodstream to the site by various chemotactic factors ingest the bacilli released from the lysed macrophages. These initial stages of infection are usually asymptomatic (Wallace *et al.,* 2005).

Two to four weeks after infection, two additional host responses to *M. tuberculosis* develop: a tissue- damaging response and a macrophage- activating response. The tissue-damaging response is the result of a delayed- type hypersensitivity (DTH) reaction to various bacillary antigens: it destroys nonactivated macrophages that contain multiplying bacilli. The macrophage-activating response is a cell – mediated phenomenon resulting in the activation of macrophages that are capable of killing and digesting tubercle bacilli. Although both of these responses can inhibit mycobacterial growth, it is the balance between the two that determine the form of tuberculosis that will develop subsequently. With the development of specific immunity and the accumulation of large number of activated macrophages at the site of the primary lesion, granulomatous lesion is formed. These lesions consist of lymphocytes and activated macrophages, such as epithlioid cells and giant cells. Initially, the newly developed tissue-damaging response is the only event capable of limiting mycobacterial growth within macrophages. This response, mediated by various bacterial products, not only destroys macrophages but also produces early solid necrosis in the center of the tubercle. Although *M. tuberculosis* can survive, its growth is inhibited within this necrotic environment by low oxygen tension, low pH, and other factors. At this point,

some lesions may heal by fibrosis and calcification, while other undergoes further evolution.

Cell-mediated immunity is critical at this early stage. In the majority of infected individuals, local macrophages are activated when bacillary antigens processed by macrophages stimulate T- lymphocytes to release interferon γ (IFN γ) and other lymphokines. These activated cells aggregate around the lesion's center and effectively neutralize tubercle bacilli without causing further tissue destruction. In the central part of the lesion, the necrotic material resembles soft cheese (caseous necrosis). Even when healing takes place, viable bacilli may remain dormant within macrophages or in the necrotic material for years or ever throughout the patient's lifetime. These healed lesions in the lung parenchyma and hilar lymph nodes may later undergo calcification. In a minority of case, the macrophage-activated response is weak, and mycobacterial growth can be inhibited only by intensified DTH reactions, which lead to tissue destruction. The lesion tends to enlarge further and the surrounding tissue is progressively damaged. At the center of the lesion, the caseous material liquefies. Bronchial walls as well as blood vessels are invaded and destroyed and cavities are formed. The liquefied caseous material, containing large numbers of bacilli drained through bronchi. Within the cavity walls, tubercle bacilli multiply well and as spread into the airways and the environment through expectorated sputum.

In the early stage of infection, bacilli are usually transported by macrophages to regional lymph nodes, from which they disseminated widely to many organs and tissue. In young children with poor natural immunity, haematogenous dissemination may result in fatal military tuberculosis or tubercular meningitis. Cell-mediated immunity confers partial protection against *M. tuberculosis* while humoral immunity has no defined role in protection. Two types of cells are essential: macrophages, which directly phagocytize tubercle bacilli, and T lymphocytes, (mainly CD4+ lymphocyte) which induce protection through the production of lymphokines especially INFγ. After the infection with *M. tuberculosis*, alveolar macrophages secret a number of cytokines: interleukin (IL) 1 contributes to fever, IL-6 contributes to hyperglobulinemia; and tumor necrosis factor α (TNF α) contributes to the killing of mycobacteria, the formantion of granulomas, and number of systemic effects such as fever and weight

loss. Macrophages are also critical in processing and presenting antigens to T lymphocytes; the result is a proliferation of CD4+ lymphocytes, which are crucial to the host's defense against *M. tuberculosis*. Qualitative and quantitative defect of CD4+ T cells explain the inability of HIV-infected individuals to contain mycobacterial proliferation. Reactive CD4+ T-lymphocytes produce cytokines of the Th1 pattern and participate in MHC class II restricted killing of cells infected with *M. tuberculosis*. Th1 CD4+ T-cells produce INFγ and IL-2 and promote cell mediated immunity. Th2 cell produce IL-4, IL-5, and IL-10 and promote humoral immunity. The interplay of these various cytokines and their cross regulation determine the host's response. The role of cytokines in promoting intracellular killing of mycobacteria has not been entirely elucidated. INFγ may induce release of nitric oxide and INFα which also seems to be important. Finally, a recently describe subset of T cells capable of recognizing lipid elements of the bacillus presented by CD1 molecules may be implicated in protection. *M. tuberculosis* possesses various protein antigens. Some are present in the cytoplasm and cell wall; others are secreted. The latter are more important in eliciting a T lymphocyte response is suggested by experiments documenting the appearance of protective immunity in animals after immunization with live, protein-secreting mycobacterium. Among the antigens with a potential protective role are 30 kDa (or 85B) and ESAT-6 antigens. Protective immunity is probably the result of reactivity to a large number of different mycobacterial antigens.

Coincident with the appearance of immunity, delayed type hypersensitivity to *M. tuberculosis* develops. This reactivity is the basis of PPD skin test, which is used primarily for the detection of *M. tuberculosis* infection in persons without symptoms. The cellular mechanisms responsible for PPD reactivity are related mainly to previously sensitized CD4+ lymphocytes, which are attracted to the skin-test site. There they proliferate and produce cytokines (Wallace *et al.*, 2005).

2.8. Humoral immune response in tuberculosis

The current understanding of humoral immune response to tuberculosis is from the serological studies that by and large aimed developing a sero-diagnostic test to identity tuberculosis. To date, the genome analysis has revealed 113 protein as secreted and 49 as

excreted proteins of *M. tuberculosis*. The exported and secreted proteins of mycobacterium are hypothesized by several researches as the key protective antigens. The most studied secreted proteins of *M. tuberculosis* are ESAT-6 (early secretory antigen target), CFP-10, 38kDa, 16kDa and Ag85 complex. The ability of these proteins to elicit serological response makes them to be utilized as the candidates for sero-diagnosis. The other proteins eliciting humoral immune response are cell wall fraction (CWF) and lipoarabinomannan.

2.9 **Mechanism of anti-tubercular drug resistance**

Resistance of *M. tuberculosis* to anti-tubercular agents may be constitutive (natural) or acquired. The mechanisms driving *M. tuberculosis* resistance to anti-tubercular drugs are genetically control. A proportion of mutants resistant to a single drug are generated spontaneously in any bacilli population, even if not exposed to any anti-tuberculosis drugs.

The natural drug resistance of *M. tuberculosis* is an important obstacle for the treatment and control of Tuberculosis. This resistance has traditionally been attributed to the unusual multi-layer cell envelope and active multidrug efflux pumps (Rossi, 2006). Recent insights into mechanisms that neutralize the toxicity of antibiotics in the cytoplasm have revealed other systems that function in synergy with the permeability barrier and efflux systems to provide natural resistance. Drugs inhibiting these intrinsic systems would enable many antibiotics, which are already available but have not been used for a tuberculosis, to gain a new potential use against *M. tuberculosis* (Lomovskaya, 2006; Nguyen, 2006).

M. tuberculosis acquired drug resistance is caused by mutations in chromosomal genes (Heym, 1994). So far, no single pleotropic mutation has been found in *M. tuberculosis* to cause a MDR phenotype. The MDR phenotype is caused by sequential accumulation of mutations in different genes involved in resistance to individual drugs, due to inappropriate treatment or poor adherence to treatment (Rattan *et al.,* 1998). However, it is important to observe that some resistance strains do not present these classic mutations, suggesting the possibility of the existence of other mechanisms such as efflux pumps and alteration in the permeability of the cell wall.

Spontaneous chromosomally borne mutation occurring in *M. tubrculosis* at a predictable rate (approximately 10^{-5} to 10^{-8}) is thought to confer resistance to anti tuberculosis drugs. A characteristic feature of these mutations is that they are unlinked. Thus, resistance to a drug is usually not associated with resistance to an unrelated drug. A tuberculosis cavity usually contains 10^7 to 10^9 bacilli. If mutations causing resistance to isoniazid occur in about1 in 10^6 replications of bacteria, and that mutations causing resistance to rifampicin occur in about1 in 10^5 to 10^7 replications, the probability of spontaneous mutations causing resistance to both isoniazid and rifampicin would be $10^6 \times 10^7 = 1$ in 10^{13}. Given that this number of bacilli cannot be found even in patients with extensive cavitary pulmonary tuberculosis, the chance of spontaneous dual resistance to rifampicin and isoniazid developing is practically remote. Thus, the fact that mutations are unlinked forms the scientific basis of antituberculosis chemotherapy (Sharma and Mohan, 2004).

The rate at which mutation emerges differs for all of the antitubercular agents. The probability of resistance is very high for less effective drugs such as thiacetazone, ethionamide, capreomycin, cycloserine, and viomycin (10^{-3}); intermediate for drugs such as INH, SM, EMB, kanamycin, and p-amino salicylic acid; and lowest for RIF (Rattan et al. 1998). The risks of mutation for rifampicin, isoniazid, streptomycin, and ethambutol are 3.32×10^{-9}, 2.56×10^{-8}, 2.29×10^{-8}, and 1.0×10^{-7} mutations per bacterium per cell division, respectively. The mutation rate, rather than the mutation frequency, is the most reliable measure, as it records the risk of mutation per cell division rather than the proportion of mutant cells (Gillespie, 2002). Consequently, the probability of a mutation is directly proportional to the bacterial load. A bacillary load of 10^{-9} will contain several mutants resistant to any one antituberculosis drug (Rattan *et al.*, 1998).

Resistance to a drug does not confer any selective advantage to the bacterium unless it is exposed to that drug. Under such circumstance, the sensitive strains are killed and the drug-resistant mutants flourish. When the patient is exposed to a second course of drug therapy with yet another drug, mutants resistant to the new drug are selected, and the patient may eventually have bacilli resistant to two or more dugs. Serial selection of drug resistance, thus, is the predominant mechanism for the development of MDR strains; the patients with MDR strains constitute a pool of chronic infections, which propagate primary MDR

resistance. In addition to accumulation of mutations in the individual drug target genes, the permeability barrier imposed by the *M .tuberculosis* cell wall can also contribute to the development of low-level drug resistance. Studies addressing resistance to SM have found evidence of such a two step mechanism for the development of drug resistance (Rattan *et al.*, 1998). Genetic loci involved in drug resistance in *M. tuberculosis* are shown in Table 2.7.

2.9.1 Isoniazid (Isonicotinic acid hydrazine, INH)

INH is pro-drug that requires processing by the bacterial catalase-peroxidase to become active . Once activated, it inhibits the biosynthesis of mycolic acids,which are essential components of the mycobacterial cell wall. This drug is bactericidal against metabolically active bacilli and bacteriostatic against resing bacilli (Rattan *et al.*, 1998). INH is active against *M. tuberculosis*, *M. bovis*, *M. kansasii*. Susceptible *M. tuberculosis* strains show minimal inhibitory concentration (MIC) between 0.02 and 0.2mg/L.

INH is bacteriostatic for resting mycobacteria and bactericidal for rapidly dividing organisms Many molecular targets of INH have been identified

- *Kat*G gene that encodes for mycobacterial enzymes which have both catalase and peroxidase activity; in fact many organisms with mutations in this gene have reduced susceptibility to INH, so the current thinking is that INH is a prodrug which requires activation by catalase and peroxidase activity.
- Two genes in mycolic acid (a critical component in the mycobacterial cell wall) synthesis: *inh*A and *kas*A.
- *inh*A is a target both for INH and also for one of the second line drugs ethionamide. *inh*A is an enoyl-acyl carrier protein reductase which catalyzes an NADH-specific reduction step which is essential to the fatty acid synthesis or elongation of those long chain fatty acids for mycolic acid synthesis.
- *kas*A, a ketoacyl carrier protein synthatase, also mediates synthesis of mycolic acid. Therefore, mutations or disabling any one of these genes seems to confer resistance to INH.

Table 2.7: Gene loci involved in conferring drug-resistance in *M. tuberculosis*

Drug	Gene	Product	Reported frequency in resistant strains (%)
Rifampicin	*rpo*B	Subunit of RNA polymerase	>95
Isoniazid	*kat*G	Catalase-peroxidase	60-70
	*oxyR-ahp*C	Alky hydro-redutase	~20
Streptomycin	*rps*L	Ribosomal protein S12	60
	rrs	16s rRNA	<10
Ethambutol	*emb* CAB	Emb CAB	69

Ref: Sharma & Mohan, 2004

Basically, INH first gets taken up by *M. tuberculosis* by a transport process. INH gets activated by catalase and peroxidase activity encoded for by the *kat*G gene and then INH blocks (actually competitively inhibits) NADH-dependent reduction *(inh*A) and /or synthesis (*kas*A*)*, which is critical to mycolic acid synthesis. Resistance to INH is mostly associated with mutations or deletions in *kat*G*;* other mutation related with INH resistance occur in the coding region of *inh*A gene (or its promoter) and *kas*A.

2.9.2 Rifampicin (RIF)

Rifampicin inhibits gene transcription by interacting with the beta submit of the ribonucleic acid (RNA) polymerase enzyme. It is bactericidal against dividing mycobacteria and also has some activity against non-dividing bacilli. *M. tuberculosis* strains are normally susceptible to 0.1-2 mg/L. The introduction of RIF, thus, allowed reduction of duration of standard anti-tuberculosis treatment from one year to nine months. This was later reduced to six months after incorporation of PZA. Rifampicin is very lipid soluble. So it can easily penetrate the mycobacterial cell wall. It then binds to bacterial, but not eukaryotic, DNA-dependent RNA polymerase. RIF specifically interacts with the beta subunit of RNA polymerase, thereby hindering transcription. RIF had long been believed to interfere in the initiation part of transcription process. But recently using purified RNA polymerase from *M. smegmatis*, it has been demonstrated that RIF specifically inhibited the elongation of full-length transcripts and had virtually no effect on the initiation of transcription. Almost all clinical isolates of *M. tuberculosis* resistant to RIF show mutations in *rpoB* gene which encodes the beta-subunit of the RNA polymerase, resulting in conformational changes that determine the low affinity of this subunit for RIF and consequently, resistance to the drug Mutations conferring resistance to RIF are clustered in three short regions in the central region of the beta-subunit gene: cluster I (amino acids 512-544) cluster II (amino acids 563-574) and cluster III (amino acids 687) (Jin, 1998; Williams, 1994).

2.9.3 Ethambutol (ETHAM)

This drug is used to treat tuberculosis and other opportunistic infections caused by non-tuberculous mycobacteria such as *M. kansasii*. The MICs of sensitive *M. tuberculosis* strains range from 0.5-8mg/L. Ethambutol is also active against dividing mycobacteria,

being bacteriostatic. Since it affects the biosynthesis of the cell wall, it has been suggested that it contributes towards increasing the susceptibility of *M. tuberculosis* to other drugs.

Until recently, ethambutol's mechanism of action was largely obscure. Ethambutol is a synthetic compound used as first-line drug for anti-tuberculosis therapy in combination with other drugs. It has been demonstrated that ethambutol acts on enzymes involved in the biosynthesis of arabinogalactan, inhibiting the polymerization of cell wall arabinan of arabinogalactan and of liporarabinomannan (Takayama, 1989; Mikusova ,1995). Synergy resulting form co-administration of ethambutol and other drugs gave further evidence for its involvement in obstructing the formation of cell wall. The synergistic effect was explained as a consequence of increased permeability of the mycobacterial cell wall due to ethambutol leading to increased uptake of other drug. Recently it has been shown that it specifically inhibits arabinosyl transfer, suggesting that arabinosyl transferase is the primary cellular target for ethambutol (Rattan *et al.*, 1998).

In *M. tuberculosis* the *emb* operon has three contiguous genes: *emb*C, *emb*A, and *emb*B, which encode mycobacterial arabinosyl transferase (Telenti ,1997). These enzymes have been considered as the drug targets for ethambutiol, since substitutions of codon 306 in the *M. tuberculosis emb*B gene have been shown to be the most frequent and predictive mutations for its resistance (Srivastava, 2006). In *M. tuberculosis*, mutations of genes other than *emb*B have been associated with its resistance. Often these mutations affect a putative regulatory sequence in the *emb*C-*emb*A intergenic region (Ramaswany, 2000).

