CHAPTER 1

INTRODUCTION

1.1 THE GENUS MYCOBACTERIUM

The genus Mycobacterium is responsible for more suffering worldwide than all other bacterial genera put together. The genus is divided into two major groups: the slow growers and the rapid growers. The best known property of the genus is the ability of the bacterial cell to resist decolorization by weak acids after staining, hence the term “acid-fast bacilli” (AFB). Currently, there are over 54 species of well-defined mycobacteria (Wayne and Kubica., 1986). As a result of their clinical importance, mycobacteria have been the focus of a lot of attention but even so they have always been difficult to distinguish from corynebacteria, nocardia and related bacteria, the organisms that have been traditionally classified based on a few morphological and staining properties (Bousfield and Goodfellow, 1976).

1.2 CHARACTERISTICS

AFB are approximately 1 to 10 μm long and 0.2 to 0.6 μm wide. They typically appear as slender, rod-shaped bacilli, but they may appear curved or bent. Individual bacteria may display heavily stained areas referred to as beads and/or alternating light and dark areas of stain producing a banded appearance. Some mycobacteria other than Mycobacterium tuberculosis may appear pleomorphic, ranging in appearance from long slender rods to coccoid forms, with more uniform distribution of staining procedures (Kubica and Wayne, 1984). Although mycobacteria may vary in form from coccobacilli to long slender rod-shaped cells they share a distinctive staining property that depends upon their lipid-rich cell walls. They are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, however, the mycobacteria resist decolorization with acidified organic solvents (Goren et al., 1978).
The members of the genus mycobacteria are generally slow growers (generation time of 18-24 hours) as compared to the members of other bacterial genera and this can be attributed to the lipid material in the cell wall that hinders permeation of nutrients into the cell. The thick cell wall of mycobacteria is extremely unique. It is composed of 4 layers. The innermost layer is the peptidoglycan layer while the next 3 surface layers are composed of lipids such as mycolic acid, glycolipids, cord factor and wax D (Asselineau and Lederer, 1950). The most rapidly growing species grow on simple media after incubation for 2 - 3 days at temperatures between 20 - 40°C. However, many of the pathogens require incubation for 2 - 6 weeks or more on complex media at restricted temperatures. Among the pathogenic species, Mycobacterium leprae has so far not been cultivated successfully in vitro.

1.3 CLASSIFICATION OF THE GENUS MYCOBACTERIUM

The genus mycobacterium can be broadly classified into tuberculous and non-tuberculous mycobacteria.

1.3.1 Tuberculous mycobacteria

The tuberculosis complex consists of M. tuberculosis, M. microti, M. africanum and M. bovis.

1.3.1.1 M. tuberculosis

This species is clearly the most important member of the genus in terms of its clinical importance. Its most distinctive features includes the accumulation of niacin, very strong nitrate reducing ability, its susceptibility to a broad range of inhibitory substances and the formation of characteristic colonies on solid medium (Kubica and Wayne, 1984).

1.3.1.2 M. microti

This organism is also known as "vole bacillus", a rarely encountered organism, which is of little practical importance. Its enzymatic behaviour is almost
similar to that of \textit{M. tuberculosis}, although it differs in its susceptibility to thiophene-2-carboxilic acid hydrazide (Kubica and Wayne, 1984).

\subsection*{1.3.1.3 \textit{M. africanum}}

This species closely resembles \textit{M. bovis}, but the difference lies in the presence of amidase reactivity (Kubica and Wayne, 1984).

\subsection*{1.3.1.4 \textit{M. bovis}}

Smith (1898) was the first one to recognise the differences in the origin between the tubercle bacilli of human and bovine. However, the genetic analysis of \textit{M. bovis} shows close similarity with \textit{M. tuberculosis}.

\subsection*{1.3.2 \textit{Nontuberculous Mycobacteria (NTM)}}

The NTM are a heterogeneous group of pigmented and non-pigmented acid-fast bacilli that were not recognised as potential human pathogens until 35-40 years ago. In general, they form an important part of the bacterial flora in our environment. Most of the bacteria are harmless but some can cause severe human disease or may be fatal (Banks, 1994).

These NTM differ in several aspects from the classical tubercle bacilli. They present varying degrees of acid-fastness and have a wide temperature range for growth and can be cultured comfortably at temperatures not found in the human body. The time taken for growth is generally less than 7 days. Many of these NTM are pigmented yellow or orange. The NTM are generally refractive to the first line antituberculous drugs like rifampicin, isonicotinic acid hydrazide (INH), streptomycin, and ethambutol.

\subsection*{1.3.3 Runyon Classification of Nontuberculous Mycobacteria}

According to Runyon, the NTM are classified into four groups on the basis of rate of growth, production of yellow pigment, or whether pigment production is in the dark or only after exposure to light (Runyon, 1958., 1959.).
Runyon Group I- Photochromogenic slow growers
Runyon Group II- Scotochromogenic slow growers
Runyon Group III- Non- photochromogenic slow growers
Runyon Group IV- Rapid growers

1.3.3.1 Runyon Group I - Photochromogenic slow growers

These are actively growing cultures, which develop yellow pigment on exposure to light, but fail to produce pigment in the dark. Cultures require 2 - 6 weeks' incubation before visible growth can be seen.

1.3.3.1.1 *M. kansasii*

This organism is usually associated with pulmonary disease in man but in contrast to *M. tuberculosis* appears to be minimally contagious. This characteristic is shared by other members of Group III and Group IV pathogenic mycobacteria. Two strains of *M. kansasii* have been described; one is associated with clinically significant human disease while the other does not appear to be pathogenic for man. The two strains may be differentiated on the basis of their catalase activity. Low or non catalase producers are non-pathogenic whereas high catalase producers are potential pathogen (Buhler and Pollak, 1953).

1.3.3.1.2 *M. marinum*

*M. marinum* is associated with disease limited to superficial portions of the body, particularly the skin. It is commonly found in swimming pools and fish tanks. The infection is often referred to as "Swimming pool granuloma". *M. marinum* may be distinguished from *M. kansasii* by its source, more rapid growth at 25° C to 35° C and its inability to reduce nitrate to nitrite (Wayne et al., 1978)
1.3.3.2 Group II - Scotochromogenic slow growers

In this group pigment can be produced both in the light and dark. Cultures require 2 - 6 weeks' incubation at the temperature ranging from 25 to 42°C before visible growth can be seen. In this group pigment can be produced both in the light and dark. Cultures require 2 - 6 weeks' incubation at the temperature ranging from 25 to 42°C before visible growth can be seen. In this group pigment can be produced both in the light and dark. Cultures require 2 - 6 weeks' incubation at the temperature ranging from 25 to 42°C before visible growth can be seen. In this group pigment can be produced both in the light and dark. Cultures require 2 - 6 weeks' incubation at the temperature ranging from 25 to 42°C before visible growth can be seen.

1.3.3.2.1 M. scrofulaceum

This organism is associated with cervical adenitis particularly in children. *M. scrofulaceum* has also been associated in rare instances with pulmonary disease where there has been severe underlying disease like emphysema or silicosis (Prissick and Masson, 1957).

1.3.3.2.2 M. gordonae

This is considered as a saprophyte and often found in tap water. *M. gordonae* is often referred to as the “tap water” bacillus and is one of the most commonly isolated nontuberculous mycobacteria (Wayne, 1970).

