Chapter 1
INTRODUCTION

1.1 INTRODUCTION

Tuberculosis (TB), a disease caused by the bacterium *Mycobacterium tuberculosis*, has affected mankind for over 5000 years and the disease continues to be a major cause of mortality and morbidity. With its presence before recorded historical period, tuberculosis has left its mark on music, art, human creativity and literature; and has influenced the advance of healthcare and biomedical sciences (Palomino *et al.*, 2007). In comparison with other microbial pathogen *Mycobacterium tuberculosis*, may have killed more persons (Daniel, 2006). Treating tuberculosis disease is not an easy task and the duration of treatment takes months. Multiple antibiotics are needed to prevent drug resistance, and side-effects are common and some drugs are expensive. The genus *Mycobacterium* is presumed to have originated more than 150 million years ago (Daniel, 2006). Three million years ago, an early progenitor of *M. tuberculosis* was probably co-evolved with early hominids in East Africa and contemporaneous (Palomino *et al.*, 2007). About 15,000 - 35,000 years ago the modern members of *M. tuberculosis* complex seem to have originated from a common progenitor (Gutierrez, 2005). As early as 5000, 3300 and 2300 years ago TB was documented in Egypt, India, and China, respectively (Daniel, 2006). World Health Organization (WHO) declared TB a global health emergency in the year 1993 and in 1998, together with the International Union Against Tuberculosis and Lung Disease (IUATLD) and other international partners, formed the Stop TB Initiative, a new global strategy to control TB that has recently evolved into a global partnership, the Stop TB Partnership (2006).

This infectious disease is very common and often deadly caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis* in humans. Lungs are prime site of attack but it can also affect other body parts also (Kethireddy *et al.*, 2010). When TB patients cough, sneeze, or spit it spreads through air. Most infections in humans result in an asymptomatic, latent infection (Kumar *et al.*, 2007) and about one in ten latent infections eventually progresses to active disease, which kills more than 50% of its victims if not treated (Mishra *et al.*, 2012). In general, small
percentage of people infected with *Mycobacterium tuberculosis* will develop TB disease; however, the probability of developing tuberculosis is higher among people infected with the human immunodeficiency virus (WHO, 2012). TB is also more common among men than women, and affects mostly adults in the economically productive age groups; around two-thirds of cases are estimated to occur among people aged 15–59 years (WHO, 2011). There are two further mycobacteria that can cause tuberculosis disease in human *Mycobacterium africanum* and *Mycobacterium bovis* (Niemann et al., 2000). The three different mycobacterium; *M. tuberculosis*, *M. africanum* and *M. bovis* (*M. microti* is non-pathogenic in humans and not discussed further) are referred to as *M. tuberculosis* complex (MTBC) (Niemann et al., 2000). Other than *M. tuberculosis* complex, rest of the mycobacteria are referred to as non-tuberculous mycobacteria (NTM) and may cause pulmonary disease resembling tuberculosis.

Tuberculosis is a disease of global importance. One-third of the world population is estimated to have been infected with *Mycobacterium tuberculosis* and eight million new cases of tuberculosis arise each year (WHO, 2011). The tuberculosis crisis is likely to escalate since the human immunodeficiency (HIV) epidemic has triggered an even greater increase in the number of tuberculosis cases (WHO, 2011).

Tuberculosis kills over two million people worldwide each year, more than any other single infectious disease, including AIDS and malaria (WHO, 2012). Transmission of tuberculosis is virtually entirely by droplet nuclei created through coughing by untreated patients suffering from pulmonary tuberculosis (the most common form) in a confined environment. Infected droplets remain airborne for a considerable time, and may be inhaled by susceptible persons. Pulmonary tuberculosis usually occurs in the apex of the lungs. These develop cavities which contain large populations of tubercle bacilli that can be detected in a sputum specimen. The diagnosis can only be made reliably on demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory (WHO, 2011).

Mycobacteria are aerobic, slightly curved or straight bacilli (*i.e.*, rod shaped), 0.2–0.6 x 1.0–10\(\mu\)m in size (Soolingen, 1998). Their cell wall has high lipid content that includes waxes comprising mycolic acids with long, branched chains. The lipid
content of the cell wall excludes the usual aniline dyes used to stain bacteria (Brennan et al., 1994). Mycobacteria are not therefore readily stained using the Gram stain method. Special staining methods are used to promote the uptake of dye and, once stained, mycobacteria are not easily decolourised; that is, they retain the stain even when washed with acid-alcohol solutions. Their resistance to decolourisation is termed acid fastness, hence the term acid-fast bacilli (AFB) (Laboratory Services In Tuberculosis Control Microscopy, 1998). Growth rates for mycobacteria are slow to very slow (e.g., *M. tuberculosis* takes 16–18 hours to undergo one cycle of replication).