2.9.4 Streptomycin(SM)

Streptomycin (SM), an anminocyclitol glycoside antibiotic produced by some strains of *Streptomyces griseus*, was the first drug with anti-tubercular activity to be discovered. It is mainly used is the treatment of tuberculosis. Most *M. tuberculosis* strains are susceptible to 1-8 mg/L of streptomycin.

It binds irreversibly to 30s- ribosomal subunit and inhibits ribosomal protein synthesis in at least three ways: It interferes with the initiation complex of peptide formation thereby, inhibiting the early stage in the initiation of protein synthesis. Second mechanism is the misreading on the recognition region of the ribosome. As a result wrong amino acid is

inserted into peptide chain leading to the synthesis of non-functional protein. Third, it causes breakup of polysomes into nonfunctional monosomes.

Mutations associated with SM resistance in *M. tuberculosis* have been identified in the 16S ribosomal RNA (rRNA) gene (*rrs*) and *rps*L gene encoding ribosomal protein S12 (Finken, 1993). The majority of point mutations producing SM resistance occur in *rps*L, and the most common mutation is an AAG->AGG change in codon 43, which result in a Lys->Arg substitution; less frequently an AAG->ACG (Lys-> Thr) substitution is observed (Bottger ,1994; Musser, 1995). The second mechanism of SM resistance in *M. tuberculosis* is mutations in *rrs*. Mutations in *rps*L and *rrs* have bee identified in 50 and 20% of SM-resistant clinical isolates, respectively, resulting in high or intermediate levels of SM resistance respectively. The low level of SM resistance may be due to changes in the cytoplasm concentration of SM due to the action of efflux pumps which could be the molecular basis of SM resistance e in these strains (Ainsa ,1998; Meier, 1996; Silva ,2001).

2.10 Laboratory diagnosis of tuberculosis

Several laboratory techniques are employed to detect mycobacteria in clinical samples. Each technique has its advantages and disadvantages. Also, the application of each method depends on logistic, trained manpower and other resources. The following section deals with each technique.

2.10.1 Detection of Acid fast bacilli by Z-N method

The cornerstone of the diagnosis of tuberculosis is the direct microscopic examination of acid fast stained sputum specimens for tubercle bacilli. This technique is simple, cheap, inexpensive and easy to perform. It may be used as a rapid method for the follow up of the progress of the patients on therapy. The disadvantage of the technique is that its sensitivity is 33% (Lipsky *et al.*, 1984). It is likely to be successful only if large number of bacilli between 5000 to10000 per milliliter of sputum is present (David ,1976; Kent and Kubica, 1985; Bass *et al.*, 1990) showed that overall sensitivity of AFB sputum smear in patients with confirmed pulmonary tuberculosis was 50-80%. Another disadvantage is that AFB microscope can not distinguish between live and dead AFB (Rieder, 1996) that results in over diagnosis of treatment failure (WHO, 2003). Furthermore, delayed smear conversion

(patients still positive at follow up before treatment failure can be declared) is often due to prolonged excretion of dead bacilli (Telzak, 1997).

Ziaee *et al.,* (2008) showed that the sensitivity, specificity, positive and negative predictive values were 51%, 100%, and 57%, 100% for the Z-N and auramine staining method respectively. Another study conducted by Ha *et al.*, (2009) showed that sensitivity and specificity of Z-N method was 28.2% and 100% respectively..

2.10.2 Isolation of mycobacteria by culture

Various culture methods are used for the isolation and identification of Mycobacterium. The organisms can be cultured in solid and liquid media with different modifications. Detail description of various culture methods with their merits and demerits are described.

2.10.2.1. Culture in solid media

L-J media: Bacteriological culture provides the definitive diagnosis of tuberculosis. Depending on the decontamination method culture can be positive even when bacterial load is as low as 10 to 100 viable tubercle bacilli per ml of sample on L-J media which is egg enriched media containing glycerol and asparagines and malachite green. *M. tuberculosis* reproduces extremely slowly (generation time 18-24 hours) and needs 2-8 weeks to have visible colonies on L-J media.

L-J medium has a number of advantages and disadvantages.

Advantages:

- It is easy to prepare
- It is less expensive and supports good growth of tubercle bacilli
- It can be stored in the refrigerator for several weeks provided culture bottle caps are tightly closed to minimize drying by evaporation.
- Susceptibility tests for anti tubercular drugs can be done.
- Can differentiate between *M. tuberculosis* complex and NTM using bio chemical reactions.

Disadvantages:

- It may take as long as eight weeks for culture becoming positive

- Sensitivity and specificity of culture on L-J media was 48% and 100% respectively (Negi *et al.*,2005).

2.10.2.2 Culture in liquid media

Liquid media are used mainly for the production of large volumes of cells or cultures filtrate for research purposes. Herman Kirchner liquid medium is the most useful and least expensive of liquid media for culture of tubercle bacilli. Dubos Tween Albumin broth, Middlebrook 7H9, 7H12 are used for sub-culturing and preparation of inoculum for drug susceptibility tests. These are also used for recovering small numbers of organisms from sterile specimens such as cerebrospinal fluid (CSF). The addition of Tween to the media allows for homogenous growth and dispersal of clumps of *M. tuberculosis* growth (Kent and Kubica, 1985). The liquid media reduces the turn-around time for isolation of acid fast bacilli to approximately 10 days compared with 17 days or longer for conventional solid media (Forbes, 2002). It has the additional advantage that it can support large inoculum.

2.10.2.3 Radiometric method-BACTEC 460 (Beckton Dickinson):

This technique is specific for mycobacterial growth, where ^{14}C labeled palmitic acid in 7H12 medium is used. This system detects the presence of mycobacteria based on their metabolism rather than visible growth. When the ^{14}C labeled palmitic acid (substrate) present in the medium is metabolized, $^{14}CO_2$ is produced and measured by the BACTEC system instrument and reported in terms of growth index (GI) value (Ramchandran and Paramasivan, 2003a). Growth index greater than or equal to 10 are considered positive (Forbes et al. 2002). Growth of mycobacteria may be detected within 5-7 days and tubercle bacilli can be differentiated from other mycobacteria within five days by using p-nitro-a-acetylamino-b-priophenone (NAP) which inhibits the growth of *M. tuberculosis* and usually does not affect the growth of MOTT bacilli (Kantor *et al.*,1998). Comparative tests have shown that the method is very successful and reliable and that confirmatory results for *M. tuberculosis* can be obtained within two weeks (Ramchandran and Paramasivan, 2003a). However, the high cost of both the apparatus and the radio-labeled medium and biohazard associated with the disposal of such medium prohibits its routine use in most high tuberculosis prevalence countries (Kantor *et al.*, 1998).

2.10.2.4 Mycobateria growth indicator tube (MGIT, Becton Dickinson):

A fluorescent compound (ruthenium metal-complex) is embedded in silicone on the bottom of 16×100 mm round-bottom tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the both. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring mycobacteria consume the dissolved oxygen and allow the fluorescence to be observed using a 365 nm UV transilluminator. Growth can also be detected by the presence of non-homogeneous turbidity or small grains or flakes in the culture medium. The medium components are substances essential for the rapid growth of mycobacteria. The tube contains 4 ml of modified Middlebrook 7H9 broth. The supplement contains casein peptone, albumin, dextrose, catalase, oleic acid, polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin. Up to 0.5 ml of the pretreated sample can be inoculated into the tube. Then they are mixed by inverting several times and incubated at 37°C. The MGIT tubes are read daily starting on day 2 or 2 to 3 times a week for 8 weeks or longer depending of the type of specimen. Fluorescence is detected as a bright orange color in the bottom of the tube and also an orange reflection on the meniscus. The isolation of the *M. tuberculosis* complex with MGIT from specimens occurs around 7 days earlier than that of egg-based media (Hanna *et al.,* 1994). BACTEC MGIT 960 detection system is an automated version of manual MGIT tube method.

2.10.2.5 MB/BacT system:

This is a non-radiometric continuous monitoring system with a computerized database management. It is based on colorimetric detection of CO_2. comparison of the performance of MB/BacT system with that of BACTEC 460 showed that the mean time for detection of M. tuberculosis by the BACTEC system was 11.6 days and 13.7 days by the MB/BacT system. It was concluded that the MB/BacT with the computerized data management system is an acceptable alternative for BACTEC 460 method, some minor disadvantages such as increased contamination and slightly longer time for detection of growth [Rohner *et al.*, 1997].

The summary of the various culture methods of *M. tuberculosis* are shown in Table 2.8.

Table 2.8: Detection of *M. tuberculosis* by different staining and culture methods

Methods	Sensitivity	Specificity	Positivity	Time required	Contamination	References
Staining method						
Z-N staining	28.2-51%	100%	63.95-84.88			Ziaee *et al.*, 2008 Ha *et al.*, 2009 Aftab *et a*l., 2008 Rishi *et al* ., 2007 Uddin *et al* 2009
Auramine rhodamin	54.6-57.0%	95-100%				Ziaee *et al.*,2008 Runa *et al.*, 2011
Culture methods						
L-J method	34.4-88.0%	100%	84-98.6%	17.3-26.2 days	5.07-27.2%	Aftab *et al.*, 2008 Querol *et al.*1995 Rishi *et al.*, 2007 Uddin *et al.*, 2009 Medoza *et al.*, 1993 Moore *et al.*, 2004 Gopinath and Singh, 2009
MGIT method	42.0-100%	100%	98.6%	8-12 day	13.4%	Ha *et al.*, 2009 Rishi *et al.*,2007 Devis *et al.*,2009 Gopinath and Singh 2009 LiLi *et al.*, 2011
BACTEC method	80-100%	81.2-98.5%	94.0-96.5%	8-12 days	-	Mendoza *et al.*, 1993 Devasia *et al.*, 2009 LiLi *et al.*, 2011
MB/Bac T method	98.6%	73.3%	9.24 days	95.34%	3.62%	Uddin *et al.*, 2009 Runa *et al.*, 2011

2.10.2.6 Molecular methods for the detection of mycobacteria

Many investigators have evaluated the efficacy of polymerase chain reaction (PCR) for the diagnosis of pulmonary and extra pulmonary tuberculosis by comparing the results with those of acid fast stained smears, culture and clinical diagnosis of pulmonary tuberculosis and concluded that PCR might be a useful tool for the diagnosis of tuberculosis within a day. Cohen et al (1998) carried out PCR by the in house and Roche technique with the sputum samples of suspected tuberculosis patients and observed sensitivity of 85% (in house PCR) and 74% (Roche technique) and specificity of 88% and 93% respectively.

Sensitivity of smear negative patients was 73% (in house technique) and 53% (Roche technique) respectively. The in house PCR detected 100% and Roche detected 95% of patient was with culture positive tuberculosis patients.

Querol et al (1995) investigated a total of 314 samples (sputum 286, bronchoalveolar lavages 28) from 242 patients by PCR and the results were compared with those obtained by acid-fast stained smears, culture and clinical diagnosis. All smear and culture positive samples were PCR Positive. The sensitivity of PCR, culture and staining was 97%, 88% and 65% respectively and specificity was 100% in all cases.

Massoud et al (2009) evaluated a total of 113 (48 pulmonary and 65 were extra pulmonary) cases tuberculosis patients and compared the result of PCR with culture and staining. The sensitivity of PCR, culture and staining was 76.99%, 43.36%, and 23.43% respectively. One hundred and fifty one clinically suspected tuberculosis patients were investigated to evaluate the diagnostic potential of PCR based detecting of *M. tuberculosis* complex in sputum by Parvez *et al*., (2003). The sensitivity of PCR was 92% with specificity 70% considering the culture result as gold standard. Jorgen et al (1996) investigated 452 respiratory samples of 204 patients by PCR. PCR had a sensitivity of 41.4% for smear positive specimen and 60.9% for smear negative specimen with a specificity of 96.1%. Analysis of 254 PCR positive but culture negative specimen with discrepant result reveal that 130 were from patients with recently diagnosed tuberculosis and 94 represented a presumed laboratory error. Similar analysis of 118 PCR negative, culture positive

specimens demonstrated that 27 discrepancies were due to presumed uneven aliquot distribution and 11 were due to presumed laboratory error. PCR inhibitors were detected in 08 specimens.

Jafarin et al (2008) conducted a study on 40 suspected tuberculosis patients and compared sensitivity and specificity of AFB, auramine-rhodamine staining and multiplex PCR for the detection of *M. tuberculosis* and non tubercular mycobacteria. The sensitivity of PCR was 65% and the specificity was 100% in case of multiplex PCR. Sensitivity and specificity was 27.5% and 100% respectively by AFB and 32.5% and 100% by Auramine-Rhodamine staining respectively. Beige et al (1995) investigated 103 clinically suspected patients of tuberculosis and compared sensitivity & specificity of PCR and culture method. The study demonstrated that the sensitivity of PCR and culture was 98% and 56.5% respectively.

Study conducted by Ginesu *et al.,* (1998) to evaluate the diagnostic value of PCR and compared PCR value with that of culture and AFB method. It was observed that sensitivity of PCR was 91.43% and specificity was 87.78% and sensitivity of culture was 96.8% and specificity was 94.4% respectively. The efficacy of PCR in the detection of mycobacteria specific nucleic acid by different studies is summarized in Table 2.9.

Table 2.9: Efficacy of PCR methods for detection of mycobacteria

Reference & Country	Sample size	PCR	
		Sensitivity	Specificity
Parvez *et.al.*, (2003), Bangladesh	151	92%	70%
Abe *et al.*, (1993),USA	135	81.3%	94.2%
Claridge *et al.*, (1993), USA	5000	83.6%	98.7%
Miller *et.al.*, (1994), USA	750	78.2%	NA
Nolte *et.al.*,(1993), USA	313	91.0%	100%
Shawar *et.al.*,(1993), USA	384	74.0%	95%
Yuen *et.al.*, (1993), USA	519	96.0%	85%
Cohen *et.al.*, (1998), Spain	85	85%	88%
Querol *et al.*, (1995),USA	314	97%	100%
Bennedsen *et al.*, (1996), UK	7064	87.9%	99.5%
Jafarian *et al.*, (2008), Iran	40	65%	100%
Beige *et al.*, (1995),USA	103	98%	70%
Ginesu *et al.*, (1998), USA	395	91.43%	87.78%
Dar *et.al.*, (1998), India	182	59%	100%
Mustafa *et al.*, (1999), Kuwait	151	88% (2 band) 75% (3 band)	100%
Rodriguez *et al.*, (1997),USA	123	75.65%	100%

2.11 Anti mycobacterial drug susceptibility testing

Early detection of drug resistance constitutes one of the priorities of Tuberculosis control programs. It allows initiation of appropriate treatment in patients and also surveillance of drug resistance. Detection of drug resistance has been performed in the past by so-called 'conventional methods' based on detection of growth of *M. tuberculosis* in the prescience of he antibiotics. However, due to the laboriousness of some of these methods, and most of all, the long period of time necessary to obtain results, in recent years new technologies and approaches have been proposed. These include both phenotypic and genotypic methods. In many cases, the genotypic methods in particular have been directed towards detection of RIF resistance, since it is considered a good surrogate market for MDR-tuberculosis, especially in settings with a high prevalence of MDR-Tuberculosis. Genotypic methods have the advantage of a shorter turnaround time, no need for growth of he organism, the possibility of direct application in clinical samples, lower biohazard risks, and the feasibility of automation; however, not all molecular mechanisms of drug resistance are known. Phenotypic methods, on the other hand, are in general simpler to perform and might be closer to implementation on a routine basis in clinical mycobacteriology laboratories. Basically there are two types of drug susceptibility tests "direct and indirect method"

2.11.1 Direct method

The direct method is a susceptibility test which is performed directly from the processed and concentrated sputum. The specimens which can be used for direct method must be AFB smear positive. The advantages of this method are less time consuming and truly reflect the susceptibility of bacilli in the body of patient. However, this test is not suitable for using in absolute and resistance ratio methods and may produce an error results if there are not sufficient growth in control media.