1.3.3.2.3 M. szulgai

This was first reported by Marks *et al.*, (1972). These are very rare, usually pathogenic and are most often isolated from sputum.

1.3.3.3 Group III - Non-photochromogenic slow growers

These organisms are a heterogeneous group containing both pathogenic and non-pathogenic species. Most are non-pigmented and are extremely slow growers.
1.3.3.3.1 *M. avium- intracellulare.*

*M. avium* complex or MAC, as it is commonly referred to, includes *M. avium* and *M. intracellulare*. They are biochemically indistinguishable. However, they can be separated by molecular methods (DNA probes) or by high performance liquid chromatography (HPLC). At one time it was considered that except for epidemiological purposes, it was not clinically significant to separate the strains. But, with the advent of Acquired Immunodeficiency Syndrome (AIDS) and its association with MAC infection, the differentiation of *M. avium* and *M. intracellulare* assumes importance because of the difference in response of these two different organisms to the available antimycobacterial drugs (Rynerson et al., 1971; Guy and Chapman, 1961).

This species of MAC sometimes causes a self-limiting cervical adenitis and is also responsible for progressive and, if untreated, usually fatal disease especially in immunocompromised patients. This organism is the most common mycobacterium strain, apart from *M. tuberculosis* isolated from patients with pulmonary disease in USA. Often the histopathologic features of the lung of a patient infected with *M. avium intracellulare* cannot be distinguished from the lesions of ordinary tuberculosis. Currently in AIDS patients, over 80% of the mycobacterial isolates are *M. avium* and *M. intracellulare*, whereas in non-AIDS patients approximately 60% are MAC. MAC is difficult to treat, as it is extremely refractory to the currently available antimycobacterial drugs (Kuze et al., 1981).

1.3.3.3.2 *M. xenopi*

Schwabacher first reported this species in 1959. Approximately 50% of the isolates have been associated with pulmonary infections in humans.

1.3.3.3.3 *M. terrae*

Up to 10% of *M. terrae* strains have been reported to be associated with human disease (Tsukamura, 1967).
1.3.4 Group IV - Rapid growers

With the exception of the *M. fortuitum* complexes, the members of mycobacterium Group IV are saprophytes and considered to be non-pathogenic. They are characterised by their ability to grow rapidly (2 - 7 days) at temperatures ranging from 25 to 42°C. Colonies are generally smooth but rough variants may occur. They may be pigmented or non-pigmented.

1.3.4.1 *M.fortuitum complex (M.fortuitum, M. peregrinum, M.abscesses, M. chelonae)*

Members of the *M. fortuitum complex* are often the cause of abscesses especially at injection sites or surgical wounds. They can be associated with pulmonary disease (usually *M. abscesses*) as well, especially in cases of decreased immune response (Wayne, 1961). They are the second most common cause of infections caused by NTM after *MAC* and like *MAC* are resistant to antituberculous drugs that are routinely used.

1.4 TUBERCULOSIS (TB)

TB is the leading killer of adults in the world today among infectious diseases, and poses a serious challenge to international public health. Thus, there is a great concern about the worldwide magnitude of the new TB epidemic. The World Health Organisation (WHO) in 1993 declared TB to be a “global emergency” – the first declaration of its kind in WHO history. The bacilli, which cause TB, *M. tuberculosis* affects about a third of the world’s population, and is responsible for the death of 3 million people worldwide annually. Western countries have enjoyed a long decrease in cases due to the control of population and crowding. However, TB is making a come back especially with the advent of AIDS epidemic. In the west 80% of the infected population are over 50 years of age and therefore their impact on social and economical aspects are limited. However, in the developing countries, 75% of the infected people are less than 50 years of age and around 6.7% of deaths occur due to TB in the age group of 15 and 49 which is economically most productive period.
TB currently kills 1,70,000 children and more adults each year than AIDS, malaria, and tropical diseases put together. The disease is especially devastating in developing countries, where it accounts for more than a quarter of all preventable adult deaths. In concert with the AIDS pandemic, TB has overwhelmed health services and devastated urban populations in parts of Africa. Asian countries, with their large cities, extremely high rates of TB bacillus infection and the growing spread of HIV, currently account for two-thirds of all TB cases. In addition to this, multi-drug resistant TB has appeared among the AIDS population posing a great threat to the general population. However, 25% of TB deaths in adults is avoidable, if diagnosed timely and treated.

1.4.1 Mode of infection

The causative organism of TB, *M. tuberculosis* is an aerobic, non-spore-forming, acid-fast bacilli that is transmitted by inhalation of infected respiratory droplets coughed or sneezed into the air. It is a slow growing bacterium and takes 4-6 weeks to grow. Thus clinicians often employ the Purified Protein Test (PPD) test to confirm exposure to TB; however, this test does not diagnose active infection. Patients with a productive cough can be diagnosed with a Ziehl-Neelson acid fast staining of the infected sputum.

Primary TB infection starts with inhalation of infected droplets and ends with a T-cell mediated immune response referred to as the Gohn complex, a calcified scar in the periphery of the lungs. This response generally controls the infection in healthy individuals. These people will have a +ve PPD, but will be asymptomatic. Secondary TB occurs within individuals who become re-infected, or in whom the previous dormant infection becomes reactivated. The main features of secondary TB are the presence of granulomas with caseous necrosis and cavities especially at the apex of the lung. This can lead to a life threatening disseminated or miliary TB. Miliary TB without a pulmonary focus often appears as a fever of unknown origin.

An active TB infection is almost always localised to the apices of one or both lung lobes. It will rarely affect the lower lobes. Its clinical course is insidious
with a gradual onset of both local and systemic symptoms. Patients often complain of general malaise, weight loss, and a low-grade remittent fever with night sweats that escalate during the progression of the disease. As local pulmonary involvement worsens, patients will have a productive cough that begins as mucoid and later becomes purulent. Half of these cases will have accompanying hemoptysis. Extension of the disease to the pleura can lead to pleuritic pain and spontaneous pneumothorax.

The prognosis of TB is generally good unless it is multi-drug resistant. Asymptomatic infected patient must go for a two or three drug regimen that usually consists of Isoniazid, Rifampicin, and Pyrazinamide. Patients with secondary active TB must take these drugs along with Ethambutol or Streptomycin for nine months to one year.

In India, among the population of more than 900 million people, an adult is infected with the tuberculosis bacilli in every second of a minute. Each year more than 2 million people develop active tuberculosis, and up to 500,000 people die. The pool of infection and the resulting risk of becoming infected with the disease are even higher (WHO report, 1997).