Mycobacteria can be detected in clinical samples after acid-fast staining and microscopic examination. Tuberculosis is acquired through airborne transmission of droplet nuclei and risk of infection increases with their concentration and with time of exposure (Bass et al., 1990). Once an individual has been infected, he remains infected for a long time, possibly progressing to active disease, sometimes years after the initial infection, in about 10% of the infected cases when no immunosuppressive disorders are present. Conditions such as emotional and physical stress or immune-suppression upon HIV infection increase the chance to develop active TB (WHO, 2011). Upon infection with *M. tuberculosis*, most individuals develop some degree of delayed-type hypersensitivity to tuberculin, providing as such a measure for infection status. However, this measure varies with age and ethnic and geographic origin of the population under study, parameters that also seems to determine the efficiency of protection of BCG vaccines against TB (Fine, 1989). Age also influences the risk for developing progressive disease after primary infection, being highest among young children, while TB in adults generally appears many years after infection. Development of progressive disease can be due toreactivating a latent form of the disease or to reinfection; the attribution of each of the two mechanisms depends on the risk of infection within the community and the immune status of the individual (Sutherland, 1976).

The symptoms of active TB of the lung are coughing, sometimes with sputum or blood, chest pains, weakness, weight loss, fever and night sweats. Tuberculosis is treatable with a course of antibiotics. It is a disease of poverty affecting mostly young adults in their most productive years.
The vast majority of TB deaths are in the developing world. Left untreated, each person with active TB disease will infect on average between 10 and 15 people every year and this continues the TB transmission (WHO, 2011).

Global efforts to control the TB pandemic have been undermined by the emergence and spread of strains that are resistant to the commonly used first line anti-TB drugs isoniazid, rifampicin, ethambutol, and pyrazinamide. Strains resistant to at least isoniazid (INH) and rifampicin (RIH), the two most efficacious TB drugs are termed multi drug resistant (MDR) (CDC, 2006). MDR-TB treatment is rather complicated as it requires second line drugs some of which are only injectable, are less efficacious, more toxic and more expensive than the first line agents (WHO, 2009). Treatment lasts for 18-24 months but only around 50%-60% of MDR-TB patients will be cured compared with 95%-97% cure rate for patients with drug-susceptible strains treated with first line agents (CDC, 2003). The recent emergency of extensively drug resistant tuberculosis (XDR-TB) defined as MDR-TB strains with resistance to a fluoroquinolone and to at least one injectable second line drug (kanamycin, amikacin, or capreomycin) has further complicated the problem of MDR-TB (WHO, 2010). The emergence of drug resistance in TB patients is mostly a result of deficient or deteriorating TB control programs. Factors related to the development of drug resistance include the following: inadequate or inefficient administration of effective treatment; poor case holding; use of substandard drugs; inadequate or irregular drug supply; ignorance of health care workers in the treatment and control of TB; interruption of chemotherapy due to side effects; non-adherence of patients to the prescribed regimens; availability of anti-TB drugs without prescription; illiteracy; low socioeconomic status of patients; massive bacillary load; laboratory delays in identification and susceptibility-testing of *M. tuberculosis* isolates; and the lack of the use of uniform laboratory methodology and quality control measures (CDC, 2006).

The global expansion of Directly Observed Treatment, Short-course (DOTS), the Stop TB Strategy, has been an enormously successful public health intervention (WHO, 2010). Millions of TB patients have been treated and millions of lives have been saved. However, TB remains a serious public health threat. More than 9 million new cases are reported every year, and the incidence rate is falling at less than 1% per year (WHO, 2010). Although many countries have met the Stop TB targets of 70%
case detection and 85% cure rate by 2005, TB incidence is not falling as expected or is falling too slowly in these countries. India is a prime example and has successfully scaled-up the DOTS strategy to cover 100% of the population, and has already achieved the targets for case detection and cure. Yet, India reported over 2 million TB cases in 2009, with 280,000 deaths (WHO, 2010). There may be many explanations for this inconsistency. TB is a disease of poverty with several social determinants. Merely diagnosing and treating patients with TB disease is insufficient, although clearly important (Lonnroth et al., 2009 and Oxlade et al., 2009). Another important reason is that TB patients are not diagnosed and cured quickly enough, and the 70/85 targets, while helpful from a programmatic perspective, are not ambitious enough (Christopher et al., 2010). Existing diagnostic approaches do not seem to quite address this challenge, and have largely failed to interrupt TB transmission in populations with a high prevalence of HIV and drug-resistant TB.