2.11.2 Indirect method

The indirect method is a susceptibility test which is performed from already grown colonies on primary isolation media. Problems of this method are duration from beginning to obtaining the result is 8-12 weeks which is too long and the process of scraping colonies

from the primary isolation media may not be able to pick up all colonies which are representative of whole culture. The indirect method is more suitable than the direct test because the amount of bacilli in inoculum can be standardized. The first WHO report in 1969 had suggested three methods for mycobacterial susceptibility test which are now called "conventional methods". These three are absolute concentration, resistance ratio and proportional methods.

2.11.3 Absolute concentration method

This method uses a standardized inoculum grown on drug free media and media containing graded concentrations of the drug(s) to be tested. Several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth (less than 20 colonies); i.e. minimal inhibitory concentration (MIC). Drug concentrations, and particularly inoculum size, must be carefully standardized with reference to wild type cultures. Variations in inoculum size are the major source of error in this method (Ramchandran and Paramasivan, 2003b).

2.11.4 Resistance ratio method

This is a refinement of the absolute concentration method that controls for variations in the MIC of a given isolate when tested on different batches of drug-resistance media. It is defined as the MIC of the test isolate divided by the MIC of a standard susceptible wild-type strain. If the ratio is two or less, or eight or more, the isolate is considered to be fully sensitive or highly resistant, respectively. Intermediate or low level resistance is difficult to measure accurately. Inoculum size needs to be standardized but the critical concentration does not need to be determined because of the direct comparison with susceptible isolates. This test is greatly affected by any variation in susceptibility of the standard strain (Drobniewski, 2007).

2.11.5 Proportion method

This method enables a precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of inoculum are planted onto both control (drug-free) and drug-containing media; at least one dilution should yield isolated countable (50-100)

colonies. When these numbers are adjusted by multiplying by the dilution of the inoculum used, the total number of viable colonies on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be estimated. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant proportion as a percentage of the total population used (Ramchandran and Paramasivan, 2003b). If growth at the critical concentration of a drug is more than 1%, the isolate is considered critically resistant (Kent and Kubica, 1985).

2.12 Liquid culture based methods

Several liquid culture-based methods for drug sensitivity of *M. tuberculosis* have been introduced during the last years. Most of them are commercially available and rely on the fact that faster growth is usually obtained in liquid medium. Recent recommendations by the WHO encourage the use of liquid culture media for cultivation and drug sensitivity of *M. tuberculosis* in low- and middle-income settings (WHO, 2007a).

2.12.1 Mycobacteria Growth Indicator Tube (MGIT)

MGIT tube culture method is utilized here to perform drug sensitivity testing. For the performance of the test a drug containing tube and a control tube are inoculated with the standardized mycobacterial suspension and incubated at 37°C (day 0). Staring on the third day (day 2), the tubes are observed daily with UV lamp for presence of fluorescence. The presence of an orange fluorescence in the drug containing tube at the same time as in the control tube or within two days of positivity in the control is interpreted as resistance to the drug; otherwise, the strain is considered to be susceptible. The test is valid if the growth control gives a positive signal until the 14^{th} day of incubation (Palomino, 1999). BACTEC MGIT 960 automated system is the automated version of manual MGIT method.

2.12.2 Radiometric BACTEC 460 Method (Beckton Dickinson)

In the presence of a certain drug, susceptibility can be measured by inhibition of he daily increases in the growth index. For the performance of the test, a test vial containing the drug under study and a drug-free control are inoculated with a standard inoculum and incubated at 37°C. The vials are then read in the **BACTEC 460** apparatus on a daily basis.

This method is considered to be the 'gold standard' for drug susceptibility testing to first-line anti-tuberculosis drugs (Roberts, 1983; Heifets, 1999).

2.12.3 The BacT/Alert 3D system

It is automated commercial liquid culture-based system originally developed for blood cultures and later applied for diagnosis and drug sensitivity of *M. tuberculosis*. Several studies have evaluated the BacT/Alert 3D system for rapid drug sensitivity of *M. tuberculosis* to first-line drugs shown good sensitivity and specificity (Ängeby *et al.*, 2003).

2.13 Microscopic observation of drug susceptibility assay (MODS)

Microscopic observation drug susceptibility assay (MODS) was developed in Lima, Peru. It uses two well known properties of *M. tuberculosis*: (i) the rate of growth in liquid media is considerably quicker than that on solid medium, and (ii) the morphology of *M. tuberculosis* in liquid culture is characteristic and recognizable, consisting of tangles and cords of organisms.

For performance of drug sensitivity test, decontaminated sputum samples are inoculated into 7H9 liquid medium in 24-well plates with or without antibiotics and incubated at 37^0 C in a CO_2 incubator. Reading of the plates is performed with an inverted microscope at 40x magnification to identify the typical cord formation of *M. tuberculosis*. Growth in the drug-containing wells and in the growth control is interpreted as resistance. MODS have been evaluated against first-line drugs and better results have been obtained for RIF and INH (Moore *et al.*, 2004; Moore *et al* 2006). This is a rapid and relatively inexpensive method which compares very well with other well established systems in terms of both sensitivity as well as specificity, and also in terms of speed of growth when compared to solid media (Ramchandran and Paramasivan, 2003a.; Caviedes *et al.*, 2000) found the sensitivity of MODS to be 92% while Moore et al. (2004) reported that overall sensitivity of detection was 94% for MODS. Mean growth detection time ranged between 9-10 days in different studies (Caviedes *et al.*, 2000; Moore *et al.*, 2004).

Although this technique may be appropriate for disease endemic high-burden countries and fairly accurate to detect resistance, especially to RIF, it requires P2 bio-safety cabinets, relatively expensive Middlebrook 7H9 broth, oleic acid albumin dextrose catalase (OADC) and anti-microbial supplements and a relatively high technical skill (Ramchandran and Paramasivan, 2003a).

2.14 Alamar blue chromogenic method

It is simple and rapid method which does not require expensive installation and it makes use of non-toxic temperature stable reagent. It is becoming the method of choice for drug susceptibility testing of MTuberculosis isolates for the areas where tuberculosis is a major problem (Franzblau *et al.,* 1997). The Alamar blue oxidation-reduction dye is a general indicator of cellular growth and viability. In oxidized state it is blue but it becomes pink upon reduction. This color change is observed by adding the indicator to inoculated test tubes after a period of inoculation. The period of incubation that is needed for sufficient metabolic activity to occur vary strain to strain. Positive and negative control tubes are used to compare the metabolic activity in the test tubes containing inocula. The control test tubes are tested after 7, 10 or 15 days of incubation by adding Alamar blue and color change is noted.

2.15 Slide-culture technique

This is an older method recently modified and evaluated for rapid detection of MDR-tuberculosis directly in sputum samples (Hamid *et al.*, 2006). It uses Sula liquid medium with added antibiotics. For each sample to be tested, sputum smears are made on one end of autoclaved slides, and placed individually in sterile glass bottles containing 7 ml of drug containing and drug free medium. All bottles are incubated at 37^0 C for 10 days, and heated at 85^0 C for 30 min before opening. Dried smears are heat-fixed, stained by Ziehl-Neelsen and examined at 100×magnification for counting acid-fast micro colonies. Any number of well-developed colonies in the drug-containing bottles with at least one colony per low-power field in the growth control is interpreted as resistant. When applied on fresh smear-positive sputum samples the slide DST technique gave an accuracy of 96% for detecting resistance to RIF. For INH, however, the accuracy was only 90% and much lower for ETHAM and SM. Although simple in its principle and interpretation of results, this

technique follows a cumbersome procedure and requires the preparation of a large number of slides for each sample making it less appropriate to analyze several samples at the same time. Furthermore, there are bio-safety concerns, since the same study found that there were still live M. tuberculosis bacilli able to grow colonies on L-J medium even after the slides were heated at 85^0 C for 30 min previous to microscopically examination.

. 2.16 Solid culture based methods

New alternative methods for rapid drug sensitivity of *M. tuberculosis* using solid culture media have also been recently proposed. The majority of these alternative procedures have been developed as 'in-house' methods with the purpose of shortening the turn around time to obtain final results.

2.16.1 The E-test

Although not developed exclusively for *M. tuberculosis* the E-test is a commercial system allowing rapid drug sensitivity test to several antibiotics. It relies on plastic strips that have an impregnated gradient of the antibiotic allowing reading of MICs directly at the zone of growth inhibition when applied on the surface of an agar plate. When testing *M. tuberculosis* an inoculums equivalent to 3.0 McFarland was recommended with results available within 5-10 days. However, the requirement of a very high inoculums concentration may represent a disadvantage, especially when dealing with potentially highly drug-resistant strains such as MDR and XDR M. tuberculosis (Wanger and Mills, 1996).

2.16.2 The nitrate reductase assay:

The nitrate reductase assay (NRA) is another simple procedure recently proposed as a rapid method for drug sensitivity test of *M. tuberculosis*. It is based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite which is then easily detected in a colored reaction. The NRA has been evaluated in recent studies to detect resistance to first-line drugs and ofloxacin showing a good concordance with the reference proportion method (Ängeby *et al.*, 2002). The NRA, also known as the Griess reaction, has been used before in the panel of biochemical tests for the identification of *M. tuberculosis* (Kent and Kubica, 1985).

2.16.3 The micro colony method

Also known as the thin-layer agar method, it is performed on Middlebrook 7H11 agar. It was originally described for the rapid detection of mycobacterial growth and later applied as a rapid drug sensitivity method (Mejia *et al.*, 1999). When tested directly on sputum samples it detected multi-drug resistance within 13 days in smear-positive samples and in 38 days in smear-negative samples (Schaberg *et al.*, 1995). Further studies in target populations are necessary to assess the feasibility of implementation and accuracy of this method for rapid detection of drug-resistance in *M. tuberculosis*. The summary of the drug sensitivity tests by various culture methods is shown in 2.10

2.17 Molecular methods for detecting drug resistance

Molecular methods for drug resistance in tuberculosis involve two basic steps: a) nucleic acid amplification such as polymerase chain reaction (PCR), to amplify the sections of the *M. tuberculosis* genome known to be altered in resistant strains; and b) second step of assessing the amplified products for specific mutations correlating with drug resistance (Garcia, 2003; Palomino, 2005). Several genotypic drug susceptibility testing methods are described.

a. Deoxyribonucleic acid (DNA) sequencing

Sequencing DNA of PCR-amplified products has become the most widely used genotypic method for detecting drug resistance in *M. tuberculosis*, it is accurate and reliable and it has become the reference standard of mutation detection. It is performed with automatic sequencers (Victor, 2001). DNA sequencing has been widely used for characterizing mutations in the *rpoB* gene in RIF-resistant strains and to detect mutations responsible for resistance to other anti-tuberculosis drugs (Telenti, 1993; Garcia, 2003; Jalava ,2004).

b. PCR Single Strand Conformation Polymorphism (SSCP)

PCR-SSCP is based on the property of single-stranded DNA to fold into a tertiary structure whose shape depends on its sequence. Single strands of DNA differing by only one or a few bases will fold into different conformations with different mobility on a gel, producing what is called a single strand conformation polymorphism (SSCP). In combination with PCR, SSCP has been applied for the detection of resistance to rifampicin, isoniazid, streptomycin and ciprofloxacin (Ramchandran and Paramasivan, 2003b).

Table 2.10: Comparison of sensitivity, specificity and time required for drug susceptibility tests by various culture methods

Methods	Sensitivity	Specificity	Time required	Reference
LJ	48.9-97.3%	100%	16-26.2 days	Aftab *et a*l., 2008 LiLi et al., 2011 Moore *et al*., 2004 Devasia *et al*., 2009
MGIT	42.3%-100%	63.0-100%	8-8.3 days	Ha *et al*., 2009
BACTEC	80-100%	81.22-100%	8-8.7 days	Johansena *et al*., 2004 LiLi et al., 2011 Devasia *et al*., 2009
MODS	39.7-100%	94.4-100%	6-13 days	Ha *et al*., 2009 Caviedes *et al*., 2000 Moore *et al*., 2004 Devasia *et al*., 2009
Slide DST	25-100%	62-98%	7-10 days	Salim *et al*., 2006

c. Real-time PCR techniques

Real time PCR has also been introduced recently for the rapid detection of drug resistance in *M. tuberculosis*. Different probes have been used for detection, such as the *TaqMan* probe, Fluorescence Resonance Energy Transfer probes, molecular beacons and bio probes (Shamputa, 2004). The main advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination and results are generally obtained in an average of 1.5-2.0 hours after DNA extraction. The main disadvantages would be the requirement of expensive equipment and reagents, and the need for skilled technical personnel.

d. LiPA (Solid phase hybridization assay)

The line probe assay or LiPA is a commercial test for the rapid detection of *M. tuberculosis* complex and rifampicin resistance. The LiPA is based on the hybridization of amplified DNA from the cultured strains or clinical specimens to ten probes encompassing the core region of the *rpoB* gene of *M. tuberculosis*, which is immobilized on a nitrocellulose strip. The absence of hybridization of the amplified DNA to any of the sensitive sequence – specific probes indicates mutations that may encode resistance. Likewise, if hybridization to the mutation-specific probes occurs, the mutation is present (Ramchandran and Paramasivan, 2003b).

The various molecular methods employed to detect drug resistance in *M. tuberculosis* is described in Table 2.11.

2.18 Application of humoral immune response in serodiagnosis of tuberculosis

It is known that in tuberculosis cell mediated immune response (CMI) is host protective. The mycobacterial antigens that are good enough to elicit CMI are also equally effective in evoking antibody mediated humoral immune response. Early diagnosis of active cases of pulmonary tuberculosis is essential for initiating prompt treatment. The routinely used test for diagnosis of tuberculosis are cumbersome and time consuming besides being less sensitive and/or specific. Attempts have been made to develop simple, sensitive, specific immunoassay for sero-diagnosis of tuberculosis. These methods are based on detection of

Table 2.11: Molecular detection of drug resistance in *Mycobacterium tuberculosis* (Drobniewski *et al.*, 2007)

Name of the drug	Targets of drug action	Mutations in gene conferring resistance	Function of genes	Molecular assays
Rifampicin	RNA synthesis	*rpo*B	DNA dependant RNA polymerase(ß subunit)	PCR-SSCP heteroduplex, Line probe sequencing (LiPa)
Isoniazid	Mycolic acid bio-synthesis	*k*atG	Catalase/peroxidase	PCR-SSCP sequencing
		*inh*A/*mab*A	Fatty acid biosynthesis	
		*ahp*C	AlkylhydroperoxideC reductase	
		*oxy*R	Oxidative stress regulator	
		*Kas*A	b-ketoacylacyl carrier protein	
Streptomycin	Protein synthesis	*rrs*	16S r RNA	PCR-SSCP sequencing
		*rps*L	Ribosomal protein S 12	
Ethambutol	Cell wall synthesis	E*mb* A,B,C	Liparabinomannan and arabinogalactan synthesis	Sequencing

antigen, antibody or circulatory immune complex. A variety of methods have been employed for detection of antibodies in tuberculosis sera directed against variety of *M. tuberculosis* antigens. Serological methods have been regarded as attractive tools for rapid diagnosis of tuberculosis due to their simplicity, rapidity and low cost. Serodiagnosis also does not require safety measures associated with handling of live bacilli in culture.

