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2.1. Definition

Tuberculosis is defined as an infectious disease of humans and other animals due to species *Mycobacterium*, marked by the formation of tubercles and caseous necrosis in tissues of any organ. Tuberculosis is derived from the word tubercle meaning a small lump or nodule. The lung is the most frequently affected organ but virtually any organ or system of the body may be involved hence it has been termed 'morbus percorpus' (Ratledge and Dale, 1999). Pulmonary tuberculosis is a contagious bacterial infection affecting the lungs caused by *Mycobacterium tuberculosis* (*M. tuberculosis*).

2.2. General Characteristics of Mycobacteria

Mycobacteria are a group of acid-fast, aerobic, slow-growing organisms whose genus includes more than ninety different species. It was first isolated in 1882 by Robert Koch who demonstrated mycobacterium as an agent of human disease (Koch, 1882). Tubercle bacilli are non-motile, non-sporing, non-capsulate, Gram-positive, straight or slightly curved pleomorphic rods. They are characterized by slow growth (*M. tuberculosis* divides every 15 to 20 hours compared to other bacteria, which tend to have division time measured in minutes), dormancy, complex cell envelope, a characteristic staining quality, intracellular pathogenesis and genetic homogeneity (Ratledge and Dale, 1999).

The microorganisms that cause tuberculosis belong to the genus *Mycobacterium* classified in the family Mycobacteriaceae of the order Actinomycetales. Together with other highly related bacteria, *M. tuberculosis*
forms a single complex, as defined by DNA/DNA hybridization studies (Imaeda, 1985). The *M. tuberculosis* complex comprises six members: *M. tuberculosis*, the causative agent in majority of human tuberculosis cases, *M. africanum*, an agent of human tuberculosis in sub-Saharan Africa, *M. microti* the agent of tuberculosis in voles, *M. bovis* which infects a very wide variety of mammalian species including humans, BCG (Bacille Calmette-Guerin) an attenuated variant of *M. bovis*; and *Mycobacterium canettii* a smooth variant rarely encountered but which can cause human disease. The complex is characterized by a lack of diversity in the bulk of its genes (Sreevatsan *et al.*, 1997).

Members of the complex differ in colonial morphology. Colonies of *M. bovis* and *M. tuberculosis* are flatter and less rugose while those of BCG tend to be raised. *M. microti* forms tiny colonies whereas *M. canettii* forms smooth colonies due to overproduction of phenolic glycolipid (PGL). Unlike the other complex members, *M. microti* and *M. bovis* require pyruvate as a growth supplement. Differences in resistance to antibiotics such as pyrazinamide and thiophen-2-carboxylic hydrazide exist due to a missense mutation in the activating enzyme pyrazinamidase (Scorpio and Zhang, 1996).

Organisms of the complex share several clinically important biologic properties (Grange, 1996). All virulent members of the complex are capable of withstanding phagocytosis and replicating within macrophages and monocytes. They are obligate aerobes and grow in tissues having highest partial pressure of oxygen such as lung apices. They are facultative intracellular pathogens preferentially utilizing mononuclear phagocytes (MP) as their habitats.
The generation time of *M. tuberculosis* in synthetic medium or infected animals is typically 24 hours which contributes to the chronic nature of the disease. Lesions thus typically evolve in a subacute to chronic course.

The cell envelope of *M. tuberculosis* contains an additional layer rich in unusual lipids, glycolipids and polysaccharides beyond the peptidoglycan layer (Kolattukudy *et al.*, 1997). Novel biosynthetic pathways generate cell wall components such as mycolic acids, mycocerosic acid, phenolthiocerol, lipoarabinomannan and arabinogalactan and several of these may contribute to mycobacterial longevity, trigger inflammatory host reactions and act in pathogenesis. The unique characteristics of the Mycobacterial cell wall, confers the property of resistance to acid and alcohol wash after staining with carbol fuchsin or certain fluorochrome dyes. *M. tuberculosis* retains certain stains after being treated with acidic solution, and is thus classified as an acid-fast bacillus (AFB).

Tubercle bacilli can remain in a state of dormancy for prolonged periods known as tuberculosis infection. The state of dormancy in which the bacillus remains quiescent within infected tissue may reflect metabolic shut down resulting from the action of cell-mediated immune response that can contain, but not eradicate the infection. The molecular basis of dormancy is genetically programmed and involves intracellular signalling pathways (Grange, 1996). As immunity wanes (through ageing or through immune suppression due to general debilitation or HIV infection), the dormant bacteria reactivate, causing disease often many years after the initial infection. This is known as reactivation (or adult
type) tuberculosis, as versus primary tuberculosis which is most often seen in pediatric cases in India.

The *M. tuberculosis* genome comprises 4,411,529 base pairs (4.4Mb), contains around 4,000 genes, and has a very high guanine + cytosine content (Cole *et al*., 1998). The genes are distributed fairly evenly between the two strands and account for >91% of the potential coding capacity. Genes were classified into 11 broad functional groups and precise or putative functions could be attributed to 52% (Camus *et al*., 2002). Fifty one percent of the genes were a result of gene duplication or domain shuffling events, and 3-4 % of the genome comprised of insertion sequences (IS) and prophages (phiRv1, phiRv2). There were fifty six copies of IS elements belonging to the IS3, IS5, IS21, IS30, IS110, IS256 and ISL3 families, as well as a new IS family, IS1535, that appeared to employ a frameshift mechanism to produce its transposase (Gordon *et al*., 1999). IS6110, a member of the IS3 family, is the most abundant element and plays an important role in genome plasticity (Gordon *et al*., 1999).

*M. tuberculosis* lacks interstrain genetic diversity and nucleotide changes are very rare (Cole *et al*., 1998). Most of the proteins are identical in all cell strains and antigenic drift is restricted. Systematic sequence analysis of twenty six loci in a large number of independent isolates, revealed that the genome of *M. tuberculosis* is either unusually inert or that the organism is evolutionary young. A large portion of the coding capacity of *M. tuberculosis* is devoted to the production of enzymes involved in lipogenesis and lipolysis, and to
two new families of glycine-rich proteins with a repetitive structure that may represent a source of antigenic variation (Sreevatsan et al., 1997).

2.3. Pathology and pathogenesis

Pulmonary tuberculosis infections are initiated by the respiratory route. Different forms of the disease usually arise from dissemination of the bacilli from infected lungs. In most cases the disease follows a general pattern into four stages (Wallgren, 1948).

- In the first stage, 3 to 8 weeks after *M. tuberculosis* is inhaled, bacteria become implanted in alveoli, and are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the primary or Ghon complex.

- The second stage, lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs including other parts of the lung. Acute disease in the form of tuberculosis meningitis or miliary (disseminated) tuberculosis can occur in this stage in some individuals.

- Pleurisy or inflammation of the pleural surfaces can occur during the third stage, lasting 3 to 7 months causing severe chest pain. This stage can be delayed for up to 2 years. This is caused by hematogenous dissemination or release of bacteria into the pleural space from subpleural concentrations of bacteria in the lung. The free bacteria or their components interact with sensitized CD4 T lymphocytes that then proliferate and release inflammatory cytokines (Kamholz, 1996).
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- The last stage or resolution of the primary complex, may take up to 3 years. In this stage, the disease does not progress and more slowly developing extrapulmonary lesions, like those in bones and joints, frequently present as chronic back pain.

Most humans infected with tuberculosis do not exhibit progression of the disease. One-third of exposed individuals become infected, and 3 to 5% of these develop disease in the first year and an additional 3 to 5% develop tuberculosis later in their lives. In most non HIV-infected patients tuberculosis is caused by reactivation of preexisting infection. HIV-positive persons infected with *M. tuberculosis* have a 50% chance of developing reactivation (post primary) tuberculosis at some time in their lives. These individuals and others who are immunosuppressed can also be newly infected with *M. tuberculosis* and in many cases show rapid progression to active disease (Garay, 1996).

2.4. *M. tuberculosis* virulence and the disease process

*M. tuberculosis* enters the alveolar passages of exposed humans and comes in contact with resident macrophages. Alternatively bacteria can be ingested by alveolar epithelial type II pneumocytes in alveoli. *M. tuberculosis* can infect and grow in these pneumocytes *ex vivo* (Bermudez and Goodman, 1996). Dendritic cells play a key role in activating T cells with specific *M. tuberculosis* antigens (Bodnar *et al.*, 2001; Gonzalez-Juarrero and Orme *et al.*, 2001). The bacteria are phagocytosed by a process initiated by bacterial contact with macrophage mannose and/or complement receptors (Schlesinger, 1993).
Glycoprotein surfactant protein A, found on alveolar surfaces, enhances the binding and uptake of *M. tuberculosis* by upregulating mannose receptor activity (Gaynor *et al.*, 1995). Surfactant protein D on the other hand, located in alveolae, inhibits phagocytosis of *M. tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface (Ferguson *et al.*, 1999). Human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake (Noss *et al.*, 2001).

On entry into a host macrophage, *M. tuberculosis* and other intracellular pathogens initially reside in the phagosome. In case of normal phagosomal maturation these bacteria can encounter a hostile environment that includes acid pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides. Intracellular pathogens have evolved many ways to avoid this hostile vacuolar microenvironment (Fenton and Vermeulen, 1996). Pathogenic mycobacteria inhibit phagosome-lysosome fusion (Frehel *et al.*, 1986) the mycobacterial phagosome is not acidified (Crowle *et al.*, 1991) due to the exclusion of proton ATPases from the mycobacterial phagosome (Sturgill-Koszycki *et al.*, 1994). Live *M. tuberculosis* cells traffic to late endosomes by opsonization with polyclonal antibodies against *M. tuberculosis* which directs bacterial binding to Fc receptors. There is a decrease in the expression of major histocompatibility complex class II proteins and in the MHC-II presentation of bacterial antigens in macrophages after *M. tuberculosis* infection. This effect is induced by the presence of secreted or surface-exposed *M. tuberculosis* 19-kDa lipoprotein, which interacts with TLR2 in the early phase of bacterial entry into macrophages (Thoma-Uszynski *et al.*, 2001).
Infected macrophages attract inactivated monocytes, lymphocytes, and neutrophils which do not kill bacteria very efficiently in the lung, through production of chemokines (Fenton and Vermeulen, 1996). Granulomatous focal lesions composed of macrophage-derived giant cells and lymphocytes begin to form thus containing the spread of the bacteria. As cellular immunity develops, macrophages loaded with bacilli are killed resulting in the formation of the caseous center of the granuloma, surrounded by a cellular zone of fibroblasts, lymphocytes, and blood-derived monocytes.