In 2010, there were 8.8 million (range, 8.5–9.2 million) incident cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB (WHO, 2011). There were 3.2 million (range, 3.0–3.5 million) incident cases of TB and 0.32 million (range, 0.20–44 million) deaths from TB among women in 2010 as per Global tuberculosis report by WHO (2012). The same report shows 5.7 million notifications of new and recurrent cases of TB, equivalent to 65% (range 63%–68%) of the estimated number of incident cases in 2010. India and China accounted for 40% of the world’s notified cases of TB in 2010, Africa for a further 24% and the 22 high-TB burden countries (HBCs) for 82%. At global level, the treatment success rate among new cases of smear-positive pulmonary TB was 87% in 2009 (WHO, 2012).

At present TB is a worldwide health threat, possibly due to the limitations of current anti-tuberculous drugs. As resistant strains of Mycobacterium tuberculosis have slowly emerged, treatment failure occurs too often, particularly in countries lacking the necessary healthcare organisation to provide the long and costly treatment adapted to individual patients (WHO, 2011). Further Multi drug-resistant TB (MDR-TB) has been reported in almost all parts of the world, primarily as a consequence of poor treatment services, which have not only increased the costs towards treatment, but also increased the risk of transmission of these resistant strains of the bacilli as per WHO global tuberculosis report (2011). Despite substantial investments and progress
made in expansion of the directly observed therapy, short course (DOTS) strategy and improved treatment completion rates, inadequate case detection remains a major obstacle to global control of tuberculosis. Efforts during the past decade to diagnose consistently and treat the most infectious cases have slowed the rate of disease incidence, but have not yielded substantial progress towards elimination (Robert et al., 2010).

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 TB. Even more alarming, a person is newly infected with M. tuberculosis every second of every day (WHO, 2011). TB hinders socioeconomic development: 75% of people with TB are within the economically productive age group of 15-54 years. Ninety-five per cent of all cases and 99% of deaths occur in developing countries, with the greatest burden in sub-Saharan Africa and South East Asia. Household costs of TB are substantial (Christopher et al., 2010 and World Health Organization, 2009). The global resurgence of tuberculosis and the increase in multidrug resistant strains has led to a concomitant increase in demand of susceptibility testing of isolates to first (streptomycin, isoniazid, rifampicin, ethambutol, pyrazinamide) and second line anti-tubercular (ATT) drugs (ethionamide, amikacin, kanamycin, capreomycin, ciprofloxacin, ofloxacin, levofloxacin, cycloserine, rifabutin etc.) (WHO, 2006). Recent surveys also reveal that drug-resistant tuberculosis is still ubiquitous and alarmingly high in several countries.

In most countries, more cases of TB are reported among men than women. This difference is partly because women have less access to diagnostic facilities in some settings, but the broader pattern also reflects real epidemiological differences between men and women, both in exposures to infection and in susceptibility to disease. In regions where the transmission of M. tuberculosis has been stable or increasing for many years, the incidence rate is highest among young adults, and most cases are caused by recent infection or reinfection. As transmission falls, the caseload shifts to the older age groups, and a higher proportion of cases come from the reactivation of latent infection (Borgdorff et al., 2002).

HIV and TB form a lethal combination, each speeding the other’s progress. HIV infection is a potent risk factor for TB. Not only does HIV increase the risk of
reactivating latent *M. tuberculosis* infection, it also increases the risk of rapid TB progression soon after *M. tuberculosis* infection or reinfection (WHO, 2013). The TB burden in countries with a generalized HIV/AIDS epidemic has therefore increased rapidly over the past decade, especially in the severely affected countries of eastern and southern Africa. TB is one of the most common causes of morbidity and the most common cause of death in HIV-positive adults living in less-developed countries, yet it is a preventable and treatable disease (Corbett et al., 2003; Aaron et al., 2004 and WHO, 2009).