As early as 1898, Arloing showed that sera from tuberculosis patients could agglutinate tubercle bacilli (cited in Daniel 1987). Interest was renewed and several groups of investigators committed themselves to find an optimal antigen for tuberculosis serodiagnosis. At the beginning, complex antigens were used in most cases, such as whole bacteria, culture filtrates, bacterial extracts, tuberculins and their purified derivatives (PPD). Most recently, individual purified antigens have also been assayed including proteins, lipopolysaccharides and glycolipids i.e. Ag 85, 38 KDa protein and LAM. To date, however no test has shown sufficient high sensitivity and specificity values for diagnostic purposes (AL Zahrani, 2000; Bothamely, 1995; Singh, 2003; Raquib, 2003; Julian, 2004; Lopez-Marin, 2003).

Surface antigens such as LAM or proteins expressed under stress conditions, such as alpha crystalline protein may be relevant. In an experiment where 17 recombinant mycobacterial protein antigens, native Ag85 complex, LAM and *M. tuberculosis* lysate were used to detect antibody responses induced by BCG vaccination. Only LAM reactive serum IgG responses were significantly increased in both BCG vaccinated individuals and active tuberculosis patients. Oral BCG vaccination leads to a significant increase in LAM reactive secretory IgA thus limiting its detection as a marker for active infection (Brown, 2003).

A study conducted by Demkow (2005) showed humoral immune response against 38KDa+16KDa and 38-KDa + LAM in bronchoalveolar fluid from patients of pulmonary tuberculosis. The mean IgG level for 38-KDa+LAM was significantly higher in the tuberculosis than that in the non tubercular group.

Kalantri et al (2005) investigated IgG and IgM antibody levels by using A-60 antigen and observed a good sensitivity (80%) and specificity (95.8%) for IgG. The efficiency and predictive values were also high. The sensitivity of IgM was low (28.5%) but specifically was high (95.7%).

A number of studies were conducted by different researches by ELISA method to assess IgG and IgM in the serum, bronchoalveolar lavage and pleural fluids of persons infected with *M. tuberculosis* and found that IgG antibody had good sensitivity and specificity. Study conducted by Kumar et al (2010) assayed IgG antibody against Ag85 complex by ELISA and observed sensitivity of 84.1% and specificity of 84.1% as compared to ESAT-6 where sensitivity was 64.9% and specificity were 88.9% in active tuberculosis patients. In another study, Kumar et al reported the humoral antibody activity of Ag 85 complex, Ag-6 and 38 KDa antigens in pulmonary tuberculosis cases and normal healthy volunteers by ELISA method and observed that the 38 KDa reacted positively with 52% of the tubercular sera and 0% of normal control sera. Sada et al (1990) found that detection of LAM reactive serum IgM by ELISA had a specificity of 91% and sensitivity of 72% for the diagnosis of active infection among the Mexicans in a group with high tuberculosis prevalence.

Proteins that are actively secreted by mycobacteria have been recognized to play a major role in the host's immunity. Based on this hypothesis culture filtrates of BCG were evaluated for their specificity and sensitivity by identifying the group vaccinated with BCG. Rani et al (2005) demonstrated that OD values of culture filtrated in vaccinated group was significantly higher ($P<0.001$) compared to that of the unvaccinated persons. Fujita et al (2005) evaluated humoral immune responses of active tubercular patients against six mycobacterial lipid antigens TDM-1, TMM, TMM-T, TMM-M, PL-2 and PL-1 and showed positive result.

Ag 85 complex proteins are major secretory products of *M. tuberculosis* and induce cellular and humoral immune response in infected human beings. Kashyap et al (2007) demonstrated Ag85 complex in the serum of tuberculosis patients by indirect Elisa method with sensitivity 82% and specificity 86%. Probha et al (2005) carried out a study to

estimate the levels of IgG and IgM antibodies for culture filtrate antigen in the serum and pleural fluid of tubercular and non tubercular patients by ELISA and found that IgG level aganst CF protein was higher in patients.

The serodiagnostic antibodies against individual antigens of *M. tuberculosis* 38 KDa, 48Kda and CFP-10/ESAT-6 antigen in tuberculosis patients revealed sensitivity of 73.6% 73.2% and 60.4% respectively with specificity of 85.4%, 77.7% and 73.8% respectively (Xuequing *et al.*, 2010). Beak et al (2005) carried out investigation to detect IgG against 6KDa, 16 KDa, 26 KDa and 38 KDa antigens by Western blot test from patients of pulmonary tuberculosis and healthy individuals and observed that reactivity of IgA against those antigens were 57%, 50.9%, 98% and 59% respectively.

Agarwal et al (1989) evaluated immune response against the antigens of intact cell (IC), sonicate supernatant (SS) and PPD. Both IgG, IgM antibodies were measured separately in sera of patients with active pulmonary tuberculosis and normal healthy controls. It was observed that against all the three types of antigens the patients developed predominantly IgG type of antibodies. The IgM response of the patients against the same antigens was comparatively much less.

Upadhye et al (2007) evaluated immune response of cocktail of Es-31, Es-43, Est-6 antigens in extra pulmonary tuberculous cases and observed a sensitivity and specificity of 72% and 91% for antibody detection. Raheman et al (1988) assessed humoral immune response against antigens (whole cell, cell wall and cytosolic antigen) in 75 cases of pulmonary tuberculosis and found that the levels of total IgG was significantly higher than the mean total of IgG levels of the healthy controls.

Many investigators assayed for humoral immune response to tubercular antigens and evaluated different antigens as candidate for serodiagnostic test to detect active tubercular infection. The success is so far variable. But till today, there is no study regarding the humoral immune response in Bangladeshi population either with active tuberculosis or in healthy people. A summary of the different studies regarding serodiagnosis of tubercular infection is given in Table 2.12

Table 2.12: Efficacy of sero-diagnosis of active tuberculosis by using different candidate antigens

Antigen used	Antibody	Sensitivity	specificity	Reference
A-60	IgG	80%	95.8%	KalantriY *et.al.*,. (2005)
	IgM	28%	95.7%	
16kDa+r38kDa	IgG	52.5%	93.3%	Senol *et.al.*, (2007)
Ag 85 complex	IgG	82%-84.1%	85.2%-86%	Kumar *et.al.*, (2010) Kashyap *et.al.*, (2007)
ESAT-6	IgG	64.9%	88.9%	Kumar *et.al.*, (2010)
CFP-10	IgG	66%	85.2%	Kumar *et .al.*, (2010)
LAM	IgG	80% - 93%	72 -100%	Brown *et.al.*, (2003) Chan *et.al.*, (2000), Sada *et.al.*,. (1992), Boechme et.al.,(2005),
Cord factor	IgG	81%	96%	Fujta *et.al.*, (2005)
CFP-10/ESAT-6	IgG	60.4%	73.8%	Xueqiongwu *et.al.*, (2010)
BCG-CFP	IgG	88.5%	77.3%	Rani *et.al.*, (2005)
38 kDa	IgG	59%-73.6%	85.4%	Xueqiongwu *et.al.*, (2010); Beck *et. al.*, (2005)
MTB 48	IgG	73.2%	77.7%	Xueqiongwu *et.al.*, (2010)
Sonicate supernatant	IgG	80.7%	NA	Agarwal *et.al.*, (1989)
PPD	IgG	94.7%	NA	Agarwal *et.al.*, (1989)
Intact cell	IgG	8.7%	NA	Agarwal *et.al.*,(1989)
26 kDa	IgG	98%	NA	Beck *et. al.*, (2005)
16 kDa	IgG	50.9%	NA	Beck *et. al.*, (2005)
6 kDa	IgG	57%	NA	Beck *et. al.*, (2005)
Ag -6	IgG	89%	NA	Raja *et.al.*, (1994)
TB 16.3	IgG	88-98%	NA	Welding *et al.*,. (2005)

2.19 Interferon gamma released assays (IGRA) for diagnosis of tuberculosis

The vast majority of tuberculosis cases reported in Africa, South East Asia and Western Pacific countries associated with spread of HIV infection has a major impact on current situation of tuberculosis (Steinbrook R *et al.*, 2007). Individuals with HIV infection are at increased risk of rapid progression of a recently acquired tuberculosis infection as well as reactivation of latent tuberculosis infection. Delayed diagnosis of tuberculosis and initiation of appropriate treatment more than 3 weeks after presentation are associated with 45-85% of deaths in HIV infected patient (Bames *et al.*, 1991). Decreased tuberculin reactivity, lower sensitivity of acid fast staining, atypical radiographic presentation and similarity in presentation with other HIV related infections hinders the diagnosis of tuberculosis in HIV infected patients. Recently introduced interferon gamma released assays (IGRA) are promising tests for diagnosis of tuberculosis infection. IGRA is available in two commercial formats namely Quantiferon Tuberculosis-Gold (QFT-G) and T-spot tuberculosis assay. Several studies have been conducted in various clinical settings on the accuracy and utility of IGRA and these have been reviewed elsewhere. Most of these studies have reported that sensitivity of IGRA is modest to detect active infection (Jafari *et al.*, 2006; Kobashi *et al.*, 2006). Kabeer et al (2009) showed that positivity of QFT-G in culture positive pulmonary tubercular, culture negative pulmonary tuberculosis and extra pulmonary tuberculosis patients were 66%, 45% and 77% respectively. Sensitivity and specificity of QFT-G in active tuberculosis in children were 79.9% and 85.8% respectively while sensitivity and specificity of tuberculin skin test were 65.4% and 89.4% respectively (Sester *et al.*, 2010). In active tuberculosis, sensitivity and specificity of QFT-G were 80% and 79% respectively and that of tuberculin skin test was 65% and 75% respectively (Sester *et al.,* 2010). In latent tuberculosis infection (LTBI) sensitivity and specificity of QFT-G was 67% and 99.4% respectively and that of tuberculin skin test (TST) was 71% and 88.7% respectively (Menzies *et al.*, 2007). Therefore, it appears that interferon gamma released assays is more applicable in detecting latent tubercular infection rather then active infection. It is a good alternative to the TST where tuberculosis is less endemic.

CHAPTER 3

MATERIAL AND METHODS

3. Materials and Methods

To address the objectives of the study, a total of 300 suspected tuberculosis cases were included in the study. Sputum and lymph node aspirates were collected for isolation and identification of mycobacteria by culture and biochemical tests. Nucleic acid of mycobacteria in sputum and culture isolates was detected by polymerase chain reaction. Susceptibility to first line anti-tubercular agents was determined by proportion method. Blood was collected from tuberculous and healthy subjects to determine antibody response to mycobacterium specific antigens by ELISA method. The detail methods are described below under different sections.

3.1 Study population and period

Two categories of patients namely suspected cases of pulmonary and extra-pulmonary tuberculosis were included. Sputum and lymph node aspirates from suspected pulmonary and extra pulmonary tuberculosis cases were collected. Sputum was collected from a total of 255 suspected cases of pulmonary TB patients who attended out patient department (OPD) of BIRDEM and Tuberculosis Control and Training Institute (TCATI), Chankharpool, OPD and admitted patients of National Institute Of Diseases of Chest and Hospital (NIDCH) ,Mohakhali, Dhaka. Lymph node aspirates were collected from 45 patients of suspected cervical and axillary tuberculous lymphadenitis attending OPD of Dhaka Medical College Hospital. About 3ml of blood was collected aseptically by venepuncture from 30 bacteriologically confirmed pulmonary TB cases and 30 healthy subjects for serological study. The study was carried out from April 2005 to September 2010.

3.2 Collection of samples

The early morning sputum sample were collected in clean, sterile wide mouthed, closed lid disposable plastic container. The quantity of the sputum collected from each patient was 2-5 ml. The lymph node aspirates were collected aseptically in 50 ml sterile Falcon tubes containing 3ml of sterile distilled water. The containers were labeled with patient's name, identification number and date. The samples were brought to the department of

Microbiology, BIRDEM, Dhaka as soon as possible, where further laboratory works were done in a class-2 bio-safety cabinet. For detection of antibody about 3ml of blood was collected from each patient after taking consent from them.

3.3. Processing of samples for AFB staining and culture

All the samples (sputum and lymph node aspirates) were digested and decontaminated of other bacteria by N-Acetyl-L-Cysteine-Sodium Hydroxide (NALC) method as described by Kent and Kubica (1985). Briefly the method is described below:

a. Fresh digestant and decontaminant solution was prepared by adding 0.5 grams of NALC powder per 100 ml of the mixture of equal volume of 4% sodium hydroxide and 2.9% sodium citrate solution.

b. 2-5 ml of sputum was taken in 50 ml sterile Falcon tube.

c. Equal volume of freshly prepared NALC sodium hydroxide solution was added to the sample in the Falcon tube. The cap of the Falcon tube was made tightened and the tube was vortexed for 10 seconds and allowed to stand for 15 minutes at room temperature.

d. The tube was then filled up to 45 ml with 0.067M phosphate buffer (pH 6.8) and was swirled by hand to mix.

e. The specimen was centrifuged at 3000 rpm for 15 minutes.

f. The supernatant was discarded by sterile pipette and the pellet was resuspended by adding phosphate buffer to achieve a final volume of 3 ml. The resuspended pellet was kept in 3 different eppendrof tubes for (i) preparation of smear for Ziehl-Neelsen (Z-N) stain, (ii) culture of mycobacteria and (iii) for rapid detection of mycobacteria by PCR method.

3.4 Staining and microscopic examination of smears

For acid fast staining new, clean, unscratched and grease free slides were used. One end of the slides was labeled with patient's name, identification number and date. Two smears were made for one sample. One with sputum without concentrated and the other was with concentrated sample. The smear was made over an area of approximately 1×2 cm of the

slide with a bacteriological loop. The smears were air dried and then heat fixed by passing the slides 3-4 times through the blue cone of a burner flame. The smears were stained by Ziehl-Neelsen method (Kent and Kubica, 1985). The stained smear was examined under oil immersion objective of light microscope. The smear was searched by making a series of three parallel sweeps to the length of the smear which included 300 microscopic fields.

3.5 Culture of *Mycobacterium*

Lowenstein-Jensen (L-J) media was used for culture of *Mycobacterium* (Kent and Kubica, 1985). L-J media in McCartney screw capped bottle was labeled with patient's name, identification number and date. Then 0.1ml of decontaminated pellet was inoculated onto the L-J media. The inoculum was spread evenly over the entire surface of the media using a sterile pipette. The media were incubated at 37°C in a slanted position with screw caps loose for 3 days to ensure an even distribution of the inoculum. After 3 days of incubation the caps were tightened to minimize evaporation and drying of the media and the media were kept in upright position. The media were examined within 7 days of incubation to detect the rapidly growing mycobacteria and to enable prompt removal of contaminated cultures. The cultures were examined weekly for 8 weeks for the evidence of growth (Kent and Kubica, 1985). On appearance of visible colonies, colony morphology, rate of growth and pigment production were noted. *M. tuberculosis* produces dry, buff to cream colored rough surfaced colonies (Appendix 1.2). The growth was confirmed by acid fast staining of colonies with Z–N staining method and the organisms were identified by biochemical tests. If no growth appeared after 8 weeks of incubation, the culture was reported as no growth.

3.6 Biochemical tests for Identification of *Mycobacterium*

All the isolates of mycobacteria were identified for species by colony morphology, growth rate, pigment production, staining and biochemical tests. Catalase, niacin, nitrate reduction test, growth inhibition test by para-nitrobenzoic acid (PNB) test and Thiophen -2-carboxylic acid hydrazide (TCH) susceptibility test were done to identify *M. tuberculosis* and other mycobacteria. Interpretation of biochemical tests was done as described elsewhere.

3.6.1 Niacin Test (Kent and Kubica, 1985): Four weeks old culture on (L-J) media was flooded with one ml sterile distilled water and the bottles were placed in slating position so that water covers the entire surface of the medium for 30 minutes. Then 0.5 ml of the fluid, which contained extracted niacin, was taken in a clean screw capped test tube. To it 0.5 ml of the 4% aniline solution (4ml aniline in 96 ml of 95% ethanol) and 0.5 ml of 10% cyanogen bromide (5 gm cyanogen bromide in 50 ml distilled water) were added and shaken well. Immediate development of yellow colour indicated positive result. No colour change indicated negative result (Fig 3.1). An extract from a known culture of reference strain of *M. tuberculosis* H37Rv was taken as positive control and extraction of fluid from uninoculated media was taken as reagent control.