*M. tuberculosis* bacilli are unable to multiply within this caseous tissue due to its acidic pH, low availability of oxygen, and the presence of toxic fatty acids. Some organisms however may remain dormant for decades. The strength of the host cellular immune response determines whether an infection is arrested or progresses to the next stages. This enclosed infection is referred to as latent or persistent tuberculosis and can persist throughout a person's life in an asymptomatic and non transmissible state.

In persons with efficient cell-mediated immunity, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial infection in the lung or if a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, aging, or other factors, the granuloma center can become liquefied and serve as a rich medium in which the revived bacteria replicate. Viable *M. tuberculosis* escapes from the granuloma and spread within the lungs and to other tissues via
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the lymphatic system and the blood. At this stage the person becomes infectious (Dannenberg and Rook, 1994).

2.5. Clinical features

Uncontrolled *M. tuberculosis* growth is associated with extensive lung damage that causes death by suffocation due to insufficient oxygen. This anoxia is caused by the obliteration of lung parenchymal cells involved in oxygen uptake as well as obstruction of bronchiolar passages by granulomatous growths and by blood released during the rupture of liquefied granulomas in adjacent lung tissue. Growth of *M. tuberculosis* elicits inflammatory host responses necessary to control infections and can also cause extensive tissue damage. In addition, *M. tuberculosis* uptake can cause apoptosis of macrophages (Keane *et al.*, 1997, Laochumroonvorapong *et al.*, 1996) resulting in adjacent tissue damage.

Uncomplicated primary disease is usually symptomless. Post primary disease causes local and generalized symptoms including coughing, production of sputum, haemoptysis and pleuritic pain. Constitutional symptoms include fatigue, anorexia, weight loss, fever and night sweats. Patients with pulmonary tuberculosis may also show other respiratory or constitutional symptoms.

2.6. Epidemiology of Tuberculosis

2.6.1. Global distribution

Tuberculosis is among the ten leading infectious causes of global mortality, and the seventh most morbidity-causing disease in the world (Murray and Lopez, 1997). It has been estimated that approximately 1/3rd of the world's
population is infected with the tubercle bacillus, and that every year eight million people develop tuberculosis.

In 2004, there were 8.9 million new cases of tuberculosis (140/1,00,000 population), of which 3.9 million (62/100,000) were smear-positive and 7,41,000 cases were in adults infected with the human immunodeficiency virus (HIV). There were 14.6 million prevalent cases (229/1,00,000) of which 6.1 million were smear-positive (95/1,00,000). An estimated 1.7 million people (27/1,00,000) died from tuberculosis in 2004, including those co-infected with HIV (2,48,000) (World Health Organization, 2006). The estimated number of new and incident tuberculosis cases occurring globally in 2004 is shown in Fig. 1 and Fig. 2.

Fig.1: Estimated number of new tuberculosis cases, 2004 (World Health Organization, 2006)
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Fig. 2: Estimated tuberculosis incidence rates, 2004 (World Health Organization, 2006)

2.6.2. Global and regional incidence

The estimated incidence, prevalence and mortality due to tuberculosis in different regions of the world are shown in Table 1. World Health Organization (WHO) estimates showed that the largest number of new tuberculosis cases in 2004, which accounted for 33% of incident cases globally, occurred in South-East Asia Region. The estimated incidence per capita in sub-Saharan Africa however was nearly twice that of the South-East Asia Region, at nearly 400 cases per 1,00,000 population. The highest number of deaths and the highest mortality per capita were in the WHO Africa region where HIV led to rapid growth of the tuberculosis epidemic and increased the likelihood of dying from tuberculosis.
Table 1: Estimated incidence, prevalence and tuberculosis mortality, 2004
(http://www.who.int/mediacentre/factsheets/)

<table>
<thead>
<tr>
<th>Region</th>
<th>Incidence</th>
<th>Prevalence</th>
<th>TB Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All forms</td>
<td>Smear-positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Number</td>
<td>Number</td>
<td>Number</td>
</tr>
<tr>
<td></td>
<td>(thousands)</td>
<td>(thousands)</td>
<td>(thousands)</td>
</tr>
<tr>
<td>Africa</td>
<td>2 573 (29)</td>
<td>1 098 (152)</td>
<td>3 741 (518)</td>
</tr>
<tr>
<td>The Americas</td>
<td>363 (4)</td>
<td>161 (18)</td>
<td>466 (53)</td>
</tr>
<tr>
<td>Eastern Mediterranean Europe</td>
<td>645 (7)</td>
<td>289 (55)</td>
<td>1 090 (206)</td>
</tr>
<tr>
<td>Europe</td>
<td>445 (5)</td>
<td>199 (23)</td>
<td>575 (65)</td>
</tr>
<tr>
<td>South-East Asia</td>
<td>2 967 (33)</td>
<td>1 327 (81)</td>
<td>4 965 (304)</td>
</tr>
<tr>
<td>Western Pacific</td>
<td>1 925 (22)</td>
<td>865 (50)</td>
<td>3 765 (216)</td>
</tr>
<tr>
<td>Global</td>
<td>8 918 (100)</td>
<td>3 939 (62)</td>
<td>14 602 (229)</td>
</tr>
</tbody>
</table>

Worldwide the incidence rate of tuberculosis grew at 0.6% per year by 2004 (Fig. 3).

Fig.3: Global trends in estimated TB incidence rates (represented in blue), and the annual change in incidence rates (in red), 1990–2004 (World Health Organization, 2006).
In 2005, incidence rates increased for most of the period since 1990 in African countries and in Eastern Europe. In African countries with high rates of HIV infection, incidence increased due to the spread of HIV, but the rate of increase fell from 14% per year to less than 3% per year by 2004. In African countries with lower rates of HIV infection, incidence increased more slowly (1–2% per year), with no signs that the increase was slowing. Incidence rate was stable or decreased in six regions (Fig. 4).

In Eastern Europe, the rate of increase reached nearly 14% annually by 1995, but halted by year 2000, and then declined. The resurgence of tuberculosis in Eastern Europe during the 1990s was associated with relatively high rates of MDR-TB among new and previously treated patients (World Health Organization, 2006). The global detection rate of new smear-positive cases increased from 11% in 1995 to 45% in 2003 (with the lowest case-detection rates in Eastern Europe and the highest rates in the Western Pacific) (Dye et al., 2005).

The total number of MDR tuberculosis cases estimated to have occurred worldwide in 2004, was 424,203 or 4.3% of all new and previously treated cases. In the same year, 1,81,408 MDR-TB cases were estimated to have occurred among previously treated tuberculosis cases alone. China, India, and the Russian Federation accounted for 62% of the estimated global burden (Zignol et al., 2006).
Fig. 4: Trends in estimated tuberculosis incidence rates (blue lines), and the annual change in incidence rates (red lines), for nine groups of countries 1990–2004 (World Health Organization, 2006)
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- **Africa - low HIV**: Algeria, Angola, Benin, Cape Verde, Comoros, Eritrea, Gambia, Ghana, Guinea, Guinea-Bissau, Liberia, Madagascar, Mali, Mauritania, Mauritius, Niger, Sao Tome & Principe, Senegal, Seychelles, Sierra Leone, Togo.

- **Central Europe**: Albania, Bosnia & Herzegovina, Croatia, Cyprus, Hungary, Poland, Serbia & Montenegro, Slovakia, Slovenia, TFYR Macedonia, Turkey.

- **Eastern Europe**: Armenia, Azerbaijan, Belarus, Bulgaria, Estonia, Georgia, Kyrgyzstan, Latvia, Lithuania, Moldova, Romania, Russian Federation, Tajikistan, Turkmenistan, Ukraine, Uzbekistan.

- **Eastern Mediterranean**: Afghanistan, Bahrain, Djibouti, Egypt, Iran, Iraq, Jordan, Kuwait, Lebanon, Libya, Jamahiriya, Morocco, Oman, Pakistan, Qatar, Saudi Arabia, Somalia, Sudan, Syrian Arab Rep, Tunisia, United Arab Emirates, West Bank & Gaza Strip, Yemen.

- **Established Market Economies**: Andorra, Australia, Austria, Belgium, Canada, Czech Rep, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Israel, Italy, Japan, Luxembourg, Malta, Monaco, Netherlands, New Zealand, Norway, Portugal, San Marino, Singapore, Spain, Sweden, Switzerland, United Kingdom, United States.

- **Latin America**: Anguilla, Antigua & Barbuda, Argentina, Bahamas, Barbados, Belize, Bermuda, Bolivia, Brazil, British Virgin Is, Cayman Is, Chile, Cuba, Dominica, Dominican Republic, Ecuador, El Salvador, Grenada, Guatemala, Guyana, Honduras, Jamaica, Mexico, Montserrat, Netherlands Antilles, Nicaragua, Panama, Paraguay, Peru, Puerto Rico, St Kitts & Nevis, St Lucia’s Vincent & the Grenadines, Suriname, Trinidad & Tobago, Turks & Caicos Is, Uruguay, US Virgin Is, Venezuela.

- **South-East Asia**: Bangladesh, Bhutan, DPR Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, Thailand, Timor-Leste.

- **Western Pacific**: American Samoa, Brunei Darussalam, Cambodia, China, China Hong Kong SAR, China Macao SAR, Cook Is, Fiji, French Polynesia, Guam, Kiribati, Lao PDR, Malaysia, Marshall Is, Micronesia, Mongolia, Nauru, New Caledonia, Niue, N Mariana Is, Palau, Papua New Guinea, Philippines, Rep Korea, Samoa, Solomon Is, Tokelau, Tonga, Vanuatu, Viet Nam, Wallis & Futuan Is.
Nine percent (7%-12%) of all new tuberculosis cases in adults (aged 15-49 years) were attributable to HIV infection. There were an estimated 1.8 million (5th-95th centiles, 1.6 - 2.2 million) deaths from tuberculosis, of which 12% (2,26,000) were attributable to HIV. The distribution of HIV amongst tuberculosis patients, showed relatively high rates in countries of eastern and southern Africa (Fig. 5). Tuberculosis was the cause of 11% of all adult AIDS deaths. The prevalence of M. tuberculosis - HIV co-infection in adults was 0.36% (Corbett et al., 2003).