It is possible that, in addition to increasing individual susceptibility to TB following *M. tuberculosis* infection, a high burden of HIV-associated TB cases also expands *M. tuberculosis* transmission rates at the community level, threatening the health and survival of HIV-negative individuals as well. In several countries, HIV has been associated with epidemic outbreaks of TB (Corbett 2003; Aaron 2004 and WHO, 2009). Many of the reported outbreaks involved multidrug-resistant (MDR) strains, which respond poorly to standard therapy of the growing burden of TB (Corbett et al., 2003; Aaron et al., 2004 and WHO, 2009).

TB is the only disease ever declared a global emergency by the WHO (Palomino, 2012). As repeatedly stated, one third of the world’s population is latently infected with *Mycobacterium tuberculosis* and 10% of these people will develop active disease at some point in their life (WHO, 2009). Almost 8.8 million new cases of TB were reported in 2005, and 1.6 million deaths were attributed to the disease. Asia and Sub-Saharan Africa accounted for 7.4 million new cases of TB worldwide (WHO, 2009). Yet, it was not long ago that we envisaged and proudly announced the elimination of TB by the end of the last millennium. Indeed, in the late ’70s and early ’80s, it was thought that TB could be eradicated from most developed and industrialized countries (WHO, 2009). TB was already regarded as a disease from the past and started to be neglected by medical doctors, scientists and agencies in charge of its control. However, this never became a reality, mainly due to the appearance of antibiotic resistance, and therefore, TB continues to be the big killer it was in the pre-antibiotic era. This unexpected re-emergence of TB in the ’90s served not only to strengthen control measures but also to fuel research on TB (WHO, 2009).
India has become the world hub for tuberculosis. According to the latest World Health Organisation (WHO, 2011) report, India leads the world with around 25 lakh TB cases, ahead of China, which reports only nine lakh patients. The report titled ‘Global Tuberculosis Control, 2011’ states that WHO registered 8.8 million cases of TB last year of which 2.5 million patients were from India. The five countries with the highest incidence in 2010 were India (2.5 million), China (1.2 million), South Africa (0.59 million), Indonesia (0.54 million) and Pakistan (0.48 million). India alone accounted for an estimated 26 per cent of all TB cases worldwide, and China and India combined accounted for 38 percent. TB remains a major killer in India, killing two persons every three minutes, nearly 1,000 a day (WHO, 2011).

Early detection of drug resistance constitutes one of the priorities of TB control programs. It allows initiation of the appropriate treatment in patients and also surveillance of drug resistance. Detection of drug resistance has been performed in the past by conventional methods based on detection of growth of $M. tuberculosi$s in the presence of the antibiotics (Garcia, 2003 and Palomino, 2005). 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. Genotypic methods have the advantage of a shorter turnaround time, no need for growth of the 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. Genotypic methods for drug resistance in TB look for the genetic determinants of resistance rather than the resistance phenotype, and involve two basic steps: nucleic acid amplification such as polymerase chain reaction (PCR), to amplify the sections of the $M. tuberculosi$s genome known to be altered in resistant strains; and a second step of assessing the amplified products for specific mutations correlating with drug resistance (Garcia, 2003 and Palomino, 2005).

There is no relevant literature available about the drug susceptible or resistant pattern of the Northeast region of India. As huge death caused by TB in its severe form of MDR and XDR, the present study is being undertaken with the aim to extend the knowledge of drug susceptibility pattern of $Mycobacterium tuberculosi$s. The study includes analysis of $Mycobacterium tuberculosi$s for their drug susceptibility using different methodology and also emphasised on an attempt to
highlight the current situation of TB, its drug resistant and diagnostic trends in Northeastern region of India. The research on this field in the region mention is poorly taken into interest by any scientific resources so I hope my study would in future attract many more researchers to take up such related or similar topics for their PhD work and give certain societal contribution from it. Phylogenetic analysis of sequenced 16sRNA samples using Bioinformatics tools will also help trace the relationship between the strains of Mycobacterial species from clinical isolates.
1.2 OBJECTIVES OF THE STUDY

The primary aim of this study is to analyse the drug susceptibility of *Mycobacterium tuberculosis* in the Northeastern region of India.

1. Analysis of samples to identify and detect the presence of Mycobacterium in the clinical samples and comparison of the methods of drug susceptibility tests.

2. Evaluate the performance of the DNA Probe Strip Test for their Drug Susceptibility Test compared with culture methods while analyzing the frequency of gene mutations associated with resistance to isoniazid (INH), rifampicin (RIH) and ethambutol (EMB) among *M. tuberculosis* isolates.

3. Strain typing the clinical samples using PCR-RFLP and phylogenetic analysis of the Mycobacterial strains using 16sRNA region.