3.6.2 Nitrate Reduction Test: The test was done as described by Kent and Kubica (1985). Briefly,

- 2 loopfuls of test organisms from a 4-weeks old culture was inoculated into a screw cap test tube containing 2 ml of $NaNO_3$ buffer substrate solution (Appendix 1) and vortexed well to make a suspension.
- The suspension was incubated upright for 2 hours at 37°C in a water bath.
- After removing from water bath 1 drop of 1:1 dilution of concentrated HCL (50 ml conc. HCL in 50 ml of distilled water) was added.
- Then 2 drops of 0.2% aqueous solution of sulfanilamide were added.
- 2 drops of 0.1% aqueous solution of N- naphthylethylene-diamine dihydrochloride were added and shaken well and the tubes were examined immediately for change in color.
- If no colour developed the test was either negative or the reduction has proceeded beyond nitrite. Small amount of powdered zinc was added to all negative tests. If nitrite was still present, it was catalytically reduced by zinc dust and a red color developed indicating true negative. If no color developed when zinc dust was added, the original reaction was positive but the nitrate was reduced beyond nitrite.
- A positive test was indicated by the change in colour, which ranged from pale pink (0) to deep red (5+) when compared with the colour standards. Only 3+ to 5+ were

Table 3.1: Biochemical properties of common mycobacteria encountered in clinical practice (Forbes et al, 2002)

Group	Species	Niacin production	Nitrate reduction	68° catalase	Growth on TCH
MTuberculosis complex	*M.tuberculosis*	+	+	-	+
	M.bovis	-	-	-	-
	M.africanum	-	-	-	V
Photo chromogen	*M.marinum*	±	-	+	+
	M.kansasii	-	+	-	-
	M.simiae	+	-	+	+
	M.asiaticum	-	-	+	+
Scoto chromogen	*M.scrofulaceum*	-	-	+	+
	M.szulgai	-	+	+	+
	M.gordonae	-	-	+	+
Non photo chromogen	*M.avium comp.*	-	-	±	+
	M.gastri	-	-	-	+
	M.malmoence	-	-	±	+
	M.ulcerans	-	-	+	+
	M.flavescens	-	-	+	+
	M.xenopi	-	-	+	+
Rapid growers	*M.fortuitum*		+	+	-
	M.cheloni	V	-	V	-
	M abscessus	V	-	V	-
	M. smegmatis	-	+	+	-

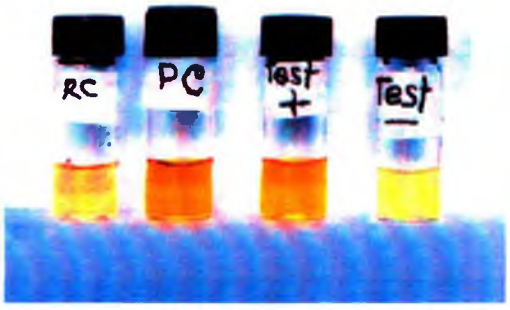


Figure 3.1: Niacin test: (RC) Reagent control; (PC) Positive control (H37Rv); (Test +) sample containg *M tuberculosis;* (Test-) sample containg no *M tuberculosis.*

Figure 3.2: Nitrate reductuion test; (RC) Reagent control; (PC) Positive control (H37Rv); (sample +) sample containg *M tuberculosis;* (sample-) sample containg no *M tuberculosis.*

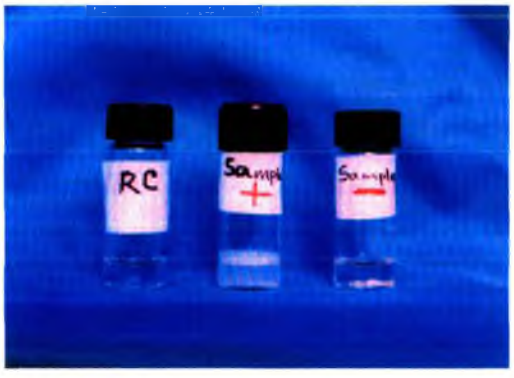


Figure 3.3: Catalase test: (RC) reagent control; (Sample +) sample containg *M tuberculosis;* (sample-) sample containg no *M tuberculosis.*

considered positive for *M. tuberculosis*. A negative test was indicated by no colour change. Fig 3.3 shows the results of nitrate reduction tests.

M. tuberculosis H37Rv was taken as positive control, tubes containing reagents without organisms were taken as negative control.

3.6.3 Heat stable catalase test: Catalase, an intracellular soluble enzyme is capable of splitting hydrogen peroxide into water and oxygen. *M. tuberculosis* and *M. bovis* does not show catalase activity when suspended in 0.067 M phosphate buffer (pH 7.0) and heated to 68°C for 20 minutes. The test was carried out as described by elsewhere **(Kent and Kubica, 1985)**

A suspension was prepared from several loopful of test organisms in 0.5 ml of 0.067M phosphate buffer solution at pH 7.0 **(Appendix 2)** in a screw capped test tube and placed in water bath at 68°C for 20 minutes. The tubes were removed from the water bath and allowed to cool at room temperature. Then 0.5 ml of Tween-hydrogen peroxide mixture (equal volume of 10%Tween 80 and 30% hydrogen peroxide) was added to the tube and then the tube was recapped gently. A positive test was indicated by the formation of bubbles (Fig 3.3). No bubbles indicated a negative test. Negative tubes were not discarded before 20 minutes.

For "negative control" *M. tuberculosis* (H37Rv) was used while phosphate buffer solution without *Mycobacterium* was used as "reagent control". *M. gordonae* was not available for "positive control".

3.6.4 Sensitivity to para-nitrobenzoic acid (PNB) (Laidlaw, 1989)

M. tuberculosis and *M. bovis* are sensitive to PNB **(Appendix 3)** and fail to grow on the PNB medium. Non-tuberculous mycobacteria are resistant and grow in PNB medium.

Test procedure:

- Two sets of L-J media were prepared, one containing PNB at a concentration of 500µg/ml and the other set as PNB free control medium.
- A large loopful of homogenized culture suspension were inoculated on the slope of one L-J media containing PNB and one PNB free control media.
- After 3 weeks of incubation both the tubes were observed for growth.
- If growth occurred in PNB free control medium but not in PNB containing medium then it was considered as *M. tuberculosis* complex. If growth occurred both in PNB

free control medium and in PNB containing medium then it was considered as non-tuberculosis mycobacteria.

In each batch of test one PNB containing tube was inoculated with reference strain of *M. tuberculosis* H37Rv as 'negative' control.

3.6.5 Growth inhibition by Thiophene -2- Carboxylic Acid Hydrazide (TCH) (Kent and Kubica, 1985)

This test was done to distinguish *M. bovis* from *M. tuberculosis*. Only *M. bovis* is susceptible to low concentration of TCH (1-5μg/ml). *M. tuberculosis* and other mycobacteria are usually resistant to the inhibitory action of this compound.

A loopful of bacterial suspension was inoculated on two tubes of L-J media, one containing sterile TCH at a concentration of 10 μg/ml and another without TCH. A set of similar test was done with reference strain of *M. tuberculosis* H37Rv as positive control. The bottles were incubated at 37°C and reading was taken after three weeks.

If there was growth in TCH free control bottle but not on TCH containing media then it was considered as *M. bovis*. If growth occurred in TCH free control bottles and on TCH containing media then it was either *M. tuberculosis* or other mycobacteria.

3.7. Drug susceptibility test

Drug susceptibility to isoniazid, rifampicin, streptomycin and ethambutol was done by the indirect proportion method (Kent and Kubica, 1985). The drugs used for susceptibility test were obtained from Aventis, Bangladesh except streptomycin which was obtained from Opsonin Chemical Industries Ltd. Dhaka with proper label mentioning manufacturing and expiry date. The potency of each antibiotics was verified by reference H37Rv strains.

3.7.1. Preperation of drug containing media

The measured amount of drug was diluted with specific diluent and sterilized by membrane filter (pore size 0.22 micron). Required amount of stock solutions of drugs were added to different media to obtain critical concentration of drugs in each medium. The concentrations of drugs used in this study are given in Table 3.2.

Table 3.2: Concentration of anti-tubercular drugs in L-J medium (Canetti *et al.*, 1969)

Drug	Concentration of drugs in L-J Medium (μg/ml)
Isoniazid	0.2 μg/ml
Rifampicin	40. 0 μu/ml
Streptomycin	4μg/ml
Ethambutol	2 μg/ml

To prepare L-J drug containing and drug free media, five 500-ml sterile flasks were taken and labeled properly, one for control and other four for each of the four drugs (isoniazid, rifampicin, streptomycin and ethambutol). To each flask required quantity of mineral salt solution-egg fluid-malachite green mixture was taken aseptically. To prepare drug-containing media, appropriate amount of the stock solution of each drug was added to the labeled flasks to have the final concentration of the drugs (Table 3.2). While adding drugs, flasks were shaken gently to mix the drugs properly with the media. Then the media was dispensed in 6-8 ml amounts in each pre-labeled McCartney bottle with aseptic precaution and then solidified in hot water bath at 85°C for 50 minutes. The media were incubated at 37°C for 48 hours as a sterility check. The media were stored in the refrigerator at 4° C with caps were tightly closed to prevent evaporation.

3.7.2 Inoculation and incubation and reading for resistance

Several loopful (2-5 mg) of growth from the primary culture bottle was taken in a sterile screw cap tube containing 3 ml of sterile distilled water and 6 to 8 glass beads. It was ensured that portions of multiple colonies were picked from the primary culture. The suspension was then homogenized by vortex for 5 minutes. Larger particles were allowed to settle down and supernatant were adjusted with the turbidity of the McFarland standard 1 (Appendix 4). Similar type of test tube was used for preparation of McFarland turbidity standard. The suspension of test organisms was further diluted to 10^{-2} and 10^{-4} with sterile distilled water. Using a micropipette 0.1 ml of both dilutions were inoculated in two sets of drug containing media and drug free control media. All media were incubated at 37°C. The media were examined at 48 hours and then weekly. The reading for drug susceptibility was taken at 3 weeks. The readings were not taken before 3weeks as resistant mutants often

grow more slowly than susceptible ones and may not produce visible colonies until the 3 weeks. Again colonies appearing on the drug containing media after 3 weeks may not represent resistant bacilli as some drugs, on prolonged incubation, may be inactivated to sub static level permitting growth of susceptible strains in drug containing media. Reference strain of *M. tuberculosis* H37Rv was used as a susceptible control each time when susceptibility test was done.

3.7.3 Interpretation

The number of colonies on control and drug containing media were counted and the percentage of the resistant organisms was calculated as follows:

$$\frac{\text{Number of colonies on drug media x100}}{\text{Number of colonies on control media}} = \% \text{ resistant}$$

If the percentage of resistant organism was 1% or more then the isolate was considered resistant to that specific drug. A set of tubes with and without drugs were inoculated with reference H37Rv strain of *M. tuberculosis* as a quality control.

3.8 Detection of nuclic acid of mycobacteria by PCR method

Sputum, lymph node aspirates and culture isolates from patients were tested to detect *Mycobacterium* specific DNA by PCR. Multiplex PCR was performed for detection of *M. tuberculosis* and MOTT.

A commercial thermostable PCR kit (EZTB-PCR kit) designed to detect both the M. tubercusolis specific and Mycobacterium genus specific DNA was used. The kit was obtained from MBDr, Biodiagnostic Research Sdn Bhd, Malaysia. The detection method was based on the use of multiplex polymerase chain reaction to detect the genomic DNA in the samples which was extracted in a few steps.

The kit contained thermostable PCR reagents and primers specific for M. tuberculosis and *Mycobacterium* genus. Five pairs of primers were used. Two pairs of primers were for *M. tuberculosis* and 2 pairs for genus specific. One pair of primer was used for internal

control. The targets for the primers and the corresponding size of the amplified products were as follows:

- IS6110 ---------- 541 bp
- HSP65---------- 127 bp
- ISB9---------- 383 bp
- DNAJ ---------- 211 bp

3.8.1 Extractions of DNA from sputum, lymph node aspirate and culture colonies

- Sputum/lymph node aspirate were digested and decontaminated by NALC- sodium hydroxide method and pellets were used for DNA extraction. Organisms from culture were directly used.
- 100 µl of DNA extraction buffer (supplied with kits) was added to the pellet and it was vortexed briefly to mix.
- Then it was heated at 100°C for 10 mins and vortexed briefly to mix.
- Then it was centrifuged at 12,000 rpm for 50 mins to precipitate resin. 50 µl of supernatant was collected into a sterile micro-centrifuge tube. Only 5 µl was used for PCR reaction. The remaining supernatant was stored at -20°C for future PCR (if necessary).

3.8.2 Procedure for PCR

- 15 µl of water (supplied) was added to each thermostabilized PCR mix tube, left for 10 mins at room temperature then it was vortexed briefly to ensure the thermostabilized PCR mix was well dissolved.
- 5 µl of extracted DNA sample was added to the thermostable PCR mix.
- Positive control was prepared by adding 40 µl of water (supplied) and then it was left for 2 mins at room temperature. And the tube was vortexed to reconstitute. Then 5 µl was added to the thermostable PCR mix. Remaining positive control was stored at -20°C for future use.
- For negative control, 5 µl of water (supplied) was added to the thermostable PCR mix.

- The tubes were placed in thermal cycler and PCR reaction was started by using PCR cycling conditions as follows:

Temperature/Time	Cycles
95°C/3 mins	1 cycle
95°C/30 sec	30 cycles
57°C/30 sec	
72°C/40 sec	
72°C/ 5 mins	1 cycle

3.8.3. Detection of amplified PCR product by electrophoresis

The PCR products were analyzed by gel electrophoresis as described below:

- 2% agarose gel was prepared in tris borate EDTA buffer (TBE buffer) pH.7.5-7.8.
- 10µl of PCR product was loaded onto the gel. Then electrophoresis was run at 60 volts for 45 mins.
- TB DNA ladder was prepared by adding 80 µl of water distilled water to the tube containing the ladder and left for 2 minutes at room temperature and the tube was vortexed to reconstitute. 10µl of TB DNA ladder was loaded into one well of the gel. The remaining TB DNA ladder was stored at -20°C for future use.)
- The product size was analyzed by Bio-Rad gel documentation system, and DNA fragment(s) size was compared with DNA ladder which was loaded together in the gel.
- Each time H37Rv was used as a positive control in addition to the positive control provided. Colonies of H37Rv was treated similar to samples for extraction of DNA and for PCR reaction. Then the results were interpreted as per Table 3.5.

Table 3.3: Interpretation of PCR results

Size of amplicon				Internal Control (663 bp)	Interpretation
541 bp	127 bp	383 bp	211 bp		
+	+	+	+	+/-	*Mycobacterium tuberculosis*
+	-	+	+	+	*Mycobacterium tuberculosis*
+	-	+	+	-	*Mycobacterium tuberculosis*
-	+	-	+	-	*Mycobacterium tuberculosis*
-	-	+	+	+	Atypical mycobateria
-	-	+	+	+	Atypical mycobateria
-	-	-	+	+	Atypical mycobateria
-	-	-	-	+	*Mycobacterium tuberculosis* and atypical mycobacteria not detected
-	-	-	-	-	Sample contains PCR inhibitors

3.9. Humoral immune response

Humoral immune response was determined by enzyme linked immunosorbant assay (ELISA) by determining the titre of IgM and IgG antbodies to 4 different *M. tuberculosis* antigens namely Ag 85 complex, cell wall fraction (CWF), culture filtrate protein (CFP) and lipoarabinomannan (LAM). The antigens were obtained from the Department of Microbiology, Immunology and Pathology, Colorado State University, 1682 Campus Delivery, Fort Collins, CO 80523. Sera from 30 confirmed active tuberculosis cases and 30 healthy individuals were tested for antibodies against the above mentioned antigens.