Fig.5: Estimated HIV prevalence in new tuberculosis cases, 2004 (World Health Organization, 2006).

2.6.3. Epidemiology of tuberculosis in India

Tuberculosis is a major public health problem in India. Incidence of tuberculosis is 168/1,00,000, 75 of which are sputum smear positive infectious
cases of tuberculosis. Prevalence rates suggest that the extent of tuberculosis is 4 per 1000 population with one third of the cases (i.e. 1.3/1000) being smear positive (i.e. infectious) cases of tuberculosis. Prevalence of infection detected by tuberculin testing increases with age and in India it is more than 40% in adults (Chakraborty, 1998). Tuberculosis incidence and prevalence trends have not changed substantially over a period of time. For infected infants the life long risk of developing tuberculosis is 10%.

Each year, 1.8 million new cases of tuberculosis occur in India, of which about 0.8 million are highly infectious new smear-positive pulmonary cases (Dye et al., 1999). An estimated 0.5 million deaths occur from tuberculosis every year and nearly 10% of all causes of crude mortality in the community were contributed to death due to tuberculosis. Deaths due to tuberculosis exceed the combined deaths from all other communicable diseases and accounted for 26 percent of all avoidable adult deaths. The highest proportional mortality in women in the reproductive group is attributed to tuberculosis (Chakraborty, 1998). The disease is most prevalent in the age group of 20 to 50 years.

The Global report of WHO in 2006 ranks India as the world’s most heavily affected country. Every year, approximately two million people in India develop tuberculosis accounting for one fourth of the world's new tuberculosis cases (World Health Organization, 2006). India has nearly 30% of the global tuberculosis burden, and two-thirds of all cases that occur in South-east Asia. Roughly 330,000 people died with tuberculosis in 2004. Table 2 shows estimates for the year 2004.
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Table 2: Tuberculosis estimates of India (World Health Organization, 2006)

<table>
<thead>
<tr>
<th>Metric</th>
<th>Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence (all cases/100 000 pop/yr)</td>
<td>168</td>
</tr>
<tr>
<td>Incidence (ss+/100 000 pop/yr)</td>
<td>75</td>
</tr>
<tr>
<td>Prevalence (all cases/100 000 pop)</td>
<td>312</td>
</tr>
<tr>
<td>Mortality (deaths/100 000 pop/yr)</td>
<td>30</td>
</tr>
<tr>
<td>Prevalence of HIV in adult TB patients (15–49 yrs, %)</td>
<td>5.2</td>
</tr>
<tr>
<td>New tuberculosis cases multidrug-resistant (%)</td>
<td>2.4</td>
</tr>
<tr>
<td>Previously treated TB cases multidrug-resistant (%)</td>
<td>15</td>
</tr>
</tbody>
</table>

To assess the prevailing epidemiological situation of tuberculosis a nation-wide tuberculin survey was conducted during 2000-03. The objective of the survey was to estimate the prevalence of infection among children one to nine years of age and to compute average annual risk of tuberculous infection (ARTI) in different parts of the country. ARTI is defined as the probability of acquiring new infection during the course of one year and is computed from the estimated prevalence of infection among younger children. It emulates the overall impact of various factors influencing the transmission of tubercle bacilli such as prevalence of infectious cases in the community and efficiency of case finding and treatment activities. It is also the first epidemiological parameter to be affected following a change in the tuberculosis situation in the community.

Results indicated that the ARTI was 1.5% ± 0.3%. Tuberculosis situation in most parts of India was found to be grave with an average of about 1,100-1,900 persons out of every 1,00,000 acquiring new tuberculous infection each year and potentially at risk of breaking down into disease any time in the future. The situation was worse in urban areas as compared to rural areas. The
ARTI estimates obtained implied that the incidence of fresh cases would continue to be high and a drastic reduction in the disease cannot be expected within a short time (Chadha et al., 2005).

![Graph of Annual risk of tuberculous infection (ARTI) in India by zone and stratum](http://www.tbcindia.org)

Studies were undertaken in different regions in India by Tuberculosis Research Centre (TRC) during 1997-2000 to determine MDR levels. Study revealed acquired MDR-TB resistance levels of 25-100% (Paramasivan, 2003). The 2004 WHO Global TB Report estimates that 4.6 percent of TB patients have HIV infection. In India, there were an estimated 5.134 million people living with HIV AIDS (PLWHA) by the end of 2004. Estimates also indicated that 5.2% of adults in the age group of 15-49 years were HIV positive and 2.4% were new multidrug resistant cases.

Approximately 4 million persons in India (<1% of the population) were found to be infected with HIV, of which approximately half also were infected with *M. tuberculosis* (World Health Organization, 2004). An additional 1,40,000 tuberculosis cases are estimated annually amongst tuberculin skin test-
positive HIV-infected persons (Swaminathan et al., 2000). The incidence of tuberculosis is 1.8 million per year.

With HIV, incidence levels could go up to two million or more per year, assuming HIV rates remain around one percent and the incidence of tuberculosis remains at 1990 levels (Potts and Walsh, 2003). More recently it was found that the predicted ratio of the prevalence of HIV in TB patients to that in the general population was 8.8 in 2000. This was in agreement with data from Pune, where the prevalence of HIV in women attending ANCs between 1999 and 2001 was 3.1% and in tuberculosis patients was 26%, giving a ratio of 8.3 (National AIDS Control Organization, 2004). The estimated prevalence for 2002 was 0.64 ± 0.05%, slightly lower than the National AIDS Control Organization estimate for that year of 0.79 ± 0.04% (National AIDS Control Organization, 2004).

The cumulative number of tuberculosis cases between 1990 and 2015 is expected to fall by 5.0 (3.4–6.1) million, but the cumulative number of deaths is not expected to change significantly (1.5 million fall to 0.5 million rise) due to the effect of HIV. In many countries of East and Southern Africa, tuberculosis notification rates have increased by five or more times as a result of the HIV epidemic (Corbett et al., 2003; World Health Organization 2006).

If this trend were to repeat in India, which accounts for 20% of the global burden of tuberculosis, the total number of cases in the world would double (World Health Organization, 2006). At present, <1% of Indian adults are infected with HIV, that is about five million people (National AIDS Control Organization, 2004). It has been suggested that the prevalence of HIV infection in Indian adults
could reach 5%, or ≈25 million people (Potts and Walsh, 2003; Nagelkerke et al., 2002).

2.7. History of tuberculosis control in India

Tuberculosis control in India began with the establishment of hospitals and sanatoria. The first concerted effort towards tuberculosis control was the organization of the King George V thanksgiving fund in 1929 which was used for preventive and educational activities, establishment of clinics, training of health visitors and preparation of health education material.

The Tuberculosis Association of India was established in 1939, with the objective of providing expert advice on the development of standard methods to deal with the disease, setting up model institutions for training tuberculosis workers, education of the public regarding preventive measures and for organizing meetings and conferences for scientific discussions (Tuberculosis Association of India, 1939). The Association conceived the idea of domiciliary treatment as early as 1940 (Tuberculosis Chemotherapy Centre, 1959). Research was taken up in collaboration with the Indian Research Fund Association, now known as the Indian Council of Medical Research (ICMR).

2.7.1. Government Initiatives

In 1946, the Health Survey and Development Committee, headed by Sir Joseph Bhore, outlined a conventional phased scheme for the management of tuberculosis (Ministry of Health, New Delhi, 1946). The Committee recommended a regular programme with measures like setting up tuberculosis clinics in the districts and mobile clinics in rural areas. In 1947, the Central
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Government established a Tuberculosis Division in the Directorate General of Health Services of the Ministry of Health.

The first nation-wide campaign against tuberculosis, the BCG campaign was introduced on a small scale in Madanapalli in 1948 and extended on a mass scale in 1954 (Baily, 1962). From 1955 to 1958 ICMR conducted a large-scale sample survey in six zones of the country, covering both urban and rural populations, to obtain precise information on the magnitude of tuberculosis in the country (Indian Council of Medical Research, 1959).

Effective drugs against tuberculosis were available around the same time (Streptomycin 1944, PAS 1946, Thiacetazone 1950, Isoniazid 1952 and Rifampicin 1966). In 1956, the Tuberculosis Chemotherapy Centre, now known as the Tuberculosis Research Centre (TRC), was established in Chennai. TRC demonstrated that sanatoria-based measures, were unimportant provided adequate chemotherapy was fully taken. The discovery of specific, potent, and readily available anti-tuberculosis drugs and the efficacy of domiciliary treatment was shown by New Delhi TB Centre and TCC Madras (Tuberculosis Chemotherapy Centre, 1959).

In 1959, the National Tuberculosis Institute (NTI) was established in Bangalore by GoI, with the active cooperation of the WHO, to develop a TB control programme. The NTI conducted operational research studies to enunciate suitable methods for the large-scale application of TB control measures. NTP was pilot-tested in Ananthpur district of Andhra Pradesh in 1961, and thereafter launched in a phased manner throughout the country.
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In 1968, a clinical trial to test the efficacy of BCG vaccination in Chingleput district of Tamil Nadu was carried out which revealed that BCG did not offer protection against pulmonary tuberculosis. However, it was continued in children as a part of the Expanded Programme of Immunization (EPI) to protect against the serious childhood forms of tuberculosis (Baily, 1980).

By 1978, conventional treatment regimens of 12-18 months were developed through chemotherapy trials, and used in the programme. Later with the availability of Rifampicin, the reintroduction of Pyrazinamide and the success of the six-month short-course chemotherapy (SCC) regimens in clinical trials, it was possible to reduce the treatment duration from twelve months to six months (World Health Organization, 1997).