The problem faced for effective TB control in India is multitudinous. There are real and potential conflicts between the interests of private physicians and the public sector. The quality and the supply of drugs are erratic. The diagnosis, which is based mainly on X-ray and sometimes symptoms alone, are common. Infectious cases are frequently missed, while other people are mistakenly diagnosed with TB and inappropriately treated. As a result, at least two thirds of TB patients drop out of treatment early, often becoming chronic sufferers and sources of infection to others. These chronic cases might become incubators for the deadly drug resistant bacterium (WHO report, 1997).
1.5 **BACILLI CALMETTE GUERIN (BCG)**

BCG has been used for over 60 years as a vaccine for tuberculosis and as an adjuvant for other purposes. Calmette and Guerin developed this strain by attenuating *M. bovis* after passaging for 231 generations through a media containing glycerine and Ox bile (Luelmo, 1982). However, the BCG vaccine has a less than satisfactory history. In controlled clinical trials, the efficacy of BCG as a vaccine for tuberculosis has varied from 80% protection to no protection at all. Contributing factors that have been identified in an attempt to explain the erratic performance of the BCG vaccine include genetic heterogeneity, “Natural” immunisation by saprophytic mycobacteria, manufacturing procedures such as lyophilization, or growth with surfactants. However, in the absence of an advanced understanding of the properties of mycobacteria which contribute to immunogenicity, efforts to develop improved vaccines have been unsuccessful (Collins, 1984). The recent development of modern techniques to insert genes of other organisms into mycobacteria has made it possible to consider using BCG as a vector for vaccines against other infectious agents (Jacobs *et al.*, 1987 and 1989; Lazraq *et al.*, 1991).

1.6 **TUBERCULOSIS AND AIDS**

The recently concluded First International Conference on “AIDS INDIA 2000” at Chennai, India, reported the estimated persons infected with HIV in the world as 20 million. By the year 2000, more than one million TB cases will occur in HIV infected persons annually. TB will accelerate the course of AIDS in these people, and potentially increase stigma and complicate efforts to provide medical care. Among individuals infected with TB, but without HIV infection, the risk of active TB is approximately 7- 10% per year. Whereas among persons with AIDS, the risk of active tuberculosis among newly infected persons may be 50% more (Frieden, 1997).

TB accelerates the course of HIV infection even if TB is brought under control by chemotherapy. Immuno suppression has long been known to hasten usually an asymptomatic *M. tuberculosis* infection to active tuberculosis (Selwyn
et al., 1989). This has become a major problem in the clinical management of AIDS patients in the world. It has been emphasised that, it is not the prevention of TB by mass vaccination but diagnosis of active TB and its prevention and / or cure that are the key to any effective TB control programme. Properly designed and co-ordinated longitudinal studies are needed to determine the true rate of TB and other mycobacterial infections in AIDS patients to serve as a background for possible intervention studies. Recent reports about the emergence of multidrug resistant TB in HIV infected individuals and their contacts reinforce the need for a stringent and more effective mycobacterial infection control programme in the clinical management of AIDS patients (Chowla et al., 1992).

In India, "The National AIDS Control Organisation" (NACO) has recorded, cumulatively, nearly 70,000 HIV infected persons since the inception of the survey programme. However, an independent estimate number it in the region of over 5 million, making India the country with the largest number of infected persons in the world. (John, 1997). The association of HIV infection and M. tuberculosis has been reported by clinical researchers from different parts of India (Sivaraman et al., 1992; Anuradha et al.,1993; Mohanty et al.,1993; Solomon et al., 1995). However, a detailed study about the incidence of TB in HIV infected persons in India is still lacking. A study on a small population of AIDS patients in South India showed 61% of patients to be infected with tuberculosis (Kumaraswamy et al., 1995), while in another study 52% of AIDS patients were found to have TB (16% pulmonary, 11% extra- pulmonary and 25% both) (Chacko et al., 1995). Banavaliker et al., (1997) reported that the HIV seropositivity in hospitalized pulmonary TB patients in Delhi, India is 1.3% by ELISA and 0.5% by western blot. A recent report by Paranjape., et al., (1997) quotes that the prevalence of HIV infection in TB patients has increased from 3.19% in 1991 to 20.1% in 1996 in Pune, India. The high prevalence of TB among the HIV patients reported here from various parts of India shows that TB has already made an impact on the HIV situation in India.

The most common mode of presentation of TB in HIV seropositive individuals is pulmonary TB. Upper lobe involvement in HIV seropositive pulmonary TB is still
the commonest mode of presentation but lower lobe involvement also may be seen in markedly immuno compromised individuals with a low CD4 count. Extra pulmonary tuberculosis presenting as tuberculosis lymphadenitis, tuberculosis pleural effusion, and miliary tuberculosis is seen occasionally (Tripathy, 1997). A comparative evaluation of the incidence of TB and HIV among the general population by Patel (1997) in Gujarat, India shows that the incidence of TB and extra pulmonary TB in HIV infected population are 3.82 times and 6.59 times higher respectively than the general population.

There are several reports about M. avium and other mycobacterial infections in AIDS patients from different research groups in the world (Blaser and Cohn, 1986; Crawford and Bates, 1986; Coker, 1992; Debrunner et al., 1992; Arbeit et al., 1993). However, little is known about this aspect among the AIDS patients in India. Members of Mycobacterium avium complex (MAC) are found in the environment and their role in the control of TB in India assumes importance not only because of lack of information on infection due to these mycobacteria but also because of the fact that these MAC organisms are resistant to conventional anti tuberculosis drugs and hence a different strategy is required to control their infection. A more extensive study is required to determine the actual rate of infection by M. avium and M. intracellulare in the general population in India particularly in HIV infected people for which a rapid diagnostic test is required.

Thus, the coincidence of TB and AIDS in time and space are overlapping, each worsening the other and poses a serious setback to TB control programmes world-wide particularly, in India. The timely identification of these cases is one of the most important factor for controlling or treating TB. Hence, the research focus should be,

a) Early detection of patients co-infected with HIV and TB, who may benefit from prophylactic therapy,

b) Rapid diagnosis and treatment of active TB,
1.7 CLINICAL SIGNIFICANCE OF MAC

MAC is the most important among the opportunistic pathogens affecting the immunocompromised patients both in the west and in the developing world. These organisms cause chronic pulmonary infections in elderly people with pre-existing lung disease, lymphadenitis in children and systemic bacterial infection in patients with AIDS in the developed world (Rosenzweig and Scheulter, 1981; Inderlied et al., 1993). MAC is a facultative intracellular organism that can invade and multiply within macrophages. The coinfection of monocytoid cells with HIV and MAC in-vitro results in reciprocal enhancement of multiplication. Hence, MAC increases HIV infection by increasing both the rate and degree of replication in monocytoid cells (Ghassemi et al., 1995).

Further, the number of distinct serovars / biotypes MAC is perhaps the largest among the mycobacterium genus. While the serovars 1-6, 8-11, 21 and 28 have been characterised as Mycobacterium avium, serovars 7, 12-20 and 25 are identified as Mycobacterium intracellulare (Saito et al., 1989; Wayne et al., 1993). It is difficult to distinguish the separate members of the MAC by serology because of cross-reactivity within the complex (Schaefer, et al., 1979). Denner et al., (1992) have stressed the need for a comprehensive approach to the serotyping of members of M. avium. Baess (1983) have analysed the DNA homologies between the organisms of MAC. Hampson et al., (1989) have demonstrated similarity among the MAC isolates from AIDS patients at the molecular level. They have also proved that these isolates differ from the isolates from healthy individuals at molecular level. Thorel et al., (1990) and Wayne et al., (1993) have reported a cooperative study of the international working group on mycobacterial taxonomy regarding the serovar determination and molecular taxonomic correlation of MAC.