3.9.1 Procedure for ELISA

- Each of the antigens was diluted with carbonate/bicarbonate buffer (pH 9.6) **(Appendix 5)** at a concentration of 5µg/ml. The working concentration of each antigen was determined by using different concentration of antigens namely 2.5, 5 and 10 µg/ml.
- To coat the wells of microtitre plate (96 well), 100 µl of diluted antigens (5 µg/ml) was added to each well. Plate was covered with plastic cover and was incubated at 37°C for 2 hours. Then antigen coating solution was removed and the plate was washed 3 times by filling the wells with 250 µl of PBS- 0.05%Tween 20 (PBS-T, pH 7.2, Appendix 7). The wash buffer was removed by flicking the plate over a sink. The remaining drops were removed by patting the plate over a paper towel.
- The remaining protein binding size of the wells was blocked by adding 200 µl of blocking buffer (2% BSA in PBS-0.05%Tween 20). The plate was covered with plastic cover and kept at 4°C for over night. Then the plate was washed 3 times with PBS –T.
- Then patient's serum and serum of healthy individuals were diluted at a concentration of 1:400, 1:800 and 1:1600 with PBS-T with 0.1% BSA. 100 µl of each diluted serum was added to antigen coated wells.
- The plate was covered with a plastic cover an incubated at 37°C for 2 hours. After incubation, the plate was washed 3 times with PBS-T
- Then 100 µl of IgM or IgG anti-human-HRP conjugate was added in each well at a dilution of 1:5000 (MP Biomedicals, USA) and kept at 37°C for 2 hours.

- The plate was washed 3 times with PBS-T
- Then 50µl of TMB substrate was added in each well and it was kept for 30 minitues at dark at room temperature.
- Then 50µl of 1 M sulfuric acid was added in each well and absorbance was read at 450nm in Human ELISA reader.

3.9.2 Interpretation of the result:

The concentration of antibody was expressed in OD at a particular dilution of both patient and healthy control samples.

3.9.3 Ethical issues

No extra samples were obtained from patients for the study as sputum and blood were collected routinely for diagnostic purpose while the patients attended the hospital. However, verbal consent was taken from the study population for obtaining history and for taking leftover samples submitted for diagnostic purpose. For collection of blood from healthy subjects verbal consent was taken from them.

CHAPTER 4

RESULTS

Table 4.1: Clinical profile of culture positive pulmonary tuberculosis cases (n=180)

Characteristics	Present	
	No.	%
Cough	180	100
Expectoration	174	96.66
Fever	172	95.55
Haemoptysis	28	15.55
Weight loss	156	86.66
Lymph node Enlargement	24	13.33
ESR(50-120mm in 1st hour)	180	100
*Positive X-Ray chest	144	80.00
History of anti TB treatment	20	11.11

**Positive X-Ray chest indicate the presence of either opacity, cavity, pleural effusion or combination.*

Table 4.2: Clinical profile of culture positive lymph node TB cases (n=20)

Characteristics	Present	
	No.	%
Cough	8	40
Expectoration	00	00
Fever	20	100
Haemoptysis	00	00
Weight loss	11	55
Lymph node Enlargement	20	100
ESR (50-120mm in 1st hour)	20	100
*X-Ray chest P/A view (suggestive of TB)	9	45
History of anti TB treatment	1	05

**Positive X-Ray chest indicate the presence of either opacity, cavity, pleural effusion or combination.*

Table 4.3: Results of culture of study samples

Specimen	Total Number	Culture of *Mycobacterium*		Samples Contaminated N (%)
		Positive N (%)	Negative N (%)	
Sputum	255	180 (70.59)	40 (15.69)	35 (13.72)
Lymph node aspirate	45	20 (44.45)	15 (33.33)	10 (22.22)
Total	300	200 (66.67)	55 (18.33)	45(15.00)

Table 4.4: Species distribution of culture positive mycobacteria in sputum and lymph node aspirates

Samples	Total culture positive	Mycobacteria	
		M. tuberculosis	MOTT
Sputum	180	176 (97.8)	4 (2.2)
Lymph node aspirates	20	16 (80.0)	4 (20.0)
Total	200	192 (96.00)	8 (4.00)

MOTT=Mycobacteria other than tuberculosis

Results of PCR test of direct sputum, lymph node aspirate and culture colonies are described in Table 5. Fig-1 shows the electrophoresis result of amplified product of PCR. A total of 47 culture positive sputum samples were directly subjected to PCR for the detection of mycobacteria specific nucleic acid. Out of 45 culture postive samples, 42 (93.3%) was positive by PCR while 3 yielded negative results. Out of 42 PCR positive, 2 were identified as MOTT. These 2 isolates of MOTT were also identified as MOTT by culture and biochemical tests. On the other hand, 20 culture negative sputum specimens were subjected to PCR. Out of 20 culture negative sputum samples, 2 (10%) were positive by PCR. In additon to sputum samples, 10 culture postive lymph node aspirate were directly subjected to PCR test and all (100%) became positive for *M. tuberculosis* by PCR. Twenty eight pure culture isolates were also tested by PCR. All 28 (100%) culture samples were positive by PCR. The pure culture was used as positive controls.

Overall susceptibility pattern of *M.tuberculosis* and MOTT to first line anti-TB drugs are depicted in Table-6. It was observed that out of the total 200 isolates, 192 were *M. tuberculosis* and 8 were MOTT. Out of 192 *M.tuberculosis* isolates 89 (46.35%) were sensitive to all of the four first line anti-TB drugs and 103 (53.65%) were resistant to any of the four first line anti-TB drugs. In case of the MOTT, all 8 (100%) were resistant to any of the first line anti-TB drugs.

The rate of drug resistance of *M. tuberculosis* isolated from newly detected (untreated) and previously treated cases are described in Table-7. Out of the total 192 *M. tuberculosis* isolates, 167 were newly detected and 25 were previously treated cases. Among the 167 newly detected cases 78 (46.71%) were resistant to any of the four first line anti-TB drugs and overall drug resistance pattern was INH 37 (22.15%), RIF 16 (9.58%), ETHAM 22 (13.17%), and SM 37 (22.15%). Among the previously treated cases all 25 (100%) were resistant to any drug and overall drug resistant pattern were INH 13 (52.0%), RIF 14 (56.0%), ETHAM 17 (68.0%) and SM 13 (52.0%).

Table 4.5: Result of PCR of direct sputum, LN aspirates and culture colony

Sample Type	Culture status	Number	PCR Result	
			Positive N (%)	Negative N (%)
Sputum	Positive	45	42[1] (93.3)	03 (10.7)
	Negative	22	02 (9.1)	20 (90.9)
LN aspirate	Positive	10	10 (100)	0
Culture colony	-	28	28 (100)	0

Note: [1]Out of 42 PCR positive, 2 were MOTT; LN = lymph node;

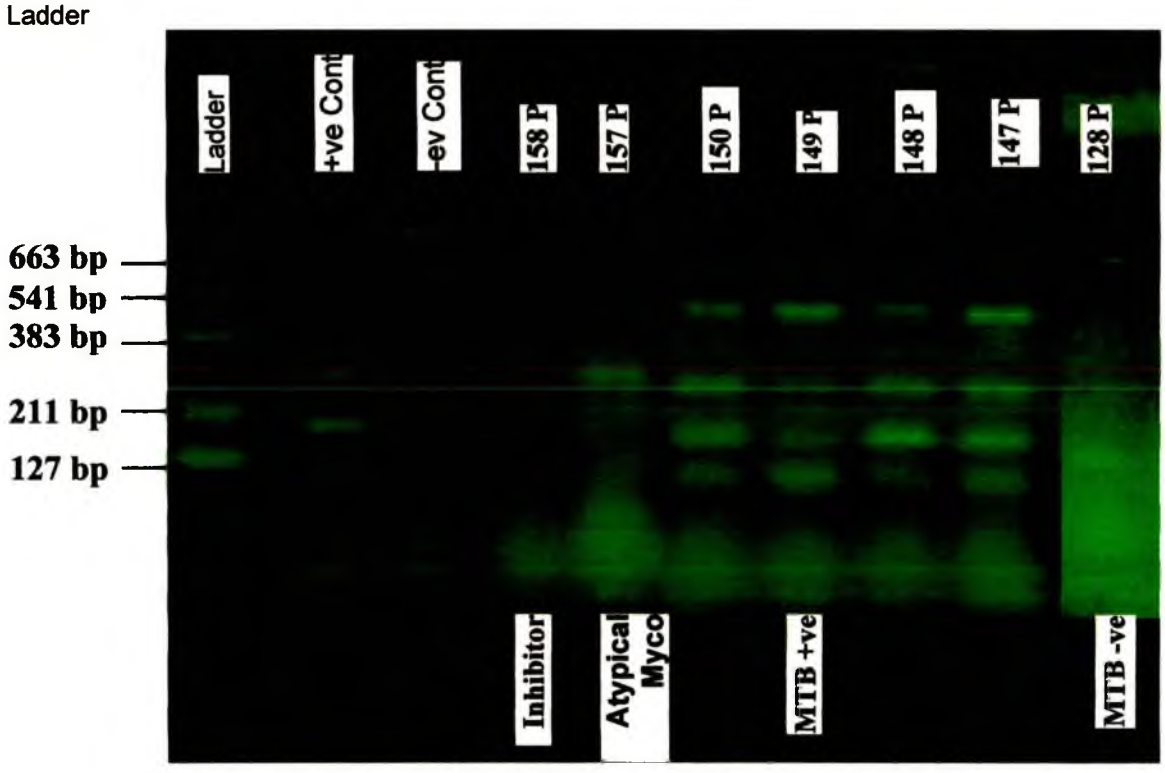


Fig 4.1: Gel electrophoresis of amplified product of PCR

Table 4.6: Overall susceptibility pattern of *M. tuberculosis* and MOTT to first line anti-TB drugs

Organism	Total Isolates	Resistant to any drug N (%)	Sensitive N (%)
MTB	192	103 (53.65)	89 (46.35)
MOTT	08	08 (100)	00

Note: Sensitive means sensitive to all drug (INH, RIF, ETHAM, SM)

Table 4.7: Rate of drug resistance of *M. tuberculosis* isolated from newly detected and previously treated tuberculosis cases

Category of cases	Total cases	Overall resistant to				Resistant to any drug N (%)
		INH N (%)	RIF N (%)	ETHAM N (%)	SM N (%)	
Newly detected cases	167	37 (22.15)	16 (9.58)	22 (13.17)	37 (22.15)	78 (46.71)
Previously treated cases	25	13 (52.0)	14 (56.0)	17 (68.0)	13 (68.0)	25(100)

Note: INH- isoniazid, RIF- rifampicin, ETHAM-ethambutol, SM-streptomycin

Table 4.8 shows resistant pattern of 167 *M. tuberculosis* isolates to 4 first line anti-TB drugs in newly detected cases. Out of the total 167 isolates 53 (31.74%) were resistant to one drug, 19 (11.38%) were resistant to two drugs, 3 (1.80%) were resistant to three drugs and 3 (1.80%) were resistant to four drugs.

Table 4.9 shows resistance pattern of *M. tuberculosis* to four first line anti-TB drugs in previously treated cases. Out of the total 25 isolates, 4 (16.0%) were resistant to one drug, 13 (52.0%) were resistant to two drugs, 1 (4.0%) was resistant to three drugs and 7 (28.0%) were resistant to four drugs.

Table 4.10 shows the rate of MDR-TB in new and treated pulmonary TB cases. Among the new cases, MDR-TB was 4.2% while it was 36.0% among the treated cases. The rate was significantly higher in previously treated group.

Table 4.11 shows the resistance pattern of isolated MOTT to four first line anti-TB drugs. Out of the 8 MOTT, resistance to INH, RIF, ETHAM and SM was 75.0%, 50.0%, 75.0% and 100.0% respectively and 12.5% was resistant to two drugs,75% was resistant to three drugs,12.5% was resistant to four drugs.

The rate of concomitant resistance pattern of rifampicin resistant *M. tuberculosis* to INH, ETHAM and SM are described in Table 4.12. It was observed that 83.3% RIF resistant *M. tuberculosis* isolates were resistant to other three drugs. The association of RIF resistance with resistance to other three drugs were significantly associated ($p<.05$). The concomitant resistance of INH, ETHAM and SM resistant *M. tuberculosis* to any other three drugs were 57.4-76.3% and the co-resistance was not significantly associated ($P>0.05$).

Table 4.13 shows the concomitant resistance rate of *M. tuberculosis* to any three first line anti-TB drugs which were sensitive to either RIF, INH, ETHAM or SM. Rate of resistance to three other drugs ranged between 34.78% to 43.21% among RIF, INH, ETHAM or SM sensitive isolates. The table indicates that a high proportion of sensitive *M. tuberculosis*

Table 4.8: Resistant pattern of *M. tuberculosis* to 4 first line anti-tubercular drugs isolated from newly detected tuberculosis cases (n=167)

No. of drugs	Name of resistant drugs	Resistant		Total	
		N	(%)	N	(%)
One drug	Only INH	18	(10.78)		
	Only RIF	5	(2.99)		
	Only ETHAM	8	(4.79)	53	(31.74)
	Only SM	22	(13.17)		
	*INH+RIF	1	(0.60)		
	INH+SM	4	(2.40)	19	(11.38)
Two drugs	INH+ETHAM	8	(4.79)		
	RIF+SM	4	(2.40)		
	ETHAM+SM	2	(1.20)		
Three drugs	*INH + RIF+SM	2	(1.20)	3	(1.80)
	*INH + RIF+ETHAM	1	(0.60)		
Four drugs	*INH+RIF+ETHAM+SM	3	(1.80)	3	(1.80)

*Note: * indicates MDR-TB*

Table 4.9: Resistance pattern of *M. tuberculosis* to 4 first line anti-TB drugs isolated from previously detected cases (n=25)

No. of drugs	Name of resistant drugs	Resistant		Total	
		N	(%)	N	(%)
One drug	Only ETHAM	02	08		
	Only SM	02	08	04	16
	*INH+RIF	1	4		
Two drugs	INH+SM	3	12		
	INH+ETHAM	1	4		
	RIF+ETHAM	4	16	13	52
	RIF+SM	1	4		
		3	12		
Three drugs	ETHAM+SM				
	*INH + RIF+SM	1	4	1	4
Four drugs	*INH+RIF+ETHAM+SM	7	28	7	28

*Note: * indicates MDR-TB*

Table 4.10: Rate of isolation of MDR-TB from new and treated pulmonary tuberculosis cases

Categories	Total Number	MDR N (%)
New cases	167	7 (4.2)
Treated cases	25	9 (36.0)

Table 4.11: Resistance pattern of MOTT to four first line anti tubercular drugs (n=8)

No. of drugs	Name of drugs	Resistant		Total	
		N	(%)	N	(%)
	INH	6	(75)		
	RIF	4	(50)		
	ETHAM	6	(75)		
	SM	8	(100)		
Two drugs	ETHAM+SM	01	(12.5)	1	(12.5)
Three drugs	INH + RIF+SM	2	(25.0)	6	(75)
	INH + ETHAM+SM	3	(37.5)		
	RIF + ETHAM+SM	1	(12.5)		
Four drugs	INH+RIF+ETHAM+SM	1	(12.5)	1	(12.5)

Note: There was no resistance to single drug alone. INH, RIF, ETHAM and SM were resistant in combination with other drugs.