In 1992, Government of India (Goi), with WHO reviewed the TB situation and the performance of the NTP (National Tuberculosis Institute, 1993). The observations revealed that no appreciable change in the epidemiological situation was seen and that the NTP, though technically sound, suffered from managerial weaknesses, inadequate funding, over-reliance on X-ray for diagnosis, had frequent interrupted supplies of drugs, and low rates of treatment completion.

2.8. Revised National Tuberculosis Control Programme (RNTCP)

In 1993, the Government decided to revitalise the NTP, with assistance from international agencies (World Health Organization, 1997). The RNTCP was pilot-tested in 1993, and further expanded in a phased manner covering the entire country by the end of 2006. The objectives of the RNTCP were to detect 70 per cent of sputum smear positive patients arising each year, and
to cure 85 per cent of these patients. The key elements of RNTCP include case detection through high-quality bacteriology, standardized treatment with supervision and patient support, effective drug supply and management system, and a comprehensive system for monitoring and evaluation.

In India, RNTCP is integrated with the general healthcare delivery systems in the states. The State TB Cell (STC) is responsible for the supervision and monitoring of the programme throughout the state. The District TB Centre (DTC) is the key organisational unit responsible for the implementation of the programme in the respective districts supported by sub-district TB Units (TUs). The TU is the lowest reporting unit under the RNTCP. To further decentralise the diagnostic and treatment services, RNTCP Designated Microscopy Centres (DMCs) and a vast network of DOT centers (treatment centers), have been established so that patients can have easy access to treatment.

RNTCP is characterized by its surveillance and information system. Data is compiled from all participating districts through e-mail, results are compiled quarterly and made publicly available on the website www.tbcindia.org. There is involvement of other sectors like non-government organizations, private practitioners, medical colleges and collaborations with the Ministry of Health and Family Welfare, Ministry of Labour, Railways, Mines and Steel in the RNTCP (Central TB Division, 2003).

In addition, coordination between the RNTCP and the National AIDS Control Programme has started in the six high HIV/AIDS prevalence states. Referral linkages between the Voluntary Counseling and Testing Centers
(VCTCs) and RNTCP diagnostic sites are being established, and reporting of HIV-TB cases at the level of VCTCs is an on-going activity.

2.8.1. Case finding under RNTCP

Findings of various studies have been instrumental for formulating the current diagnostic paradigm of the RNTCP. Studies conducted in the 1970s by NTI, Bangalore, demonstrated that nearly 70 percent of the cases diagnosed and put on treatment on the basis of X-ray alone did not have tuberculosis (Gothi et al., 1974). An international study on X-ray classification demonstrated high levels of disagreement amongst experts on the interpretation of chest radiographs (Nyboe, 1968).

Other studies showed that nearly half of the cases in the community reported at the nearest general health institutions on their own in search of treatment (Banerjee and Andersen, 1963). Furthermore, NTI also demonstrated the ability of the laboratory technicians to perform sputum smear microscopy effectively with minimal training and regular supervision (Rao et al., 1971; Nagpaul et al., 1968).

Sputum smear examination with a sensitivity of 82% and specificity of 99% was found to be a good confirmatory tool. Based on these studies, sputum microscopy for self-reporting chest symptomatics is the revised strategy made applicable throughout the country as a case-finding policy under the National Tuberculosis Programme (World Health Organization, 1997).

The various screening tools available for screening chest symptomatics were chest symptom inquiry, X-ray and tuberculin test. Symptom
inquiry is a useful screening tool although it has low sensitivity and specificity. X-ray examination having sensitivity of 92% and specificity of 88% is a good screening tool. Tuberculin test has little role as a screening tool though highly sensitive (95%) as about half of the population is infected with *M. tuberculosis*. Studies also indicated that to increase the efficiency of identifying cases, a combination of tests should be used, so that more cases could be identified (Chakraborty and Gothi, 1979; Balasangameshwara and Chakraborty, 1993).

### 2.8.2. Diagnosis paradigm of RNTCP

Based on these and other findings all patients with chest symptoms (i.e., three weeks of cough) or other symptoms suggestive of tuberculosis are advised to undergo three sputum examinations for acid-fast bacilli. Patients with two or three positive smear results are diagnosed as having sputum smear-positive pulmonary TB and are started on anti tuberculosis treatment (ATT). Those with only one positive smear examination, are advised to get a chest X-ray done and, if found to be compatible with TB, are also treated as sputum smear-positive pulmonary TB cases. Patients, in whom all three samples are negative, are prescribed broad-spectrum antibiotics such as co-trimoxazole, for 10-14 days. Those who improve with antibiotics are diagnosed not to have tuberculosis. However if the symptoms persist, the patient is re-evaluated by repeat sputum examination and X-ray. Thereafter, if in the opinion of the treating physician, the patient is suffering from tuberculosis, treatment is initiated.
2.0 Review of Literature

Cough for three weeks or more

3 Sputum smears

2-3 positive

3 negatives

Antibiotics for 10-14 days

Cough persists

Repeat sputum examination

1 positive

X-ray

Suggestive TB

Negative TB

Sputum smear positive tuberculosis. ATT started

Negative TB

Non Tuberculosis

X-ray

2-3 positive

Sputum positive tuberculosis. ATT started

Negative TB

Sputum smear negative tuberculosis. ATT started.

Fig. 7: Diagnostic algorithm for diagnosis of pulmonary tuberculosis under RNTCP

(www.tbcindia.org)
2.8.3. Treatment under RNTCP

India has contributed to a significant degree to pioneering research for the treatment of tuberculosis. The necessity and feasibility of treatment supervision in the community now called Directly Observed Treatment, and the efficacy of intermittent chemotherapy for TB were demonstrated in studies conducted at TRC, Chennai (Fox, 1961, Tuberculosis Chemotherapy Centre, 1964). These scientific findings formed the basis for the decision to adopt intermittent, short course chemotherapy regimens under direct observation as the treatment norm under the RNTCP.

In 1944, streptomycin, was isolated from *Streptomyces griseus* and used in humans (Schatz et al., 1944). In 1949 it was discovered that paraaminosalicylic acid (PAS) in combination with streptomycin prevents the emergence of drug resistance. In 1952 isoniazid was found to be inexpensive, effective and of low toxicity. These and other findings opened new prospects for nationwide treatment programs (Santha et al., 1989).

The drugs usually contained in a standard regimen are isoniazid with or without streptomycin accompanied by PAS or thioacetazone. The short course regimen was introduced in 1972 enabling the conventional duration of treatment to be approximately halved without lowering the therapeutic effect.

Tuberculosis treatment regimens have an initial intensive phase of approximately two months and a continuation phase of approximately four months. During the initial phase, there is rapid killing of bacilli. Infectious patients become non infectious within two weeks and symptoms improve. In the
continuation phase (4-6 weeks) fewer drugs are necessary, but they are to be taken for a longer time. The drugs eliminate the remaining bacilli thus preventing relapse after completion of treatment (Tomam, 1979).

**Table 3: Essential anti tuberculosis drugs and their mode of action**
(Tomam, 1979)

<table>
<thead>
<tr>
<th>Essential drug</th>
<th>Mode of action</th>
<th>Potency</th>
<th>Recommended Dose (Mg/ Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Daily</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>bactericidal</td>
<td>high</td>
<td>5</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>bactericidal</td>
<td>high</td>
<td>10</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>bactericidal</td>
<td>low</td>
<td>25</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>bactericidal</td>
<td>low</td>
<td>15</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>bacteriostatic</td>
<td>low</td>
<td>15</td>
</tr>
<tr>
<td>Thiacetzone</td>
<td>bacteriostatic</td>
<td>low</td>
<td>3</td>
</tr>
</tbody>
</table>

The mode of action of the various bactericidal drugs are as follows: Isoniazid kills 90% of the total population of the bacilli during the first few days of treatment. It is most effective on metabolically active continuously growing bacilli. Rifampicin can kill the semi dormant bacilli which isoniazid cannot. Rifampicin acts as a sterilizing drug which kills all the bacilli and prevents relapse. Pyrazinamide kills bacilli in an acid environment inside the macrophages. Its effectiveness thus makes short course chemotherapy possible (Tomam, 1979).
2.9. Diagnostics for tuberculosis

2.9.1. Routinely used diagnostics and their limitations

i) Microscopy

The microscopic detection of acid-fast rods (AFB) in stained smears of clinical specimens from suspected cases, serves as a presumptive diagnosis of tuberculosis (Allen and Hinks, 1982). Microscopy has the advantage that it is relatively inexpensive, rapid, and can be done without major infrastructural inputs (Murray et al., 1990; Rieder et al., 1998). Microscopy for AFB is currently the most cost-effective method of diagnosis of pulmonary tuberculosis in high prevalence developing countries. Another advantage is that it is also be used to assess response to treatment and to establish cure or failure at the end of treatment (World Health Organization, 1998.)

The major limitation of microscopy however is its lack of sensitivity. Between 5,000 and 10,000 tubercle bacilli per milliliter of sputum are required for direct microscopy to be positive (Tomam, 2004). Although it has been estimated that, under optimal laboratory conditions, a positive smear can be obtained with only 100–1,000 organisms per ml, a more practical estimate is about 10,000 organisms (Wolinsky, 1994). A single smear examination of sputum has a reported sensitivity of only 22–43% and 50–70% when 2–3 smears are examined over 2 days. The incremental yield of acid-fast bacilli from serial smear examinations has been shown to be 80-83% from the first, 10-14% from the second and 5-8% from the third specimen (Baily et al., 1967).
2.0 Review of Literature

Sputum smear microscopy has been shown to have a sensitivity of 96.5%, specificity of 34.9%, positive predictive value of 17.3% and a negative predictive value of 99.9% in Grzybowski’s series (Grzybowski et al., 1975). In patients with culture confirmed pulmonary tuberculosis the sensitivity of AFB microscopy ranges from 22% to 80% (Kim et al., 1984).

Concentration of the bacilli by use of cytocentrifuge increases the sensitivity of microscopy to that of culture in solid media (Fodor, 1995). Other methods like addition of sodium hypochlorite followed by centrifugation too increases sensitivity though this method, also known as the “bleach” method is not used routinely in many settings (Angeby et al., 2004).