Tsang et al., (1992) have discussed the clinical and epidemiological importance of typing of MAC isolates. Kunze et al., (1992) have reported that the M. avium strains could be differentiated on the basis of the possession of the insertion element IS901. Thierry et al., (1993) have reported the cloning and
sequencing of two DNA fragments from *M. avium* serotype 2 for use in the identification of the members of *MAC*.

Frothingham and Wilson. (1993), (1994) have reported substantial internal transcribed spacer (ITS) sequence differences among the *MAC* strains, which proves that sequence analysis may be generally useful for the identification of clinically meaningful groups of strains within individual bacterial species. Roiz et al., (1995) have reported that the Restriction Fragment Length Polymorphism (RFLP) of the insertion sequences could serve as a genetic marker for typing the *M. avium* strains from AIDS patients. Hellyer et al., (1991) have reported that the plasmid profiles are useful epidemiological markers for the identification of repeat isolates of the same strain of *MAC*. However, this is confounded by the inherent variability of plasmids as extrachromosomal elements and by their relatively frequent selective acquisition or loss due to environmental factors (Arbeit et al., 1993).

De Smet et al., (1995) have reported the sequence homology in the ITS region between the 16S and 23S rRNA of *M. avium intracellulare* isolates form AIDS patients. De Smet et al., (1996) have compared the serotyping and genetic analysis of *MAC* and reported that species classification on the basis of genetic analysis was similar to serovar typing with only minor discrepancies.

**1.8 ROLE OF NON-TUBERCULOUS MYCOBACTERIA (NTM) IN HUMAN HEALTH**

Several members of the genus *Mycobacterium* which were originally thought to be only saprophytic environmental organisms are now found to be associated with human diseases either as potential pathogens or as causative organisms (Grange, 1991). Included in this list are *Mycobacterium fortuitum chelonae* complex, *M. scrofulaceum*, *M. kansasii*, *M. xenopi* which were once believed to be only environmental. Exposure of an individual to these mycobacteria is variable and it is greatly influenced by the environment (soil and water) and one’s life style. The relevance of environmental mycobacteria in human health and disease lies in the fact that clinically it is difficult to differentiate
any one of them as specific etiological agent of pulmonary disease (Grange, 1980) and many of these mycobacteria have a positive immunomodulatory role in man. It has been shown at least in experimental animals that infections with some NTM can offer the same level of protection as BCG against TB (Edwards et al., 1982; Orme and Collins, 1984).

Innate resistance to antibiotics has been reported in several of these environmental mycobacteria. Kuze et al., (1981) have studied the drug susceptibility of atypical mycobacteria to various drugs in-vitro and in-vivo. Reports from many parts of the world have emphasised that the frequency of mycobacteria other than tuberculosis is increasing in both actual numbers and in the proportion of total mycobacterial disease. Therefore, the NTM could significantly influence the control strategies of mycobacterial diseases like leprosy and TB particularly in the context of the global re-emergence of TB, emergence of multidrug resistance in leprosy and TB and our lack of knowledge of the exact mechanism of pathogenesis in these two diseases. The etiological role, if any, played by these NTM in the disease process as opportunistic pathogens assumes greater significance in devising effective control strategies.

The different species of NTM vary in their pathogenic potential for humans. MAC and M. kansasii are well recognised pathogens in persons infected with HIV. In some areas the isolation rate for MAC has surpassed that for M. tuberculosis (Woods and Washington II, 1987). A recent report from Yates et al., (1997) quotes that the annual number of isolates of environmental mycobacteria from both HIV positive and negative patients is increasing both absolutely and relatively to isolates of M. tuberculosis.

Very little information is available regarding the prevalence of NTM in India. Isolation and identification of environmental mycobacteria form soil, water, dust and sputum samples have been reported by Kamala et al., (1994a) and Kamala et al., (1994b). Karak et al., (1996) has reported the prevalence of atypical mycobacterium in-patients form Calcutta, India as 17.4%. 
1.9 CLINICAL MANAGEMENT OF MYCOBACTERIAL INFECTION

The molecular mechanisms of the action of anti mycobacterial compounds on *Mycobacterium sp.* have only recently been revealed. Broad-spectrum antibacterials, including streptomycin, rifampicin, and fluoroquinolones have been demonstrated to act on the same targets in *Mycobacterium sp.* as they do in *E. coli.* Resistance to these agents results from single mutagenic events that lead to amino acid substitutions in their target proteins. The mechanisms of action of the unique anti-tubercular drugs, including isoniazid, ethambutol, and pyrazinamide have also recently been defined. Resistance to INH can be caused either by mutations in the katG-encoded catalase-peroxidase, the enzyme responsible for drug activation (Zhang *et al.*, 1992), or by mutations in inhA, the gene encoding for long chain enoyl-ACP reductase (Banerjee *et al.*, 1994). Ethambutol appears to block specifically the biosynthesis of the arabinogalactan component of the mycobacterial cell envelope, and pyrazinamide has no known target. With the resurgence of TB and the appearance of strains, which are multiple resistant to the above compounds, clinical management of TB is threatened. Hence, identifying the molecular mechanisms underlying the resistance for these drugs would pave way for better clinical management of TB and other mycobacterial diseases.

The following are the drugs, which are currently recommended for the treatment of tuberculosis.

1st line drugs:

INH, Streptomycin, Rifampicin, Ethambutol, and Pyrazinamide

2nd line drugs:

Capreomycin, Cycloserine, Ethionamide, Para amino salicylic acid, Kanamycin, and more recently, Amikacin, Ciprofloxacin, Clofazimine, Ofloxacin, and Sparfloxacin
1.9.1 Drug Susceptibility Testing

It is customary to perform drug susceptibility testing of the isolated organism from the patient to the various antimycobacterial drugs before therapy. This susceptibility test for *M. tuberculosis* is important since

a) Results can be used as a guide to initiate therapy.

b) Testing may be of value in confirming drug resistance in a patient showing unsatisfactory response to treatment.

c) Testing may be useful in evaluating trends of primary and acquired drug resistance within a community.

1.10 DIAGNOSIS OF MYCOBACTERIAL INFECTION

It is apparent from the above review that the timely diagnosis of mycobacterial infection is important both for clinical treatment of infected individuals and to identify persons who are at risk. The problem of correct species identification is made more acute by the AIDS epidemic and the consequent increase in associated non-tuberculous infections such as *M. avium* and *M. intracellulare* in these patients (Braun et al., 1990). Because of the toxicity associated with some anti-tuberculosis drugs, early definitive species diagnosis is important for preventing unnecessary exposure of the patients to the side-effects caused by these drugs (Kopanoff et al., 1978). In some cases, such as TB meningitis or AIDS infection, delay of diagnosis / treatment for several weeks can be fatal (Kramer et al., 1990).

1.10.1 Acid-fast test

Most mycobacteria grow at a relatively slow rate; therefore, the acid-fast test plays an important role in the early diagnosis of mycobacterial infections. Microscopy is the oldest, easiest, most rapid, and inexpensive procedure that can be performed in the laboratory to detect the presence of acid-fast bacilli (AFB) (Grange, 1983). However, the acid-fast smear should not be used in place of
culture for diagnosis. Acid-fast test requires 105 AFB per ml of sputum for detection by direct microscopy. In spite of this quantitative discrepancy in sensitivity, examination of stained smears of sputum or other clinical material has the following advantages.

a) It provides a presumptive diagnosis of mycobacterial disease within a short period.

b) Smear positive patients are rapidly identified and are the most infectious cases.

c) It may be used to follow the success of chemotherapy of tuberculosis patients.