Table 4.12: Rate of concomitant resistance pattern of RIF resistant mycobacteria to INH, ETHAM and SM

Resistant to	No	Resistant to 3 other drugs	No of isolates concomittently resistant tof			
			RIF	INH	ETHAM	SM
RIF	30	25 * (83.3) p<0.05	-	17 (56.67)	19 (63.33)	18 (60.0)
INH	49	32 ** (65.31) p>0.05	16 (32.65)	-	19 (38.71)	21 (42.86)
ETHAM	38	29 (76.3)	16 (42.1)	19 (50.0)	-	15 (39.5)
SM	54	31 (57.4)	20 (37.0)	21 (38.89)	16 (29.6)	-

Note: * χ^2 test between RIF resistance and resistance to other three drugs (P<0.05);
** χ^2 test between INH resistance and resistance to other three drugs (P>0.05)
RIF-rifampicin, INH-isoniazid, ETHAM-etambutol, SM-streptomycin

isolates (sensitive to RIF, INH, ETHAM and SM) could be resistant to any of the three other first line anti-TB drugs and it could not predict that if an isolate sensitive to any single first line drug would simultaneously be sensitive to other three drugs.

Table 4.13 shows the concomitant resistance rate of *M. tuberculosis* to any three first line anti-TB drugs which were sensitive to either RIF, INH, ETHAM or SM. Rate of resistance to three other drugs ranged between 34.78% to 43.21% among RIF, INH, ETHAM or SM sensitive isolates. The table indicates that a high proportion of sensitive *M. tuberculosis* isolates (sensitive to RIF, INH, ETHAM and SM) could be resistant to any of the three other first line anti-TB drugs and it could not predict that if an isolate sensitive to any single first line drug would simultaneously be sensitive to other three drugs.

Time required for the growth of mycobacterium (n=200) in L-J media has been depicted in Table 4.14. It shows that 148 (74%) samples yielded growth of *Mycobacterium* within 22-28 days of incubation and 60 (30%) grew within 15-21 days. None of the sample became culture positive within first 7 days of incubation.

Antibody response to four mycobacterial antigens namely Ag 85 complex, culture filtrate protein (CFP), cell wall fraction (CWF) and lipoarabinomamannan (LAM) was determined in sera of 30 confirmed cases of tuberculosis and 30 healthy subjects. IgM and IgG response were determined by ELISA method and concentration of antibodies was expressed in terms of OD value. Initially checkerboard method was used to optimize working serum dilution for respective antigens and conjugates. IgM and IgG antibody response to culture filtrate protein antigen in tuberculosis patients and healthy subjects are described in Table 4.15. It was observed that mean OD values of serum IgM and IgG against CFP were significantly higher in patients (P=0.0006; 0.0001) than that of healthy control subjects.

IgM and IgG antibody response to Ag 85 complex antigen in tuberculosis patients and healthy control subjects are depicted in Table 4.16. The mean serum IgM and IgG antibodies against Ag 85 complex was significantly higher (P=0.0001) in TB patients than that of healthy control subjects.

Table 13: Rate of concomitant resistance of RIF/INH/ETHAM/SM sensitive *M. tuberculosis* to corresponding drugs

Sensitive to	No.	Resistant to other 3 drugs		No. (%) of isolates concomitantly resistant to			
		No.	%	RIF	INH	ETHAM	SM
RIF	162	70	(43.21)	---	30 (18.52)	21 (12.96)	36 (22.22)
INH	143	52	(36.36)	13 (9.09)	---	19 (13.28)	34 (23.77)
ETHAM	154	63	(40.90)	14 (9.09)	29 (18.83)	---	38 (24.67)
SM	138	48	(34.78)	12 (8.69)	29 (21.01)	23 (16.66)	---

Note: INH- isoniazid, RIF- rifampicin, ETHAM - ethambutol, SM - streptomycin

Table 14: Time required for growth of *Mycobacterium* in L-J media. (n=200)

Time required for growth (Days)	No. (%) of samples positive (cumulative)	
	No.	%
<7	00	00
8-14	5	2.5
15-21	60	30
22-28	148	74
29-42	200	100

Table 15: IgM and IgG antibody response to culture filtrate protein antigen in tuberculosis patients and healthy control subjects

Study population	Total No.	Antibody to culture filtrate protein	
		IgM $\bar{x}$ OD ±SE	IgG $\bar{x}$ ±SE
Patients	30	0.775 ±0.102	1.324 ±0.112
Healthy control	30	0.387 ±0.032	0.569 ±0.071
P value		p=0.0006	p=0.0001

Note: Serum dilution at 1:400 and conjugate dilution 1:5000

Table 16: IgM and IgG antibody response to Ag 85 complex antigen in tuberculosis patients and healthy control subjects

Study population	Total	Antibody to Ag 85 complex antigen	
		IgM $\bar{x}$ OD ±SE	IgG $\bar{x}$ ±SE
Patients	30	1.176 ±0.091	1.505 ±0.134
Healthy control	30	0.557 ±0.03	0.867 ±0.073
P value		p=0.0001	p=0.0001

Note: Serum dilution of IgM at 1:400 & Serum dilution of IgG at 1:1600 and conjugate dilution 1:5000

Antibody response (IgM and IgG) to LAM antigen in tuberculosis patients and healthy control subjects are shown in the table 4.17. It was shown that mean IgM antibody (P=0.04) to LAM was higher in the serum of healthy control subjects than that of tuberculosis patients. But mean OD of IgG antibody to LAM was significantly higher (P=0.0001) in the serum of patients than that of healthy control subjects.

Antibody (IgM and IgG) response to CWF antigen in tuberculosis patients and healthy control subjects are shown in the Table 4.18. It was found that mean OD of IgM in healthy subjects was significantly higher (P=0.0001) than that of patients suffering from tuberculosis. But mean OD of IgG in tuberculosis patients was not significantly higher (P=0.445) than that of healthy controls.

In order to diagnose active tuberculosis cases by detecting IgM and IgG antibody response against specific antigens, a cut off OD value was determined. The cut off OD value was determined by the following formula:

Cut off OD value= Mean antibody titer of healthy subjects against specific antigen+2 × SD

Any OD value above the cut off was considered as positive for respective antibodies.

The result of cut off OD values for IgM and IgG antibodies are described in Table 4.19. It was observed that among 30 healthy subjects cut off OD value of anti-Ag 85 complex IgM was 0.877 and anti Ag85 complex IgG was 1.655. Among 30 healthy subjects cut off OD value of anti CFP (IgM) was 0.745 and anti CFP (IgG) was 1.355 at serum dilution of 1:400 for IgM and 1:1600 for IgG to Ag 85 complex.

The Table 4.20 describes serodiagnosis of tuberculosis patients by detecting IgM and IgG antibodies against Ag 85 complex and CFP antigens. It was observed that among the total number of 30 patients Anti-Ag 85 complex (IgM) was positive in 66.67% patients and it's sensitivity and specificity was 75.0% and 96.7% respectively. The anti-Ag 85 complex IgG was positive in 70.0% of patients and sensitivity and specificity was 76.9% and 93.75% respectively. Among the total number of 30 patients, anti-CFP IgM was positive in 33.3% of patients and sensitivity and specificity was 60.0% and 96.7% respectively. Anti-CFP IgG was positive in 56.7% and sensitivity and specificity was 69.8% and 93.75% respectively.

Table 4.17: IgM and IgG antibody response to LAM antigen in tuberculosis patients and healthy control subjects

Study population	Total	Antibody to LAM antigen	
		IgM $\bar{x}$ OD ±SE	IgG $\bar{x}$ ±SE
Patients	30	0.911 ±0.051	1.842 ±0.067
Healthy control	30	1.053 ±0.049	1.151 ±0.054
P value		p=0.04	p=0.0001

Note: Serum dilutions1:6400; conjugate dilution 1:5000

Table 4.18: IgM and IgG antibody response to cell wall fraction (CWF) antigen in tuberculosis patients and healthy control subjects

Study population	Total	Antibody to CWF	
		IgM $\bar{x}$ OD ±SE	IgG $\bar{x}$ ±SE
Patients	30	0.605 ±0.059	1.501 ±0.122
Healthy control	30	1.190 ±0.882	1.384 ±0.091
P value		p=0.0001	p=0.445

Note: Serum dilution at 1:400 and conjugate dilution at 1:5000

Table 4.19: Diagnostic cut off OD values for IgM and IgG antibodies as calculated from response in healthy control subjects

No of healthy subjects	Antibody	$\bar{x}$ OD ±SD	Cut off OD values ($\bar{x}$ + 2×SD)
30	Anti-Ag 85 complex antibody		
	• IgM	0.557 ±0.16	0.877
	• IgG	0.867 ±0.394	1.655
	Anti-culture filtrate protein antibody		
	• IgM	0.387 ±0.179	0.745
	• IgG	0.569 ±0.393	1.355

Note: Serum dilution for IgM at 1:400 except for IgG to Ag 85 complex at 1:1600

Table 4.20: Serodiagnosis of tuberculosis patients by detecting IgM and IgG antibodies against Ag 85 complex and culture filtrate protein (CFP) antigens

Antibody	Total cases	Patients positive No.	%	Sensitivity (%)	Specificity (%)
Anti-Ag 85 complex antibody					
• IgM	30	20	(66.67)	75.0	96.7
• IgG		21	(70.0)	76.9	93.75
Anti-CFP* antibody					
• IgM	30	10	(33.3)	60.0	96.7
• IgG		17	(56.7)	69.8	93.75

Note: True positive indicates- culture positive tuberculosis cases
True negative indicates- healthy subjects with no history of tuberculosis and no clinical feature for TB.
*CFP= culture filtrate protein

CHAPTER 5

DISCUSSION

5. Discussion

Approximately one-third of the world's population is infected with *M. tuberculosis* and 8 million new cases of active tuberculosis and 3 million deaths are estimated to occur every year (WHO, 2003). The majority of the TB cases occur in developing countries with limited resources. Currently, tuberculosis control is potentially difficult worldwide due to the emergence of MDR-TB, XDR-TB (WHO, 2006). More recently, the appearance of totally drug resistant tuberculosis (TDR-TB) has made the situation worse (Velayati, 2009).

Despite the all advances made in the diagnosis, treatment and management, tuberculosis still remains as one of the main public health problem particularly in developing countries (Paramasivan and Venkataraman, 2004). Monitoring of drug resistance pattern, early diagnosis and initiating prompt treatment has been the mainstay to interrupt the transmission of tuberculosis. In this context, the present study was designed to determine the drug resistance pattern of mycobacteria, rapid detection of mycobacteria by PCR and to assess the antibody response to specific antigens of mycobacteria in tubercular infection.

In the present study, out of 255 total sputum samples, 180 (70.59%) were culture positive on L-J media. Various authors have reported similar findings ranging from 59.72 to 87.2% positive by culture in L-J media (Lu *et al.*, 2002; Hanna *et al.*, 1999; Alcaide *et al.*, 2000). Study conducted by Gopinath and Singh (2009) reported culture positive rate in L-J medium as 34.4% while Rishi et al (2007) reported the rate as 50.6%. Runa *et al.,* (2011) reported that 55.6-66.7% of the sputum samples of suspected TB cases were positive by culture on L-J, medium which is also lower than that of the present study. In contrast to the findings of the present study, Uddin et al (2009) from Pakistan reported higher rate of isolation of *M. tuberculosis* (85.06%) in sputum samples of suspected TB cases. Out of 45 lymph node aspirate samples, 20 (44.4%) were positive by culture. The failure to isolate mycobacteria in about 30-56% sputum and lymph node aspirates was due to contamination of media or damage to organisms during decontamination process. In our study, 13.7% and 22.2% culture became contaminated out of 255 sputum and 45 lymph node aspirate samples respectively. Contamination rate reported by others ranged between 1.2% to 27.2% (Uddin *et al.*, 2009; Hanna *et al.*, 1999; Alcaide *et al.*, 2000; Somokovi *et al.,* 2000; Chien *et a*l., 2000). However, much lower rate of isolation (15%) of *M. tuberculosis* from lymph node aspirate was reported from Pakistan in L-J medium (Aftab *et al.*, 2008). Therefore, the isolation rate of mycobacteria can be increased if contamination is prevented and sample processing procedure is further improved.

Out of the 200 isolates of mycobacteria, 192 (96.0%) were *M. tuberculosis* and 8 (4.0%) were MOTT. Earlier, a study in Dhaka by Miah *et al.*, (2000) reported 95.3% isolates as *M. tuberculosis* and 4.7% as MOTT which were similar to the findings of the present study. Out of 192 isolated *M. tuberculosis*, 103 (53.65%) were resistant to any anti-tubercular drugs and 89 (46.35%) were sensitive to any anti-tubercular drugs.

Of the 167 *M. tuberculosis* isolated from the newly detected cases, 78 (46.71%) were resistant to any first line anti-tubercular drugs while the rate of resistance among previously treated cases was 100%. Previously, Miah et al (2000) from Bangladesh reported that 29.7% of *M. tuberculosis* was resistant to at least any one of the first line anti-tubercular drugs which is lower than the findings of the present study. In 2007, Rahim *et al.*, reported rate of resistance to any single first line anti-tubercular drug as 31% among patients attending TB clinic in Sunamganj, a district located about 250 km north east of the capital, Dhaka. Therefore, it appears that in last ten years the rate of resistance of *M. tuberculosis* has increased from 29% to 53% in the selected population of urban areas. This high rate of resistance among cases in Dhaka could be due to the fact that complicated cases are actually referred to Dhaka.

In Pakistan, the rate of resistance of *M. tuberculosis* to any first line anti-tubercular drug was 53.0% which is similar to the present study (Iqbal *et al.*, 2003). In India, rate being 30-58% (Pereira *et al.*, 2005; Deodhar *et al.*, 1999). World wide reported resistance to any anti-tubercular drugs ranged between 9.8-39.3% in South East Asia, 7.1%-16.1% in Afria, 9.7%-24.6% in Americas, 9.9-63.3% in Eastern Mediterranean, 8.4-15.5% in Europe and 11.4-32.8% in western pacific region (WHO, 2006).

In our study, of the 167 newly detected *M. tuberculosis*, the resistance to INH, RIF, ETHAM and SM were 22.15%, 9.58%, 13.17% and 22.15% respectively. Studies carried out earlier in Bangladesh reported that 15.8-23.0% was resistant to INH, 2.0-10.9% were resistant to RIF, 2.9-10.0% were resistant to ETHAM and 6.9-18.0% were resistant to SM (Miah *et al.*, 2000; Rahim *et al.*, 2007). The resistance pattern of first line anti-tubercular drugs observed in the present study was almost similar to the resistance pattern reported previously in 2000 and 2007.

In India, Pereira et al (2005) observed that resistance to INH, RIF, ETHAM and SM were 30%, 10%, 10% and 23.3% respectively. This study showed that resistance to INH and SM was higher in comparison to the present study. However, resistance to RIF and ETHAM was almost similar. In India another study conducted by Deodhar et al (1999) showed that

resistance to INH, RIF, ETHAM and SM as 30.41%, 58.55%, 3.67% and 46.95% respectively. In Pakistan, Iqbal et al (2008) reported the resistance pattern of *M. tuberculosis* as 26.0% for INH, 28.0% for RIF, 15.0% for ETHAM and 24.0% for SM. This resistance pattern was higher than that of present study. However, another study by Iqbal et al (2003) showed that resistance to INH, RIF, ETHAM and SM were 23.0%, 26.6%, 11.8%, 19.5% respectively. They observed that the resistant rate became higher in 2008 than that of 2003. In Turkay resistance to INH, RIF, ETHAM and SM from 1992-1999 were 14.4%, 10.6%, 2.4% and 21.1% respectively. This resistance rate was lower than the present study. This might be due to the fact that the study was carried out earlier in 1992-1999.