The use of auramine as a fluorescent method to detect mycobacteria in sputum was proposed many years ago and re-evaluated later using a combination of auramine-O and rhodamine (Ba and Rieder, 1999). It is associated with a higher rate of detection, since slides can be examined at lower magnifications. Studies have shown that it performs better than ZN staining or its Kinyoun modification (Somosko’vi A’ et al., 2001). The requirement of a fluorescent microscope however is an expensive limitation to the usage of this test (Nolte and Metchcock, 1995; Laszlo, 1999).

Quality assurance studies in eight state tuberculosis laboratories in India showed that consistency of positive microscopy results ranged from 38% to 100% and false positivity rate varied from 2% to 7% (Paramasivan et al., 2003). Occasionally, a sputum specimen contains food particles, waxes, oils, precipitates, other microorganisms, inorganic materials, and artifacts that are acid-fast and
resemble tubercle bacilli and cause false positive results (World Health Organization, 1998; Van Deun et al., 1999).

False negative results may be obtained due to inadequate quality of sputum, inadequate staining, under or over decolourisation or inspection of too few fields. Overly thick smears can obscure the presence of AFB, or AFB may fall off the slide (Van Deun et al., 1999). Microscopy cannot distinguish between different mycobacterial species. In disease endemic countries however microscopic examination of stained sputum smear remains the primary laboratory supporting tool in case detection as it is inexpensive, is very specific in high prevalence settings, and detects the most infectious subset of patients.

Fig.8a,b: Mycobacteria stained after acid fast staining

**ii) Culture**

Isolation of mycobacteria from clinical samples by culture confirms diagnosis of tuberculosis by establishing the viability and identity of the bacilli (World Health Organization, 1998). Culture methods are more sensitive than microscopy for detection of bacilli. Paucibacillary disease can be confirmed
2.0 Review of Literature

by mycobacterial culture which requires approximately 100 mycobacteria per ml
for a positive result (Tomam, 2004).

Culture increases the number of tuberculosis cases often by 30-50%
and detects cases often before they become infectious. The gain by culture over
microscopy is estimated to be about 25% in high-prevalence countries, with or
without HIV (Mitchison, 1982). It also provides necessary material to distinguish
between different mycobacterial species as well to perform drug susceptibility
testing (World Health Organization, 1998).

The primary drawback of culture is that the mean incubation time
required for the mycobacteria is very long (4-8 weeks). Drug susceptibility tests
require an additional 4 weeks. This limits its use as a first line diagnostic.
Furthermore the only media that allow abundant growth of *M. tuberculosis* are
egg-enriched media with glycerol and asparagine (viz., Lowenstein-Jensen) or
agar based media supplemented with bovine albumin (viz., 7H10 or 7H11)
(Jensen, 1995).

*Fig.9: M. tuberculosis colonies cultured on Löwenstein Jensen medium*
(http://www.ann-clinmicrob.com/)
iii) Tuberculin

Tuberculin test is a skin test used for the diagnosis of tuberculosis, especially in children. It is also useful for epidemiological investigations of infection. Tuberculin is composed of the secretory proteins of the tubercle bacillus. The immunological reaction is seen as an induration, which can be measured, and the response expressed quantitatively. The Mantoux tuberculin skin test is the preferred type of skin test because it is the most accurate.

The tuberculin test is practically the only tool currently available for the diagnosis of tuberculosis infection. It does not distinguish latent infection from infection associated with active disease and a positive result only marginally increases the probability that the patient has tuberculosis. The test therefore has little role in the diagnosis of tuberculosis disease in adults.

The tuberculin test is falsely negative in 10 – 47% of patients with active disease (Al Zahrani et al., 2000; Rooney et al., 1976; Holden 1971). In the presence of co-infection with HIV, a far higher proportion of patients with active disease will have a false-negative test result. A false-negative tuberculin test will be seen in 30% of patients with a CD4 T-lymphocyte cell count of >500/ml, compared with close to 100% of patients with a CD4 T-lymphocyte cell count of <200/ml (Graham et al., 1992, Markowitz et al., 1993).

It may be positive in individuals who have had prior BCG vaccination (Menzies and Vissandjee, 1992). In persons vaccinated at an older age, such as in primary school, 15–25% will remain positive for as long as 20–25 years (Horwitz O and Bunch-Christensen, 1972; Menzies and Vissandjee, 1992).
A false-positive test also commonly results from cross-reacting sensitivity to non-tuberculous mycobacterial antigens (Palmer et al., 1959) common in tropical and subtropical climates (Edwards et al., 1969).

iv) Radiography

The classical radiographic hallmarks of pulmonary tuberculosis are cavitation, apical distribution, bilateral distribution, pulmonary fibrosis, shrinkage and calcification (Lobue et al., 2000). No pattern however is absolutely diagnostic of tuberculosis. Interpretation of chest X-rays is subject to considerable inter and intra observer variation by radiologists and chest physicians and depends on clinical experience. Considerable subjectivity exists in the identification and in the interpretation of the radiographic abnormalities.

Studies have shown that only 20% of the patients identified by X-ray were confirmed by sputum examination (Harries et al., 1997). Patients with HIV infection may have normal chest X-rays despite active tuberculosis or atypical radiographic findings such as infiltrates without cavitation and hilar lymphadenopathy. The radiographic presentation is related to the CD4 lymphocyte count (Kelper et al., 1995; Post et al., 1995).

The WHO-led diagnostic initiative is centered on enhancing the sensitivity of AFB microscopy, or finding an alternative tool equivalent to AFB microscopy (Foulds and Brien, 1998). Current methods for the diagnosis of both smear-positive and smear-negative disease are often non-specific and insensitive, leading to both under and over-diagnosis. The incidence of smear-negative tuberculosis has increased due to the human immunodeficiency virus (HIV)
which presents an expanding diagnostic challenge (Colebunders and Bastian, 2000). Due to lack of a sensitive diagnostic for smear negative tuberculosis, diagnostic algorithms are used for diagnosis of smear negative tuberculosis (Fig. 7). The consequences of using an indirect method of diagnosis are manifold.

**i) Delay in diagnosis and economic consequences**

Due to the lack of a direct diagnostic, there is a delay of greater than ten days for the diagnosis of smear negative cases. The symptomatic is subjected to a battery of clinical tests, broad spectrum antibiotics and radiological examination before being diagnosed as a case of tuberculosis. Studies conducted, at Tuberculosis Research Centre, Chennai, found that a patient suffering from tuberculosis incurs an average total loss Rs.3,469 for diagnosis and treatment (Rajeshwari et al., 1999). Another study found that Indian workers with tuberculosis lost an average of 83 work days because of the disease, 48 of which were lost for want of correct diagnosis. Considering that two million cases are reported annually in India, the national loss per year works out to be 166 million lost work days, at a cost of Rs.694 crore. In addition, the debts incurred by patients because of tuberculosis amounted to Rs.416 crore (Dholakia, 1996).

**ii) Transmission of disease by smear negative cases**

Diagnostic delay is a significant issue in the management of tuberculosis patients, particularly those with smear negative disease. It may lead to deterioration of the individual patient's health and further spread of disease to contacts. Delayed case finding poses a problem in areas where the prevalence of HIV/AIDS is very high. In HIV positive patients, diagnostic delay may have more
serious consequences, as infection may lead to an irreversible increase in viral load, with subsequent progression of HIV/acquired immune-deficiency syndrome (Hudson et al., 2000; Lawn and Griffin, 2001). In this time period the patient is infectious and spreads the disease.

The infecting dose is estimated to be fewer than ten organisms (Riley et al., 1957; McPhedran and Opie, 1935). Recent studies using restriction fragment length polymorphism (RFLP) analysis indicated that the rate of transmission of smear negative culture positive tuberculosis was around 17% (Behr et al., 1999). The number of infections generated by smear negative pulmonary tuberculosis case was found to be 10 –20% of that generated by a smear positive case (Loudon, 1958). Another study done in Vancouver estimated that the proportion of episodes of transmission from smear negative patients ranged from 17% to 22% (Hernández-Garduño et al., 2004).

A substantial percentage of tuberculosis cases in poor countries are smear negative (Parry, 1993). Delayed diagnosis has a harmful effect on individual patients, who develop more advanced disease with permanent sequel, such as loss of pulmonary function before treatment can be initiated. In both resource-poor and resource-rich countries, clinical algorithms (based mainly on patient characteristics and radiographic findings) can raise the pretest likelihood for tuberculosis to 50% or more and clinical features alone cannot provide a sound basis for diagnosis of tuberculosis on a widespread basis (Wilkinson et al., 1997; O'Brien and Talbot, 2003).
iii) Differential diagnosis

Non-tuberculous chest symptomatics cannot be easily distinguished from a smear negative case of tuberculosis. In order to diagnose cases, a large number of non tuberculous cases have to be screened. Response to a trial of broad spectrum antibiotics is used in the diagnostic algorithm. Failure to respond to a trial of antibiotics, may be due to infection with a resistant organism. On the other hand, failure of a non tuberculosis patient to respond to a trial of antibiotics could be due to other superimposing infections or multiple superimposing infections especially in the case of HIV infected people (Oyewo et al., 1999).

In a study done by Wilkinson et al the diagnostic sensitivity of the algorithm was found to be 80%, and specificity was 78% (Wilkinson et al., 1997) A lot of other diseases mimic smear negative tuberculosis (Table 4). Studies have shown that the diagnostic yield of different broad-spectrum antibiotics is different in different communities and should be systematically assessed in different communities (Parry, 1993). A symptomatic response to antibiotics does not exclude the diagnosis of tuberculosis, as up to 50% of patients with smear-negative tuberculosis have been reported to respond to broad spectrum antibiotics (O'Brien and Talbot, 2003).