There are three procedures commonly in use for the staining of AFB. Two methods, Ziehl-Neelsen and Kinyoun staining use light microscopy to view the AFB stained red by carbol fuchsin. The third method uses a fluorescent microscope to view AFB stained as golden fluorescence by a fluorochrome dye such as auramineO. The advantage of using the fluorescent staining method is that fluorescent stained slides can be examined under low magnification allowing much larger areas of the smear to be examined in a short period of time.

Although the ability to retain aryl methane dyes such as carbol fuchsin and auramineO after washing with alcohol or weak acids is a primary feature of the genus mycobacteria, it is not entirely unique to the genus. Other bacteria, which contain mycolic acids, such as Nocardia also, exhibit this feature. Moreover, It is well known that pulmonary tuberculosis in HIV infected individuals is frequently smear negative and the progress of the culture positive or culture negative case to a smear positive case results in severe infection until the patient is diagnosed. Thus, sputum analysis, though very simple, apart from having poor sensitivity, poses difficulties due to contaminating microorganisms (including mycobacteria other than M. tuberculosis) and non-availability of sputum in some AIDS patients. In addition to that microscopy does not enable species identification of the
mycobacterial pathogen. Hence, the AFB staining cannot be considered as a specific tool to diagnose mycobacterial infection.

1.10.2 The Tuberculin Test

The commonly used tuberculin is referred as "purified protein derivative" (PPD) (Nassau and Nelstrop, 1976). Classically the term PPD has been applied to culture filtrates of *M. tuberculosis* that has been precipitated with either trichloro acetic acid or ammonium sulphate. Tuberculin as a diagnostic reagent was first applied subcutaneously. This resulted in a generalised as well as local reaction. Since a generalised reaction was undesirable, a variety of tests were developed to restrict the reaction to the area around the site of injection. These tests involved the intracutaneous or percutaneous administration of tuberculin.

For this test, a standard quantity (5 TU) of PPD of tuberculin is injected intracutaneously. A 10 mm induration was considered to be an indication of a positive reaction. This criteria was based on the results of studies in which reaction sizes to graded amounts of PPD administered to healthy individuals. The sensitivity and specificity of the tuberculin skin test have been the subjects of extensive investigation, with widely variant results. The rate of false-negative reactions for patients with active tuberculosis has varied from 5% to 25% (Woolinsky, 1994).

The tuberculin test indicates whether sensitivity due to past or present mycobacterial infection exists in a person. The range of reactivity may vary from very strong to frankly negative with all intermediate-size gradients detectable. However, no tuberculin test is perfect, either from the view of effectiveness or safety.
1.10.3 Diagnosis by means of culturing

Definitive diagnosis of the etiologic agent can be accomplished only after the organism has been recovered on primary isolation media and subjected to a number of biochemical tests. As few as 10 to 100 colony forming units/ml (CFU/ml) can be detected in culture. The most commonly used media for the cultivation of mycobacteria are the Lowenstein-Jenson (LJ) media and Middlebrook 7H-10 media. Culture methods, though specific, require 3-8 weeks for growth. The biochemical typing to identify the correct species should be done after culturing. The interval can be decreased to 5-12 days with the BACTEC TB system and to 12-19 days with SeptiCheck AFB, Bactec culture system. However, these systems cannot differentiate MAC from other atypical mycobacteria which are important in the clinical management of AIDS patients.

Van Griethuysen et al., (1996) have compared the three available culture systems for detection of mycobacteria viz. fluorescent BACTEC 9000 MB system, Septic- Chek AFB system and Lowenstein- Jenson medium for their usefulness. Casal et al., (1997) have evaluated the Mycobacteria Growth Indicator Tube (MGIT) and compared with the BACTEC 460 TB system and Lowenstein- Jenson media for isolation capacity and detection time of mycobacteria. They have also reported MGIT as the rapid, easy- to- use system.

However, from the clinicians point of view, he cannot keep a patient suspected of having TB without treatment until the culture results are available. Hence, there is a need for a diagnostic method, which would be specific, sensitive and fast.

1.10.4 Immunodiagnosis of TB

Mycobacteria are considered as the archetypical intracellular pathogens because of the capacity of these bacilli to invade and multiply within macrophages; thus, the cellular immune response to mycobacterial infection has been a subject of considerable study. Phagocytosis and processing of antigens
by macrophages or B-lymphocytes trigger a specific cellular immune response, including the activation of T-helper cells, macrophages, T-cytotoxic cells, and NK cells. Antigen processing occurs after infection with mycobacteria and leads to a complex host response involving multiple arms of the immune system (Colston, 1990).

Several mycobacterial protein antigens of molecular weights ranging from 10 kDa to 85 kDa have been reported previously (Engers et al., 1986; Bothamley et al., 1988; Ljungqvist et al., 1988; Coates et al., 1989, and Young et al., 1992). Among these, the 14 kDa antigen identified by Bothamley et al., (1988) and Verbon et al., (1992) have been found to be immunogenic in the early stages of M. tuberculosis infection. The 19 kDa secretory antigen reported by Jackett et al., (1988) is also immunodominant and has limited cross-reactivity with atypical mycobacteria. Sada et al., (1990) have discussed the use of the 30/31 kDa antigens which are secreted fibronectin binding proteins corresponding to antigens 85A and 85B of M. bovis BCG, in the serodiagnosis of smear-positive pulmonary TB. The 38 kDa antigen was reported as the most useful antigen in the serodiagnosis of multibacillary pulmonary TB by Bothamley et al., (1992). Wilkins and Ivanyi, (1990) have reported the TB72 monoclonal antibody epitope as a precise test for extra pulmonary and paucibacillary pulmonary disease. Bothamley et al., (1992) have reported the 19 kDa antigen as the best of all the purified antigens with a sensitivity of 60% and specificity of 95%. Kolk et al., (1989) have discussed the production and characterisation of serotype-specific and M. avium-intracellulare scrofulaceum (MAIS) complex specific monoclonal antibodies by immunisation with a mixture of M. avium serotypes. Amara and Sachidanandam, (1996) have reported the differential reactivity of the recombinant proteins of M. tuberculosis to the serum from patients with early and late phases of tuberculosis infection.

The specific B-cell response after a mild exposure to M. tuberculosis or BCG immunisation have been studied by few workers. However, the antibody responses (IgG & IgM) to 30/31 kDa & the 65 kDa heat shock protein was low in the vaccinated individuals (Daniel et al., 1991; Drowart et al., 1991; Bothamley
This shows that there is only a low level of humoral response to the weak exposure of mycobacteria and thus more sensitive assays are required for the measurement of these responses.

1.10.4.1 Enzyme-Linked immunosorbent Assay (ELISA)

ELISA is a primary binding test, which has been successfully adapted to many serological systems. In the first step, the antigen is coated onto a plastic surface. The serum sample from the patient is added and possible antibodies are allowed to bind. The visualisation of the reaction between antibody and antigen is accomplished by the application of an enzyme-conjugated anti-immunoglobulin preparation. After completion of the reaction between the conjugate and the antibody, the enzyme is allowed to interact with its substrate. The product of this interaction is quantitated colorimetrically. The colour intensity is proportional to the amount of antibody, which originally reacted with the antigen-coated surface.