So far we have discussed the overall drug resistance rate among our isolated *M. tuberculosis*. It is important to understand the drug resistance pattern in newly detected and previously treated TB cases as there is increased rate of resistance among previously treated cases. Of the 167 *M. tuberculosis* isolated from newly detected cases, 31.74% showed resistance to one drug, 11.38% to 2 drugs, 1.80% to 3 and 4 drugs in the present study. On the other hand, of the 25 *M. tuberculosis* isolated from the previously treated cases, 16.0% were resistant to one drug, 52.0% were resistant to 2 drugs, 4.0% were resistant to 3 drugs and 28.0% were resistant to 4 drugs. In this study, resistance to 2 and more drugs was found to increase. This increased resistance to multiple drugs among *M. tuberculosis* isolated from previously treated cases might be due to increased presence of MDR-TB in treatment failure cases. In the present study, of the 25 *M. tuberculosis* isolated from previously treated cases, 52% were resistant to INH, 56.0% were resistant to RIF, 68.0% were resistant to ETHAM and 52.0% were resistant to SM. Therefore, the resistance rate was much higher among the isolates from treated cases compared to untreated cases. Study conducted in Pakistan, observed that resistance of *M. tuberculosis* to INH, RIF, ETHAM and SM were 33.0%, 44.0% 19.0% and 32.0% respectively in treated cases (Iqbal *et al.*, 2003).

In this study 7 (4.2%) out of 167 *M. tuberculosis* isolated from new cases and 9 (36.0%) of the 25 *M. tuberculosis* isolated from treated cases were MDR-TB. MDR-TB was significantly more among treated cases than newly detected cases.

Global prevalence of MDR-TB among new cases ranged from 0.4 to 1.4% and it was 4.7%-48.3% among previously treated cases (WHO, 2006). In South East Asia, the prevalence of MDR-TB was 1.3% in new cases and 20.4% in treated cases (WHO, 2006).

The rate of MDR-TB in the present study was higher among the treated cases. It is has been estimated that globally 3.3% of all TB cases were MDR-TB in 2009 (WHO, 2011) which is closer to the findings of the present study. In accordance with the findings of the present study, earlier Miah *et al.*, in 2000 reported MDR-TB as 4.95% in new cases. But in 2007, Rahim *et al.*, reported MDR-TB as only 2% in new cases in Bangladesh. The low rate reported by Rahim *et al.,* could be due the fact that his site and study population was different from that of ours. Rahim et al (2007) conducted the study at Sunamganj which was a semi-urban area and located far from the capital Dhaka, while our study population was recruited from referral centers in Dhaka.

Khoharo and Shaikh (2011) reported MDR-TB as 5.88% in new cases and 57.5% in previously treated cases in Pakistan which is higher than the present study. This higher MDR-TB detection in the study of Khoharo and Shaikh (2011) might be due to the fact that cases were recruited from chronic and hospitalized patients. In our study, all the patients were recruited from out patient department. Similar higher MDR-TB was reported by several authors. In a study carried out in Bangladesh in a tertiary care hospital of Dhaka city, showed that 13% of the new cases and 87% of the previously treated cases was MDR-TB (Rahman *et al.,* 2009). This higher rate of MDR-TB compared to ours was due to the fact that the study was conducted on patients, admitted on specialized tuberculosis hospital in Bangladesh. Also a study in Karachi (Pakistan) reported 47% MDR-TB (Butt *et al.*, 2004).

It is important to note that all the 8 isolated MOTT in our study was resistant to all first line anti TB drugs. Out of 8 MOTT, 1 (12.5%) was resistant to 2 drugs, 6 (75.0%) was resistant to 3 drugs and 1 (12.5%) was resistant to 4 drugs. and none showed resistance to one drug.

In the present study, out of 30 RIF resistant *M. tuberculosis*, 25 (83.3%) were also concomitantly or cross resistant to other three first line anti-tubercular drugs ($p<0.05$; Table-11). Of the 49 INH resistant *M. tuberculosis*, 32 (65.31%) were concomitantly or cross resistant to other three first line anti tubercular drugs ($p>0.05$) while for ETHAM and SM the rate was 76.3% and 57.4% respectively. It is observed that 83.3% *M tuberculosis* which were resistant to RIF, were also cross resistant to INH, ETHAM or SM. Resistance to RIF in *M tuberculosis* occurs in a frequency of 1 in 10 and mono resistance to RIF is rare, where as mono resistance to INH is common (Somoskovi *et al.*, 2001). It has been proposed that resistance to RIF can be used as a surrogate marker for MDR-TB as nearly 90% of the RIF resistant strains are also INH resistant (Somoskovi *et al.*, 2001, Traore *et*

al., 2000). It has been reported that some strains become RIF dependent and grow better in the presence of RIF (Zhang *et al.,* 2009). These are not true RIF resistant strain as they grow very poorly in the absence of RIF. The mechanism of development of these strains is not clear, however, they may develop upon repeated treatment with RIF in re-treatment patients (Merza and Masjedi, 2010). In the present study, out of 162 *M. tuberculosis* isolates which were sensitive to RIF, only 43.21% was concomitantly resistant to other 3 drugs (Table 12). This finding suggests that RIF sensitive strains are less likely to be concomitantly resistant to other 3 drugs. Similarly, the rate of resistance to other anti-tubercular drugs concomitantly among INH, ETHAM and SM sensitive *M tuberculosis* isolates were 36.6%, 40.90% and 34.78% respectively. Therefore, it appears that the sensitive strains are less likely to be resistant to other ant-tubercular drugs and hence is unable to predict cross resistance accurately.

In the present study, attempt was made to evaluate the efficacy of PCR to detect mycobacteria in sputum and lymph node aspirate samples. Multiplex PCR using four sets of primers were used to detect *M. tuberculosis* as well as *Mycobacterium* other than tuberculosis (MOTT). The commercial kit which was used in the present study contained thermostable reagents for PCR. The *taq* polymerase and oligonucleotides used were made thermostable so that the kit can be used in places where there is no facility for low temperature storage. Therefore, the kit had the advantage of detecting the genus *Mycobacterium* as well as *M. tuberculosis* species. In the present study, multiplex PCR was performed with 45 culture positive sputum samples. Out 45 sputum samples, 42 (93.3%) were positive and 3 (6.7%) were negative by PCR. The rate of positivity was 100% in case of lymph node aspirates. Negativity of PCR in some of the samples might be due to the primers used in the kit were unable to detect the target sequence of the mycobactaeria present or it might be due to the presence of inhibitors in the sputum samples. Therefore, it was not possible to calculate the specificity of PCR as two culture negative cases became PCR positive. We could not consider those 2 culture negative cases as 'false positive' because both were clinically diagnosed as tuberculosis cases and responded to anti-tubercular treatment. It may be mentioned that out of 42 PCR positive cases, 2 were identified as MOTT by PCR. Theses two isolates were confirmed as MOTT by colony morphology and biochemical reactions. Therefore, PCR is a rapid method for identifying species other than *M. tuberculosis*.

Several investigators, with different sets of primers, have investigated the role of PCR to diagnose tuberculosis with variable results. The sensitivity of PCR method ranged from 59-98% and specificity ranged from 70-100% have been reported from different countries of the world. (Pervez *et al.*,2003; Hajia *et al.,* 2009; Gopinath *et al.,* 2009; Abe *et al.*, 1993; Cohen *et al.,* 1998; Querol *et al.,* 1995; Bennedsen *et al.*, 1996; Jafarian *et al.*, 2008; Beige *et al.,* 1995; Ginesu *et al.*,(1998; Dar *et al.,* 1998; Mustafa *et al.*, 1999; Rodriguez *et al.*,1997).

Immune response to *M. tuberculosis* in human is mainly cell mediated though antibody responses to different bacterial components are also observed. Humoral immune response may be used as a sero-marker to diagnose active cases of tuberculosis as well as to understand their role in immunity or susceptibility to infection. In view of this, attempt has been made in this study to determine the humoral immune response to defined mycobacterial antigens. In the present study, 30 confirmed cases of tuberculosis and 30 healthy subjects were selected to detect the IgM and IgG antibody titers against four mycobacterial antigens namely, Ag 85 complex, culture filtrate protein (CFP), cell wall fraction (CWF)and lipoarabinomannan (LAM).

IgM and IgG anti-CFP response was found significantly higher ($p< 0.001$) among patients with active tuberculosis compared to that of healthy control subjects indicating that host induced antibody response to CFP (Table-15). Similar significantly higher IgM and IgG response was observed for Ag 85 complex in active tuberculosis cases (Table-16). The presence of low level of IgM and IgG antibodies to the above antigens in the sera of healthy subjects could be due to exposure to the organisms early in the life. The wide spread exposure to *M. tuberculosis* is indicated by high percentage of tuberculin positivity rate in our population.

But, contrary to the antibody response to CFP and Ag 85 complex, it was found that the IgM antibody response to LAM and CWF was higher in healthy subjects compared to that of cases with active tuberculosis. It is hypothesized that the individuals become susceptible to develop active tuberculosis if they are unable to mount initial antibody response against these two antigens. In other words, if anybody exposed to *Mycobacterium* may not develop active diseases if the individual is capable of mounting antibody response to either LAM or CWF. Therefore, studies may be done to explore the possibilities further.

In our study, IgM antibody to CWF and LAM was significantly higher in the serum of healthy subjects (Table- 17 and 18) while the IgG antibody was almost similar. This higher

background antibody titre in the healthy subjects might be due to endemicity of tuberculosis in our country.

Attempt has been made to use antibody responses to theses antigens as serological markers for diagnosis of active tuberculous infection. For sero-diagnosis, diagnostic cut off value was determined by adding 2×SD to the mean OD of antibody response to respective antigens. It was observed that among the total number of 30 patients, anti-Ag 85 complexes IgM was positive in 66.67% of patients and its sensitivity and specificity was 75.0% and 96.7% respectively (Table- 20). The anti-Ag 85 complex IgG was positive in 70.0% of patients and sensitivity and specificity was 76.9% and 93.75% respectively. Anti-CFP IgM antibody was positive in 33.3% patients and sensitivity and specificity was 60.0% and 96.7% respectively. Anti-CFP IgG was positive in 56.7% of patients and sensitivity and specificity was 69.8% and 93.75% respectively. It appeared that IgM and IgG antibody response to Ag 85 complex was better compared to that of CFP. Therefore, determination of IgM and IgG against Ag 85 complex could be used as a serological marker for diagnosis of active tuberculosis in cases where other tests do not give conclusive information. It is particularly applicable in children where they are unable to provide sputum samples for either staining or culture. Many authors investigated antibody response against Ag 85 complex, CFP and LAM and found sensitivity and specificity similar to our findings (Kumar *et.al.*, (2010); Kashyap *et al.*, (2007); Brown *et al.,* (2003); Chan *et al.,* (2000); Sada *et al.,*(1992); Boechme *et al.*,(2005); Xueqiongwu *et al.*,(2010). Further studies, are therefore, required to refine the serological tests for accurate diagnosis of active tuberculosis.

The present study, therefore, revealed that high level of drug resistance exists to individual anti-tubercular drugs and MDR-TB was an emerging problem particularly in treated cases. Rifampicin resistance could be used as a marker for drug resistance to other drugs and could obviate the necessity of doing susceptibility test with other drugs in a resource constraint situation. Multiplex PCR in a thermostable format may be applicable for rapid diagnosis of tuberculosis in laboratories at district level. Serological tests utilizing specific antigen may be used as an adjunct test for diagnosis of tuberculosis where other diagnostic tools are lacking or ineffective. Lack of response to some antigens indicates underlying pathophysiology of tubercular infection.

CHAPTER 6

BIBLIOGRAPHY

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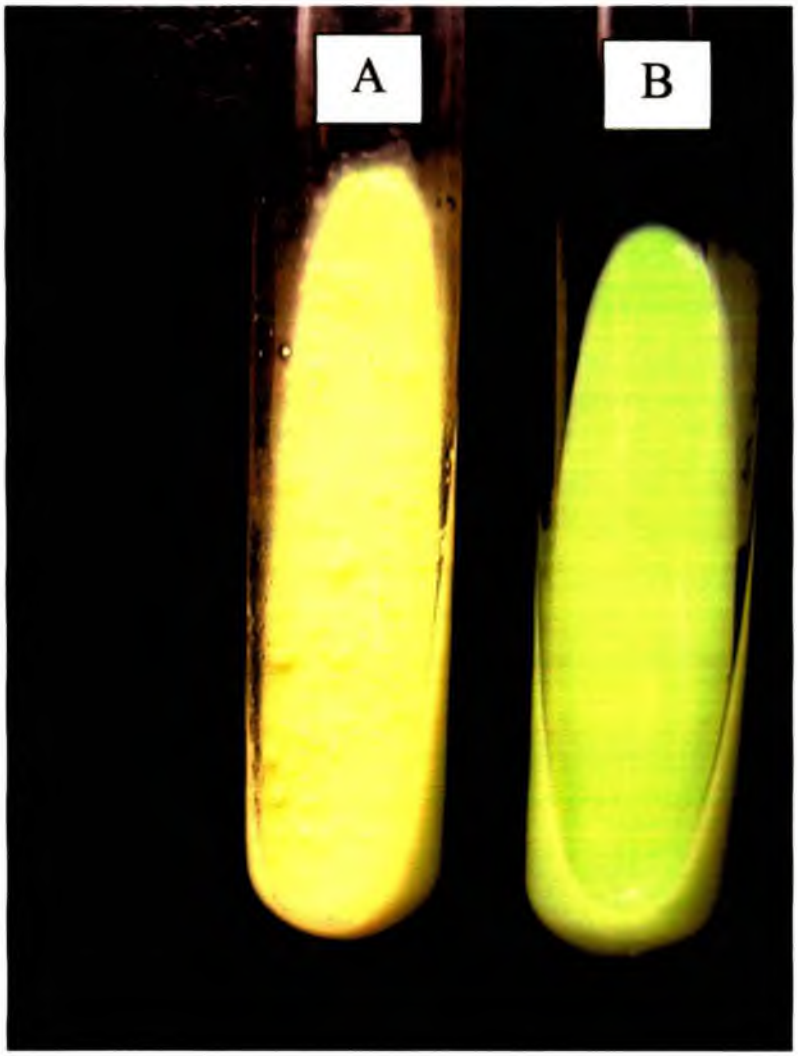
CHAPTER 7

APPENDICES

Appendix 1



Culture of *M tuberculosis* in L-J medium
Tube A contains growth of *M. tuberculosis*, Tube B contains no growth

Appendix –2

$NaNO_3$ Buffer substrate solution

Phosphate buffer solution (pH 7.2)	100 ml
Distilled water	200 ml
$NaNo_3$	25.5 gm

2 ml of $NaNo_3$ substrate solution is dispensed in each screw capped tube (13×100 mm) and autoclaved.

Appendix 3

Phosphate Buffer

1. Disodium Phosphate (Na_2HPO_4)	2.37 gm
2. Monopotassium Phosphate (KH_2PO_4)	2.27 gm

Desolved in 500 ml of distilled water (pH 6.8) and autoclaved and kept at 4^{O}-8^{O} C.

Appendix 4

McFARLAND TURBIDITY STANDARD

1. 1% aqueous Barium chloride (1 gm barium chloride in 100 ml of distilled water) and 1% aqueous sulfuric acid solution (1 ml of conc. Sulfuric acid in 99 ml distilled water) was prepared.
2. The prepared solutions were kept in a clean dry tube to be used for density determinations.
3. The tubes were sealed and labeled.

McFarland. Standard	Barium Chloride 1% (ml)	Sulfuric acid 1% (ml)
1	0.1	9.9
2	0.2	9.8
3	0.3	9.7
4	0.4	9.6
5	0.5	9.5

Appendix 5

Carbonate bicarbonate buffer

Anhydrous Sodium Carbonate Na_2Co_3	1.15 gm
Sodium bi Carbonate	4.13 gm

These were dissolved in 1000 ml of Distilled water

pH was adjusted at 9.6

Appendix 6

Phosphate buffer Saline (PBS)

Sodium Chloride (NaCl)	8.0 gm
Potasium Chloride (KCl)	0.2 gm
Disodium Phosphate (Na_2HPO_4)	1.44 gm
Potasium Di Hydrogen Phosphate (KH_2PO_4)	0.24 gm

These were dissolved in 800 ml of distilled water and were made upto 1000 ml and pH was adjusted at 7.2, sterilized by autoclaved.

Appendix 6

Blocking Solution:

Prepared by PBS-Tween (0.05%) + 2% BSA. pH was adjusted to 7.2-7.4