Table 4: Important conditions affecting differential diagnosis of smear negative pulmonary tuberculosis in developing countries
(Colebunders and Bastion, 2000)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Frequency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial pneumonia</td>
<td>14 - 41</td>
<td>Daley * et al., 1996</td>
</tr>
<tr>
<td>Emphysema</td>
<td>2</td>
<td>Daley * et al., 1996</td>
</tr>
<tr>
<td>Pulmonary nocardiosis *</td>
<td>0-4</td>
<td>Lucas * et al., 1993; Batungwanayo et al., 1994</td>
</tr>
<tr>
<td>Pneumocystis carinii pneumonia *</td>
<td>1-33</td>
<td>Abouya et al., 1992, Kamanfu G et al., 1993</td>
</tr>
<tr>
<td>Cryptococcal pneumonia*</td>
<td>0-13</td>
<td>Abouya et al., 1992, Kamanfu G et al., 1993</td>
</tr>
<tr>
<td>Histoplasmosis</td>
<td>3</td>
<td>Lucas et al., 1993</td>
</tr>
<tr>
<td>Pulmonary Kaposis sarcoma *</td>
<td>1-9</td>
<td>Abouya et al., 1992, Kamanfu G et al., 1993</td>
</tr>
<tr>
<td>Interstitial pneumonitis</td>
<td>38</td>
<td>Greenberg et al., 1995</td>
</tr>
<tr>
<td>Cytomegalovirus pneumonitis</td>
<td>1.5</td>
<td>Malin et al., 1995</td>
</tr>
<tr>
<td>Gram negative bacteraemia *</td>
<td>9-10</td>
<td>Grant et al., 1998</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>1</td>
<td>Grant et al., 1998</td>
</tr>
</tbody>
</table>

* Conditions of importance in HIV positive individuals.

Chest radiography has been included in the algorithm for selecting patients with smear negative pulmonary tuberculosis. X-ray fails to diagnose 10%–15% of culture-positive patients, and diagnoses nearly 40% of patients as having tuberculosis who had negative cultures and likely not to have active tuberculosis (Garland, 1959). Reviews of diagnostic accuracy of X-rays showed that readings were discordant for 30% of X-rays read by different experts, and 21% of experts reading the same film at two separate times. Consistency in reading by highly experienced radiologists was only slightly better (Jones *et al.*, 1996).
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1997). Radiography, whilst widely used at one time, has limited use since no chest X-ray pattern is absolutely typical of active pulmonary tuberculosis, rendering radiography a non-specific tool. The experience of many decades of detailed data collection and analysis indicate that chest radiography for diagnosis or follow-up of pulmonary tuberculosis cases, with or without HIV co-infection, is unreliable (Harries et al., 1997).

Diagnosis is especially difficult in case of tuberculosis HIV co-infection. As HIV related immunosuppression increases, the clinical pattern of TB changes. As the level of immunocompromise increases with advancing HIV disease atypical pulmonary features predominate due to other infections and smear examinations are of no use (Alpert et al., 1997). Overall population trends and most clinical based studies suggest that HIV positive patients have a high rate of smear negative disease. The presentations of pulmonary tuberculosis in the early and late stages of tuberculosis are shown in Table 5.

Table 5: Presentation of pulmonary tuberculosis in early and late stage of HIV infection (www.tbcindia.org)

<table>
<thead>
<tr>
<th>Features of pulmonary tuberculosis</th>
<th>Stage of HIV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early</td>
</tr>
<tr>
<td>Clinical presentation</td>
<td>Often resembles post</td>
</tr>
<tr>
<td></td>
<td>primary tuberculosis</td>
</tr>
<tr>
<td>Sputum smear result</td>
<td>Often positive</td>
</tr>
<tr>
<td>Chest X-ray appearance</td>
<td>Often cavities</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Health services thus face problems like under diagnosis of sputum smear positive TB and over-diagnosis of sputum smear-negative PTB in populations where HIV/TB is common. TB shortens the survival of patients with
HIV infection and accelerates the progression of HIV. There is a six to seven fold increase in the HIV viral load in TB patients. Worldwide TB is the leading cause of death in People living with HIV-AIDS (PLWA) and late diagnosis contributes to increased death rates in PLWHA (Horsburgh and Pozniak, 1993; Braun et al., 1993; Perneger et al., 1995; Ackah et al., 1995).

Though TB programmes in the high burden countries have expanded geographically, the fraction of the estimated number of sputum smear-positive cases have remained constant at 40-50%. Unless case detection systems can be improved, case detection will not rise much above 40% in the world even when the geographical coverage of DOTS is nominally 100%. It was estimated that inspite of full DOTS coverage, three-quarters of undetected smear positive cases will be living in India, China, Indonesia, Nigeria, Bangladesh and Pakistan. Substantial efforts are therefore needed to develop new case finding methods (Dye et al., 2003).

Enhanced diagnostic techniques like rapid molecular testing are projected to reduce TB prevalence and mortality by 20% or more. The impact of TB diagnostics will be sensitive to the quality of existing diagnostic standards and the level of access to diagnostic services, but is robust across a wide range of population parameters including HIV and TB incidence. Enhanced TB diagnostic techniques may have substantial impact on TB morbidity and mortality in HIV-endemic regions. As tuberculosis rates continue to increase enhanced diagnostic techniques merit further consideration as TB control strategies (Dowdy et al., 2006).
2.10. Newer diagnostics

WHO initiated the Tuberculosis Diagnostic Initiative in 1997, to develop new tools to diagnose tuberculosis (World Health Organization, 1997). It is now working in collaboration with the Foundation for Innovative Diagnostics (FIND), a Bill and Melinda Gates Foundation funded initiative for the development, evaluation and demonstration of new diagnostic methods (www.finddiagnostics.org).

The two main approaches to diagnose tuberculosis include detection of Mycobacteria or its products and the indirect approach of measurement of humoral and cellular responses of the host against tuberculosis. The priority of disease control in disease non endemic countries is identification of latent infection in risk groups, early diagnosis, detecting outbreaks and identification of patients with NTM. In endemic countries however the priority lies with labour intensive easy methodology, and use of minimum infrastructure and equipments. Focus is on improved microscopy, use of liquid culture for extrapulmonary and childhood disease, and detection of paucibacillary condition by NAA assays, phage assays and antigen capture.

Culture is currently the only widely available technology that allows for drug susceptibility testing and serves as a confirmatory diagnosis for viable bacilli. Broth based culture systems such as BACTEC, MGIT (a non radiometric method), MB/BacT, Septi-Check, and ESP, when combined with DNA probes for rapid species identification, give positive results in two weeks or less for the vast majority of sputum smear-positive specimens, and within three
weeks for smear-negative specimens (Sharp et al., 2000; Sharp et al., 1997; Kanchana et al., 2000).

Phenotypic and genotypic methods are available for detection with modifications for diagnosis of tuberculosis. Phenotypic methods include techniques like Luciferase reporter gene assay where the sample is placed into medium and then transfected with a lucerifase containing mycobacterial phage. Viable M. tuberculosis present in the sample take up the phage and the luciferase gene functions producing visible light when luciferin is added to the assay. Drug susceptibility testing can be obtained by inoculating the clinical sample into antibiotic containing medium (Jacob et al., 1993). Various studies have been carried out to describe the use of this technology (Mc Nerney 2001; Albert et al., 2002; Muzaffar 2002; Takiff and Heifets, 2002). This approach is technologically simple and inexpensive and has potential to be used in countries that have limited financial and personnel resources.

An assay for tuberculosis that employs the technology of molecular beacons has been described (El-Hajj et al., 2001; Varma-Basil et al., 2004). Molecular beacons are molecules that emit light when a chemical reaction occurs (Leone et al., 1998). This reaction will occur only when primers with DNA specificity bind their appropriate target region in PCR amplicons. In this way, rapid and sensitive diagnosis can be established. Recent studies demonstrated both the sensitivity and specificity of this assay not only in making a diagnosis of tuberculosis, but also in rapidly identifying mutations associated with antibiotic
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resistance (Piatek et al., 1998; Piatek et al., 2000). However, molecular beacon assay requires expensive equipment that is not yet widely available.

Serological tests are useful as screening tests but have low sensitivity in smear negative HIV infected patients. They are expensive require training and are not useful to distinguish between \textit{M. tuberculosis} and NTM. Antigen detection assay like capture antigen ELISA test based on capture antibody from a murine source (murine monoclonal antibody against lipoarabinomannan) have been described. Rabbit antiserum against \textit{M. tuberculosis} is used to detect antibody (Del Prete et al., 1998).

Tests for Latent tuberculosis infection like assays of interferon production by peripheral blood mononuclear cells are available. Both CD4 and CD8 T lymphocytes, are capable of producing the proinflammatory cytokine interferon in response to stimulation with \textit{M. tuberculosis} (Schluger and Rom, 1998). Peripheral blood mononuclear cells (PBMCs) separated from blood samples drawn from patients with known infection with \textit{M. tuberculosis} can be simulated in vitro with purified protein derivative (PPD) and production of interferon by PBMCs can then be measured easily by enzyme-linked immunosorbent assay (ELISA). The few published studies of this assay indicate that this may be an accurate method of detection of latent tuberculosis infection (Pottumarthy et al., 1999; Fietta et al., 2003; Taggart et al., 2004)

Compared to tuberculin skin testing, it does not require a return visit by the patient for interpretation of test results, although it is more expensive and technically complex. Studies of this approach to the diagnosis of tuberculosis
have been limited by the lack of a gold standard for the detection of latent tuberculosis infection. The initial experience with the tuberculin-stimulated interferon-assay was in patients with possible or probable latent tuberculosis infection and a sensitivity of 90% and a specificity of 98% was reported (Streeton et al., 1998). A trial of this assay has been reported, suggesting that this assay may be able to discriminate between true infection and BCG vaccination.

In addition, the assay displayed some ability to distinguish between infection with *M. tuberculosis* and NTM. Increased specificity for IFN release assays may also be provided by stimulating cells with early secreted antigen-6 (ESAT-6), a mycobacterial product that distinguishes *M. tuberculosis* from other mycobacteria, including BCG (Lalvani et al., 2001; Lalvani et al., 2001).