Several methods of immunodiagnosis have been developed and reported for TB. This includes a latex-particle agglutination method (Chandramuki et al., 1985), sandwich ELISA (Watt et al., 1988), and inhibition ELISA (Radhakrishnann and Mathai, 1991). These assays have been validated in different specimens such as sputum, urine, bronchial lavage and pleural fluid (Yanez et al., 1986; Dhand et al., 1988; Raja et al., 1988; and Singh et al., 1991).

However, the drawback of this system is the cross reactivity. If the serum from a patient contains antibodies, which react with a *M. tuberculosis* antigen preparation, these antibodies are not necessarily a result of an infection by *M. tuberculosis*. The different species within the genus mycobacterium have a considerable number of antigen in common. Some of these antigens are also common to other bacterial genera such as *Nocardia* and *Corynebacterium*. Hence, it is very difficult to avoid cross-reactivity.
1.10.5 Diagnosis of mycobacteria using High Performance Liquid Chromatography (HPLC) analysis

Identification of mycobacterium species by reverse-phase HPLC of mycolic esters have been demonstrated to be a rapid, reproducible, species-specific method. It could also be used to differentiate other mycolic acid containing bacteria (Butler et al., 1986, 1987, 1991; Butler and Kilburn, 1988, 1990; Glickman et al., 1994). Even though these systems have their own merits, the interpretation of HPLC-generated chromatographic data requires some expertise. Each chromatogram must be visually assessed, and often, hand calculations and flow charts are necessary to validate the identifications for many mycobacterial species. Savic et al., (1992) have compared the gas chromatography-mass spectrometry (GC-MS) of tuberculostearic acid with that of Polymerase Chain Reaction (PCR) for the detection of M. tuberculosis in sputum and shown that PCR is more sensitive and specific than GC-MS analysis.

1.10.6 DNA probes for the detection of mycobacterial infection

The often fulminate nature of TB in patients with AIDS, the risk of spreading the disease to contacts like health care workers, and the spectrum of Multi Drug Resistant (MDR) TB together emphasise the importance of early diagnosis. The fact that sputum smears are typically negative when noncavity pulmonary TB occurs in otherwise normal persons and that TB in patients with AIDS are usually without cavities raises the concern that diagnosis would be difficult in this population. It appears that the use of DNA probes holds the key for this problem. Recently, Yates et al., (1997) have reported that the prevalence of NTM among the TB and AIDS patients are increasing steadily and hence there is a need to use DNA based diagnostic methods which would efficiently discriminate the NTM from M. tuberculosis.

DNA probes have a number of advantages over protein based (immunological) diagnostic methods. The stability of DNA probes and their targets, as compared to protein-based systems have advantages both in transport and storage of reagents as well as samples. The specificity of DNA
probes with their theoretical ability to differentiate between two organisms differing in only a single base substitution, may be useful in differentiating closely related mycobacteria. Moreover, with the Polymerase Chain Reaction (PCR) based methods, DNA probes have the capability of exquisite sensitivity that is unlikely to be matched by immunological assays. The DNA probe may be the whole mycobacterial genome or a small cloned fragment from the genome or a synthetic oligonucleotide. If oligonucleotides are used, small differences in the DNA sequences between the probe and the target sequence in different species or strains could dramatically affect the hybrid stability and therefore the specificity of the probe would be high. In the case of a larger DNA probe the specificity would be comparatively less. Thus, the choice of the length of the probe is crucial in determining the specificity.

1.10.6.1 Restriction Fragment Length Polymorphism (RFLP) analysis

An alternative approach to the identification, characterisation and sequence determination of individual genes has been the use of a technique termed RFLP analysis. This technique was developed as a means to detect very small differences (i.e., single base pair changes) in chromosomes of organisms that are very closely related by conventional genomic analysis such as total chromosomal DNA-DNA hybridisation or sequence determination of highly conserved genes. As the name implies, DNA restriction endonucleases are used to generate numerous fragments from chromosomal DNA and the fragments are separated electrophoretically on the basis of their size.

Among DNA from closely related species, differences in the length of a particular restriction fragment could be due to genotypic variations that result from differences in one or more individual bases so that cleavage sites for a given endonuclease could be lost or gained. Alternatively, differences could also be the result of insertions or deletions of blocks of DNA within the fragment. Using small, radioactive or non radioactive labelled restriction fragments as DNA probes, variants among restriction fragments in total chromosomal DNA can be detected using the method of Southern. (1975).
The large amount of input DNA required for genomic RFLP analysis and the need for more sensitive detection techniques has led to the development of the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. In this method a specific region of the genome is amplified, and then restriction analysis of the PCR product is used to determine microheterogeneities of a single locus. In contrast, genomic RFLP analysis is based on the heterogeneous distribution of a genetic marker throughout the genome. Haas et al., (1993) have described a mixed-linker PCR-RFLP for the rapid finger printing of *M. tuberculosis* isolates. They have also demonstrated that this system could be useful in identifying any tuberculosis outbreaks. Plikaytis et al., (1992) have reported a two-step assay that combines amplification of a mycobacterium genus-specific amplicon with RFLP to differentiate the most commonly encountered mycobacterial pathogens to the species level. They have also suggested that a comparison of the restriction fragment pattern of an unknown with the database of known patterns should allow identification of the unknown to the species level.

RFLP studies have been conducted among members of the *M. tuberculosis* complex (Eisenach et al., 1986, 1988; van Sooligan et al., 1992) the *M. avium* group (McFadden et al., 1987) and among the isolates of *M. leprae* (Clark- Curtiss and Walsh, 1989). Eisenach et al., (1988), observed repetitive DNA sequences in *M. tuberculosis* DNA in their RFLP studies using the M13mp18 *M. tuberculosis* library. They have also identified three different probes which hybridised to multiple bands of *M. tuberculosis*. These probes were approximately 1.5 to 2.0 kb in size, hybridised to *M. tuberculosis* and *M. bovis* DNA but produced different hybridisation patterns for different isolates, thereby allowing differentiation between *M. tuberculosis* and *M. bovis*, and also within the different clinical isolates of *M. tuberculosis*.

Although, the direct examination of restriction endonuclease digests of genomic DNA is useful and relatively simple to perform, it is limited by the resolving power of the gels. An improved method to resolve very large DNA fragments generated by restriction enzymes recognising rare sites in
mycobacterial DNA, was used by Slutsky et al., (1994). This method produced simpler and more easily interpretable genomic fingerprints that can be used to differentiate between mycobacterial species. This method known as Pulsed Field Gel Electrophoresis (PFGE), proved to be highly useful in large scale mapping of mycobacterial genomes. However, this requires costly apparatus and technical expertise.

1.10.6.2 Diagnosis of Mycobacterium sp. by PCR based methods

One of the most powerful molecular biology techniques currently in use is DNA amplification by PCR using the thermostable DNA polymerase isolated from *Thermus aquaticus*. The invention of PCR has revolutionized the ability to amplify and manipulate a nucleic acid sequence *in vitro*. The commercial rewards of this revolution have driven the development of other nucleic acid amplification and detection methodologies like Nucleic Acid Sequence- Based Amplification (NASBA), Ligase Chain Reaction (LCR), Strand Displacement Amplification (SDA), Q-beta replicase (QBR), Cycling Probe Reaction (CPR) and branched DNA (bDNA).