Many of these are better tests in terms of overall sensitivity, specificity, and statistical accuracy than AFB smears for the diagnosis of pulmonary tuberculosis although only one, nucleic acid amplification, has come into general use.
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### Table 6: New diagnostics for tuberculosis

<table>
<thead>
<tr>
<th>Test</th>
<th>Methodology</th>
<th>Time</th>
<th>Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth property of mycobacteria</td>
<td>Radiometric</td>
<td>7-12</td>
<td>High sensitivity and specificity</td>
</tr>
<tr>
<td></td>
<td>Non radiometric</td>
<td>&lt; 7 days</td>
<td></td>
</tr>
<tr>
<td>Mycobacteria specific phages</td>
<td>Non engineered phages</td>
<td>24-48 hrs</td>
<td>Low sensitivity, high specificity</td>
</tr>
<tr>
<td></td>
<td>Engineered phages</td>
<td>24-48 hrs</td>
<td>Higher sensibility than non engineered phages</td>
</tr>
<tr>
<td>Nucleic acid based tests</td>
<td>Polymerase chain reaction</td>
<td>6-8 hrs</td>
<td>High sensitivity and specificity</td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>48 hrs</td>
<td></td>
<td>High sensitivity and specificity</td>
</tr>
<tr>
<td>Peptide nucleic acid based probes</td>
<td>48 hrs</td>
<td></td>
<td>High sensitivity and specificity</td>
</tr>
<tr>
<td>Immunodiagnostics</td>
<td>12-24 hrs</td>
<td></td>
<td>High sensitivity, low specificity</td>
</tr>
</tbody>
</table>

2.11. Polymerase Chain Reaction

#### 2.11.1. Principle of PCR

PCR is a method of DNA amplification that involves denaturing of DNA using heat, annealing of a specific primer to the DNA fragment and extension of the primer using a thermostable DNA polymerase. The starting material for PCR is a gene or segment of DNA which can be amplified a million fold. (Mullis et al., 1987; Saiki et al., 1988). The principle of the method is shown in figure 10.
To amplify a specific segment by PCR, it is not necessary to know the nucleotide sequence of the target DNA. Two small stretches of known sequences that flank the target are used. The complementary strands of a double-stranded molecule of DNA are separated by heating. Two small pieces of synthetic DNA, each complementing a specific sequence at one end of the target sequence, serve as primers. Each primer binds to its complementary sequence. Polymerase starts at each primer and copies the sequence of that strand.

Exact replicas of the target sequence are produced within a short time. In subsequent cycles, double-stranded molecules of both the original DNA and the copies are separated, primers bind again to complementary sequences and the polymerase replicates them. At the end of many cycles, the pool is greatly enriched in the small pieces of DNA that have the target sequences (Guyer and Koshland, 1989). Amplification using PCR has become a powerful tool for the rapid and specific detection of many infectious agents especially when the pathogen is difficult to culture like *M. tuberculosis*. PCR was first described in 1988, it was used to detect *M. tuberculosis* a year later (Schochetman and Jones, 1988).
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2.11.2. Nucleic acid amplification tests

Nucleic acid amplification (NAA) tests amplify target nucleic acid regions that uniquely identify the *M. tuberculosis* complex. Because NAA tests can be directly used on clinical specimens (such as sputum), they are also called...
"direct amplification tests." NAA tests are categorized as commercial kits or in-house assays. The polymerase chain reaction (PCR) is the best-known and most widely used NAA test.

Commercial kits include the Amplicor® MTB tests (Roche Molecular Systems), the Amplified Mycobacterium tuberculosis Direct Test® (MTD) (Gen-Probe Inc), the LCx® kit (Abbott Laboratories), BD ProbeTec ET assay (BD Diagnostic Systems) and INNO-LiPA RIF.TB assay (Innogenetics).

The characteristics of each of these tests are summarized in Table 7.

Table 7: Commercial kits and their characteristics
(Piersimoni and Scarparo, 2003)

<table>
<thead>
<tr>
<th>Test</th>
<th>Method</th>
<th>Target</th>
<th>Detection</th>
<th>Assay time</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMTD2*</td>
<td>TMA</td>
<td>16SRNA</td>
<td>Chemiluminescence</td>
<td>2.5</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Amplicor*</td>
<td>PCR</td>
<td>16SDNA</td>
<td>Colorimetric</td>
<td>6</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>LCx</td>
<td>LCR</td>
<td>PAB</td>
<td>Fluorimetric</td>
<td>6</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>DTB</td>
<td>SDA</td>
<td>IS6110</td>
<td>Fluorimetric</td>
<td>3</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>LiPA</td>
<td>Nested PCR</td>
<td>RpoB gene</td>
<td>Colorimetric</td>
<td>12</td>
<td>+++</td>
<td>++++</td>
</tr>
</tbody>
</table>

TMA transcription mediated amplification, LCR ligase chain reaction, SDA strand displacement amplification, PAB protein antigen, * FDA approved

The AMPLICOR MTB assay is a PCR amplified qualitative test which targets a 584-bp segment of the 16S rRNA gene shared by all the members of the genus Mycobacterium. It can be performed manually or automatically and includes an internal amplification control (IAC). Detection is by a colorimetric reaction and results are available within 6 to 7 hrs. It is approved by the FDA for testing smear-positive respiratory samples. Overall Amplicor specificity ranges
from 91.3 to 100%. False-positive results in comparison with culture were observed for specimens collected from patients receiving anti-tuberculosis chemotherapy (Gamboa et al., 1998; Reischl et al., 1998) or due to cross-reactions with NTM (Cohen et al., 1998; Tortoli et al., 1999). Literature data confirm good performances with smear-positive respiratory specimens. Its main drawback however, is the low sensitivity with smear-negative respiratory samples.

The AMTD2 assay produced by Gen-Probe, is an isothermal transcription-mediated amplification method in which the target (mycobacterial 16S rRNA) is amplified by DNA intermediates. The entire process is autocatalytic, no IAC is included and it performed at 42°C with a heat block. Detection is with an acridinium ester-labeled MTB complex-specific DNA probe. Test results, are available within 2.5 h from specimen submission and this method is approved by the U.S. FDA for testing on smear-positive and smear-negative respiratory samples.

The overall sensitivity in respiratory specimens compared with culture and clinical diagnosis ranged from 85.7 to 97.8%. Sensitivity was found to be higher for smear-positive specimens (91.7 to 100%) and lower (65.5 to 92.9%) for smear negative specimens. Specificity ranged from 92.1 to 100% and false-positive results ranged from about 1 to 7.1% (Della-Latta et al., 1998; Jorgensen et al., 1999; Alcala et al., 2001; O’Sullivan et al., 2002; Piersimoni et al., 2002).

Disadvantage of AMTD2 is the absence of detecting inhibitors in 1 to 5% of clinical samples (O’Sullivan et al., 2002; Piersimoni et al., 2002;
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Bergmann et al., 1999; Scarparo et al., 2000; Woods et al., 2001). Test lacks predictivity and cross-reactions with mycobacteria other than MTB have also been reported (Alcala et al., 2001; Tjhe et al., 2001; O'Sullivan et al., 2002; Piersimoni et al., 2002).

Commercial tests have the advantage that they are very well standardized and uniform in their methodology however they are expensive and demand a high level of expertise from experienced technologists. The specificity of CDATs has been shown to be high while the sensitivity varied, positive and negative likelihood ratios vary with the pretest probability of tuberculosis. To determine the clinical utility of CDAT result in individual patients, it is essential to consider the degree of clinical suspicion. Given the additional expense of CDATs, their use with patients for whom the likelihood of tuberculosis is either very high or very low may represent an improper use of healthcare resources (Barnes, 1977; Minh et al., 2000). Implementation of CDATs should take into account the level of laboratory service and should also be based on cost-effective analysis. Finally, test expense is very important and may be prohibitive, especially when comparing the average cost of CDATs with in-house tests.

Various laboratory developed PCR assays where the investigators put together their own protocols have been described. They vary in their extraction procedures, targets, sample input, and PCR conditions. Extraction procedures may either be based on physical or chemical based methods. Various procedures like boiling of DNA, use of glass beads or resins, sonication to lyse bacteria followed by extraction with phenol choroform have been described.
Procedures vary from simple techniques like boiling of DNA (Tanil et al., 1995; Sritharan and Barker, 1991) to other complex protocols. Metaanalysis have shown that method of DNA extraction and use of any chemical reagent for DNA extraction does not substantially affect diagnostic efficiency. No difference was seen in those studies that used phenol-chloroform versus any other DNA extraction method (Flores et al., 2005). Amplification methods may vary like use of nested (Kambashi et al., 2001), semi nested, regular, or multiplex PCR (Ginesu et al., 1998). Detection may be either probe based or by gel UV method. Analysis also suggested that the methods used for DNA extraction and signal detection are not critical.

The specificity of PCR is based on the sequence of the two primers. Any segment of genomic DNA or RNA is a potential target for a PCR diagnostic assay. A suitable target for amplification may be a single copy gene in the mycobacterial genome or present as a repeated sequence. The choice of target and design of primers within the gene target are equally important in terms of assay sensitivity and specificity. Both genus specific and species specific gene targets are utilized. Many primers which amplify specifically the DNA of *M. tuberculosis* have been designed and successfully used for identification of this microorganism from culture and also from clinical samples.

Some of the targets include the genes for insertion elements IS6110 (Thierry et al., 1990; Hashimoto et al., 1995), IS986 (Kolk et al., 1992), 32 Kda protein, (Soini et al., 1992, Soini et al., 1996), 65 kDa protein (Pao et al.,
1990; Negi et al., 2005), ribosomal sequences 16S rRNA, 23S rRNA (Verma et al., 1994) and gene targeting MPB 64 (Dar et al., 1998).

Predominant among these is the insertion sequence IS6110/IS986 which are present in multiple copies. Of all the target sequences the most commonly used are the bacterial insertion sequences (IS) elements. Bacterial insertion sequences are transposable genetic elements present in multiple copies in a genome and capable of movement to new locations in the genome. IS elements exhibit variable degrees of specificity in the selection of insertion sites on the genome, with some being highly specific and others quite random.

Many, however, are between these two extremes (Fang et al., 2001). IS6110, a member of the IS3 family, was identified in the M. tuberculosis complex. It is usually present in multiple copies in the genome, and this along with other characteristics has led to its use as a powerful genetic marker for strain differentiation. Metaanalysis have indicated that increased accuracy of in house based tests was associated with the use of IS6110 as a target of amplification (Flores et al., 2005).