Despite the differences in their processes, these amplification systems can be separated into two broad categories based on how they achieve their goal. Sequence based amplification systems, such as PCR, NASBA and SDA, amplify a target nucleic acid sequence. Signal-based amplification systems such as LCR, QBR, CPR, and bDNA, amplify or alter a signal from a detection reaction that is target dependent. Each of these systems have their own merits and demerits (Compton, 1991; Sooknanan and Malek, 1995).

Nucleic acid amplification based detection system has found its application in the diagnosis of many infectious diseases. PCR has become the tool of choice for rapid and direct identification of mycobacterial pathogens in clinical specimens, though the validation and comparison against the culture is still considered as the "gold standard". Keihn, (1993) has stressed the need for new diagnostic methods for the diagnosis of mycobacterial infection. Optimization of PCR conditions is a crucial step in designing a good PCR assay. Parameters
such as primer design and annealing temperature need to be optimized before performing the PCR reaction (Rychlik et al., 1990).

Two criteria must be met before mycobacterial specimens can be tested by DNA amplification methods: (i) the sample must be rendered noninfectious, and (ii) the organisms must be lysed to free the DNA. Zwadyk et al., (1994) have reported that heating the samples below 100°C may not considerably kill mycobacteria. They have also reported that heating at 100°C for 30 minutes consistently lysed mycobacteria to produce short fragments of DNA for PCR amplification.

The sample preparation for PCR analysis of *M. tuberculosis* is problematic because it is necessary to disrupt the thick waxy coat of the tubercle bacillus and remove the inhibitors of PCR found in many sputum and blood samples. Various sample preparation methods have been described by different workers. Buck et al., (1992) have reported sonication as the most efficient method for the processing of samples for PCR. Victor et al., (1992) have reported that treating the sputum samples with 50% sucrose improves detection of *M. tuberculosis* by PCR. Wilson et al., (1993a) have described the use of filter papers to collect and transport the sputum samples. Wilson et al., (1993b) have discussed processing of sputum samples by chaotrope-silica and by chloroform method. Folgueira et al., (1993) have compared the sonication and lysis method of sample processing with nonionic detergents and proteinase. Kocagoz et al., (1993) have reported that heating the sample in a boiling water bath to break the cell wall and releasing the DNA was better than enzymatic lysis of bacteria and phenol-chloroform extraction of DNA.

Genus specific PCR for the identification of mycobacterium has been reported by Hance et al., (1989); Fries et al., (1990); Kirschner et al., (1993); Takewaki et al., (1993) and Soini et al., (1994). Sritharan et al., (1994) have evaluated the usefulness of the genus and species specific probes for the identification of mycobacterial infection in clinical samples. Verma et al., (1994) have reported a mycobacterium genus-specific, non-radioactive test based on the 23S rRNA gene sequence of *M. tuberculosis*. They have also suggested that the
instability of 23S rRNA could be a useful property to exploit for determining whether the mycobacteria in a given specimen are viable or dead. Kapur et al., (1995) reported the use of automated sequencing of a portion of hsp65 for the rapid identification of *M. tuberculosis* and other mycobacterial species.

PCR based detection of *M. tuberculosis* in clinical samples has been reported by many workers although the specificity and the sensitivity varies from one report to another. (Patel et al., 1990; Sjobring et al., 1990; Manjunath et al., 1991; Portillo et al., 1991; Sritharan and Barker, Jr., 1991; Altamirano et al., 1992; Kolk et al., 1992; Claridge et al., 1993; Mayazaki et al., 1993; Nolte et al., 1993; Ross and Dwyer., 1993; Walker et al., 1994; Kadival et al., 1996).

The use of the insertion element *IS6110* (Thierry et al., 1990a) for PCR based detection of *M. tuberculosis* has been reported by many workers (Eisenach et al., 1990; Thierry et al., 1990b; Kolk et al., 1992; Nolte et al., 1993; Forbes and Hicks, 1993; Haas et al., 1993; Palittapongarnpim et al., 1993; Noordhoek et al., 1994). Furthermore, these IS elements serve as useful tool in epidemiology of TB because of their highly variable copy number and the great variability of insertion sites in the chromosome of different *M. tuberculosis* strains. The use of PCR/DNA probes to study the epidemiological difference between the *M. tuberculosis* strains have been reported by van Sooligan et al., (1991), Mazurek et al., (1991), Linton, (1994) and Yang et al., (1995).

Eventhough DNA has been the target for most PCR based detection methods, there are a few reports, which describe the use of RNA as a potential target for PCR amplification. (Rogall et al., 1990; Cox et al., 1991; Giessen et al., 1992; Jonas et al., 1993; Greisen et al., 1994; van der Vliet et al., 1994; Verma et al., 1994; Abed et al., 1995; Kox et al., 1995). Evans et al., (1994) have reported the possibilities of identifying the rRNA of *M. tuberculosis* in mouthwash samples from patients with TB. Though it is possible to identify *M. tuberculosis* on the basis of the presence of rRNA in the mouthwash samples, the poor sensitivity and specificity of the technique suggest that it is unlikely to be useful for diagnosis of *M. tuberculosis*. A 16S- 23S region based Random Amplified
Polymorphic DNA (RAPD) analysis for the identification of \textit{M. tuberculosis} strains was reported by Abed \textit{et al.}, (1995).

Blood samples are easily obtainable from patients and detection of \textit{M. tuberculosis} in blood is a definite indication of active infection. The PCR based identification of \textit{M. tuberculosis} from blood samples was reported by Schluger \textit{et al.}, (1994). They have also reported that they could amplify mycobacterial DNA only when they extracted DNA from the buffy coat. The DNA extracted from the serum alone did not yield any detectable signal. Their results suggested that escape of organisms from the alveolar space to the bloodstream may be more common than previously thought, and in fact circulating host defenses may have a larger role in limiting the infection to the lungs.

PCR has found its use in the detection of extrapulmonary TB also. Lombard \textit{et al.}, (1994) have reported the use of PCR in detecting \textit{M. tuberculosis} in bone marrow aspirates. Salo \textit{et al.}, (1994) reported the extraction and identification of DNA unique to \textit{M. tuberculosis} in lesions from the lung of a 1000 year old human remains from southern Peru which provides evidence for the pre-Columbian presence of human TB in the New World. Gamboa \textit{et al.}, (1997) have reported the detection of mycobacteria from the bone marrow aspirates by the amplification of a specific DNA region of the 16S rRNA gene.

An \textit{et al.}, (1995) have demonstrated a Q- Beta replicase assay for the diagnosis of \textit{M. tuberculosis} and have reported that it is both sensitive and semiquantitative. Recently Smith \textit{et al.}, (1997a) and (1997b) have described the detection of \textit{M. tuberculosis} 23S rRNA targets directly from sputum using Q- Beta replicase amplification and reversible target capture (RTC) background reduction technology on the Galileo analyzer. The sensitivity, specificity and positive and negative predictive values of this study were 79, 98, 97 and 85% respectively. A multiplex PCR assay which could identify up to 12 mycobacterial species including the noncultivable mycobacterium \textit{M. leprae} in the clinical specimen was reported by Kox \textit{et al.}, (1997).
Reports regarding the development and use of PCR based molecular diagnosis for *M. tuberculosis* in India is scanty. Reddy *et al.*, (1993) reported PCR based identification of *M. tuberculosis* and *M. bovis* using a 169 bp region of 1.4 kb single copy fragment of *M. tuberculosis*. Verma *et al.*, (1994) described the use of 23S rRNA based PCR assay for the detection of mycobacteria in general. Khandekar *et al.*, (1994) reported the evaluation of PCR based identification of *M. tuberculosis* in clinical samples. Narayanan *et al.*, (1992) and Sahadevan *et al.*, (1995) have also reported DNA probes for the detection of *M. tuberculosis*. A 38 kDa protein sequence based PCR for the diagnosis of *M. tuberculosis* has been reported by Kadival *et al.*, (1996). The detection of *Mycobacterium tuberculosis* in the lymph node of children was reported by Francis *et al.*, (1997).

Reports on DNA probes for the diagnosis of *M. avium* and intracellulare are very limited compared to the wealth of information available for the diagnosis of *M. tuberculosis* infection. The Gen- probe kit developed and marketed by Gen-Probe Inc, San Diego, USA, though widely used, cannot differentiate between different strains of *M. avium* and *M. intracellulare*. The probe developed by Hance *et al.*, (1989), and that reported by Fries *et al.*, (1990) though shown to be specific are yet to be tested on clinical settings and their predictive value is not yet known. PCR / DNA probe based identification of *M. avium* has also been reported by Saito *et al.*, (1989), (1990); Iralu *et al.*, (1993); and Sritharan *et al.*, (1995). Thierry *et al.*, (1993) have reported the development of DNA probes for the identification of *M. avium* and *M. intracellulare*. Two fragments DT1 and DT6 were identified by them from the cosmid library of *M. avium* serovar 2 (ATCC 25291) which are specific for *M. intracellulare* and *M. avium* respectively. Yamasaki and Nakamura, (1995) have reported PCR based DNA probes for the identification of *M. intracellulare*. However, these probes have not been validated in clinical samples.

A multiplex PCR for the identification of *M. avium* and *M. intracellulare* was described by Cousins *et al.*, (1996). Musial *et al.*, (1988), have evaluated the *M. avium* and *M. tuberculosis* probes developed by Gen-Probe, Inc., San Diego,
CA, USA. Reisner et al., (1994) have evaluated the M. avium and other mycobacterial probes directly on the BACTEC cultures.

When PCR is used in clinical diagnosis, controls for inhibition are indispensable. The use of an internal control would enable detection of inhibition and simultaneously permit the quantification of the PCR results. The use of an internal control for PCR was reported by few workers. (Celi et al., 1993 and Cottrez et al., 1994). Although there are a few reports concerning the absolute quantification of the PCR products (Zachar et al., 1993; Cottrez et al., 1994) there are no reports pertaining to mycobacterium. The method reported by these authors - competitive PCR, stress the use of an internal control for the purposes of quantitation. In competitive PCR, a DNA fragment containing the same primer template sequence as the target is used to compete for primer annealing and amplification. PCR products are then distinguished by size, restriction sites or southern blot analysis. Competitive PCR methods require that the target gene and competitive fragment amplify with equal efficiency. Different methods for PCR quantitation have been reported by Higuchi et al., (1993); Zachar et al., (1993); Friedhoff et al., (1993) and Miele et al., (1994).

Kolk et al., (1994) have described a semiquantitative approach for the PCR based detection of M. tuberculosis using genetically engineered M. smegmatis as control. They have also reported that this system would be helpful in detecting any inhibition in the amplification process. However, this method cannot be adopted for the quantitation of mycobacteria from clinical specimens. In a clinical set up where a large number of samples have to be screened and evaluated this method becomes cumbersome and will be prone to contamination.

A few reports are available on the use of PCR for assessing the treatment response in patients undergoing chemotherapy (Kennedy et al., 1994; Palenque et al., 1995 and Hellyer et al., 1996). Yuen et al., (1993) reported that among 41 cases of culture-documented pulmonary tuberculosis, 71% remained positive by PCR after 4 weeks of treatment whereas 39% and 32% remained positive by acid-fast smear and culture respectively thus proving the high sensitivity of PCR. Levee et al., (1994) have reported the use of PCR to monitor treatment response
in 13 patients who were receiving 6 months chemotherapy. After 2 months of treatment all the cases became smear and culture negative, whereas 23% remained PCR positive. Kennedy et al., (1994) reported that the PCR assay was able to detect conversion from positive to negative 1–2 months after culture.

A few commercial kits are available for the detection of *M. tuberculosis*. Gen-Probe, San Diego, CA, USA has developed an isothermal transcription-mediated amplification system which detects *M. tuberculosis* complex directly in respiratory specimens and provides billionfold amplification of the rRNA targets-Amplified Mycobacterium tuberculosis Direct Test (AMTDT). This has been validated by few workers (Abe et al., 1993; Pfyffer et al., 1994 and Bodmer et al., 1994). Roche Molecular Systems Somerville, NJ, USA have come up with a PCR based direct test for *M. tuberculosis* called AMPLICOR MTB. The kit contains primers that target a DNA sequence within the 16S rRNA - encoding gene present in all mycobacteria. After amplification, the resulting DNA copies are hybridised with *M. tuberculosis* specific oligonucleotide probes coated onto a microwell tray. Immobilised PCR product is detected in an ELISA-like procedure, and the results are available within eight hours of receiving the clinical specimen. Beavis et al., (1995) have evaluated this system on clinical samples.

1.11 OBJECTIVES OF THE PRESENT STUDY

Hence, considering the available information in the literature, following objectives were outlined for the present study.

a) to develop a simple protocol for processing blood samples for PCR analysis.

b) to establish PCR based diagnosis of mycobacterial infection in clinical samples from this part of India.

c) to explore the possibilities of using PCR to monitor the treatment response in patients undergoing chemotherapy.

d) to develop a specific probe for *M. avium* and to validate it in the clinical specimens.

e) To study the incidence of NTM in leprosy and tuberculosis patients.
### Table 1.1 A sampling of Tuberculosis diagnostics

<table>
<thead>
<tr>
<th>Company</th>
<th>Amplification Technology</th>
<th>Approximate cost per test ($US)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roche Molecular Systems (AMPLICOR MTB)</td>
<td>Polymerase Chain Reaction (PCR)</td>
<td>$30-50 depending on country</td>
<td>Manual procedure taking ~8 hours. Calorimetric detection in ELISA-like microwell format</td>
</tr>
<tr>
<td>Gen-Probe (AMTDI)</td>
<td>Proprietary transcription-based amplification of ribosomal RNA sequences</td>
<td>$25-40 depending on country</td>
<td>Semi-automated procedure taking ~4 hours. Amplification and detection in same tube (Machine read chemiluminescence)</td>
</tr>
<tr>
<td>Becton Dickinson</td>
<td>Strand displacement amplification</td>
<td>Not available</td>
<td>Fully automated and enclosed sample processing and detection.</td>
</tr>
</tbody>
</table>

(Source: Yule., 1994).