However some of the shortcomings of using this sequence are that the general diversity of IS6110 RFLP patterns observed in M. tuberculosis isolates suggests its insertion is random in the genome. Some genomic regions like the locus ipl are preferential loci for its insertion. Based on these finding Fang et al concluded that conclusions based on particular insertion sites should be approached with caution (Fang et al., 2001). The high copy number of IS6110 is
thought to result in increased sensitivity but given the scale of amplification it is unlikely to be a significant factor.

Within IS6110, the choice of primers can affect the PCR results. Mc Hugh et al. found a homology between DNA from mycobacteria other than tuberculosis strains (MOTT) and a central region of IS6110 and have urged caution in using diagnostic tests based on this target (Kent et al., 1995; Mc Hugh et al., 1997). The IS6110 PCR of Eisenach et al. has been shown to have a false-positive rate of 3% with specimens containing MOTT (Eisenach et al., 1991). As IS3 sequences are widely distributed in organisms found in oropharyngeal secretions and this flora is heterogeneous, false-positive results at low frequency can be expected. There have been reports of false positives with the IS 6110 and discrete bands were demonstrated with Aspergillus, Streptococcus pneumoniae, and Streptococcus pyogenes when PCR products were hybridized with a probe derived from the 181-bp fragment.

Another potential problem is that although most of the M. tuberculosis strains carry multiple copies of IS6110, strains with a single copy or no copy have been reported in many countries. A potentially more serious problem is existence of strains that lack IS6110 in patients with advanced HIV infection. A study done at TRC, India showed that 42.7 per cent isolates of M. tuberculosis were shown to either lack or have only a few copies of IS6110 (Das et al., 1995).

MPB64 gene for immunogenic protein is found only in culture filtrates of M. tuberculosis and occasional isolates of M. bovis BCG. Various
studies, including those in India have been described using primers specific for MPB64 and these primers have shown potential especially in Indian conditions (Table 8).

Experience with in house developed PCR tests has demonstrated overall sensitivities and specificities in the range of 70%-100% (Table 8). Various in house tests with sensitivities and specificities comparable to commercial tests or even greater than commercial tests have been described (Schirm et al., 1995; Eing et al., 1996). A Metaanalytical review of 84 in-house PCR-based studies to detect *M. tuberculosis* in sputum samples showed a summary receiver operator characteristic (SROC) of 97%, indicating an overall high accuracy of these tests. The main advantage of in house based tests is that they are cost effective and can be standardized according to local conditions. However, the main shortcoming is that there is substantial heterogeneity in both sensitivity and specificity of in-house NAA tests (Flores et al., 2005). In spite of these shortcomings a well standardized in house test has been useful in frontline practice (Cohen et al., 1998; Aslanzadeh et al., 1998; Kaul, 2001).
### Table 8: Studies done using NAA tests

<table>
<thead>
<tr>
<th>Study</th>
<th>No of samples</th>
<th>Method/primers</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abe et al., 1993</td>
<td>135</td>
<td>IS 986</td>
<td>81.3</td>
<td>94.2</td>
</tr>
<tr>
<td>Shawar et al., 1993</td>
<td>384</td>
<td>IS 6110</td>
<td>74</td>
<td>95</td>
</tr>
<tr>
<td>Yuen et al., 1993</td>
<td>519</td>
<td>IS 6110</td>
<td>86</td>
<td>95</td>
</tr>
<tr>
<td>Claridge et al., 1993</td>
<td>&gt;5000</td>
<td>IS 6110</td>
<td>83.6</td>
<td>98.7</td>
</tr>
<tr>
<td>Nolte et al., 1993</td>
<td>313</td>
<td>IS 6110</td>
<td>91</td>
<td>100</td>
</tr>
<tr>
<td>Beige et al., 1995</td>
<td>103</td>
<td>MPB 64</td>
<td>98</td>
<td>70</td>
</tr>
<tr>
<td>Schirm et al., 1995</td>
<td>504</td>
<td>Amplicor</td>
<td>70</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In house PCR</td>
<td>93</td>
<td>98</td>
</tr>
<tr>
<td>Eing et al., 1996</td>
<td>103</td>
<td>IS 6110</td>
<td>100</td>
<td>81.8</td>
</tr>
<tr>
<td>Della Latta and Whittier, 1998</td>
<td>1385</td>
<td>AMTD2</td>
<td>97.1</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>1385</td>
<td>PCR</td>
<td>96.7</td>
<td>100</td>
</tr>
<tr>
<td>Gambao et al., 1998</td>
<td>450</td>
<td>Amplicor</td>
<td>92.4</td>
<td>100</td>
</tr>
<tr>
<td>Cohen et al., 1998</td>
<td>112</td>
<td>IS 6110</td>
<td>85</td>
<td>55</td>
</tr>
<tr>
<td>Dar et al., 1998</td>
<td>143</td>
<td>MPB 64</td>
<td>81.8</td>
<td>91.5</td>
</tr>
<tr>
<td>Ginesu et al., 1998</td>
<td>375</td>
<td>IS 6110</td>
<td>91.4</td>
<td>87.7</td>
</tr>
<tr>
<td>Tortoli et al., 1999</td>
<td>697</td>
<td>PCR</td>
<td>75.5</td>
<td>99.8</td>
</tr>
<tr>
<td>Araj et al., 2000</td>
<td>82</td>
<td>IS 6110</td>
<td>91</td>
<td>99.8</td>
</tr>
<tr>
<td>Scarparo et al., 2000</td>
<td>296</td>
<td>AMTD2</td>
<td>85.7</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>296</td>
<td>PCR</td>
<td>94.2</td>
<td>100</td>
</tr>
<tr>
<td>Prasad et al., 2001</td>
<td>255</td>
<td>CDRI primers</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>Tansuphasiri et al., 2001</td>
<td>231</td>
<td>IS 6110</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>Piersimoni et al., 2002</td>
<td>273</td>
<td>AMTD2</td>
<td>92.8</td>
<td>99.4</td>
</tr>
<tr>
<td>Lim et al., 2003</td>
<td>168</td>
<td>IS 6110</td>
<td>77</td>
<td>100</td>
</tr>
<tr>
<td>Ozkutuk A et al., 2006</td>
<td>173</td>
<td>Amplicor</td>
<td>73</td>
<td>100</td>
</tr>
</tbody>
</table>
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2.11.3. Limitations of nucleic acid amplification tests

PCR has some shortcomings. Contamination of amplification reaction with products of a previous PCR, exogenous DNA or other cellular material can create problems. A number of precautions are discussed in Kwok and Higuchi. In general, careful procedures and use of multiple negative controls are necessary for checking contamination. Other approaches like UV irradiation of the reaction tube to damage contaminating sequences, use of modified dNTPs, in order to distinguish PCR products from sample template DNA can also be used.

PCR detects the presence or absence of a particular nucleic acid target. It will only detect a microbe if its nucleic acid is present in the particular specimen. PCR detects nucleic acids from living or dead microbes which must be taken into consideration if PCR is used to monitor response to therapy. The reliability of NAA tests for tuberculosis has been evaluated in several large, multicentric studies of inter laboratory reliability. Studies have involved analyses of blinded clinical specimens with known amounts of M. tuberculosis bacilli.

Variability in the PCR results obtained by various laboratories (for the same batch of specimens) is reflective of interlaboratory reliability. Many published studies have reported problems with false-positive PCR results, at rates ranging from 0.8% to 30% (Rattan, 2000). Various multicentre evaluations and quality control tests for Mycobacterium tuberculosis have been performed (Noordhoek, 1996, 2004) where both in house and commercial tests were used.

All studies revealed a great need for standardization and quality controls since upto 50% of the participating laboratories had unsatisfactory
results. Summary of the outcome was that strict quality assurance and quality testing is necessary and that a laboratory with high quality standards can be successful either with in house or commercial tests. Overall, these studies underscored the need for good laboratory practices to ensure the reliability of NAA tests.

2.11.4. Utility of nucleic acid amplification tests

In respiratory specimens that are AFB smear positive, the sensitivity of PCR is approximately 95% with a specificity of 98%. In specimens that contain fewer organisms and are AFB smear-negative, results are positive in 48 to 53% of patients with culture-positive tuberculosis and the specificity remains approximately greater than 95%. In summary, the sensitivity of a good quality PCR is in the range of 90–100% and 60–70% on smear positive and negative culture positive respiratory samples, respectively (Dunlap et al., 2000; Catanzaro et al., 2000; Watterson, 2000).

Sensitivity estimates have been variable across studies. Sensitivity of NAA tests has been maximal in smear-positive pulmonary tuberculosis and lower in paucibacillary forms of tuberculosis. NAA methods can be applied to clinical specimens within hours. They are helpful in the earlier definitive diagnosis of the smear-negative cases which impact clinical treatment and public health decision making like isolation and contact investigation.

The results of various studies have indicated that PCR should be performed in conjunction with microscopy and culture, and result must be interpreted within the overall clinical setting. More rapid detection of *M.*
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tuberculosis by PCR may translate into improved clinical care. The tests enhance diagnostic certainty but should be interpreted in a clinical context. Implications may differ for public health and individual clinical decisions.

At present, the main shortcoming of NAA tests is the cost, given their cost such use would strain laboratory budgets unbearably. If the cost of these assays were low enough and if the specificity of the NAA was equal to or greater than that of the acid-fast bacillus (AFB) smear, they could replace sputum smears for the purpose of making a diagnosis of pulmonary tuberculosis in previously untreated patients, which may in fact be the case in certain areas.

However, in certain settings such as centralized laboratories to which a large number of specimens can be quickly and easily referred, these tests may in fact economically feasible and clinically useful. Some analyses also suggest that there are scenarios in which use of NAA, at certain price points, might be cost effective in resource-poor countries (Van Cleeff et al., 2005).

There is an urgent need for new tests that can contribute to improving case detection and reducing diagnostic delay. The performance of new diagnostics must be evaluated in settings that reflect their intended application, and performance must be evaluated to investigate whether such new technology is cost effective and can be implemented within the existing infrastructure in high-burden countries.

2.12. Aim and Objectives

The aim of this study is to determine the comparative efficiency and cost effectiveness of an in house PCR test to other routine diagnostics like
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sputum smear microscopy, culture, and radiological examination for detection of *M. tuberculosis* for pulmonary tuberculosis in settings of routine clinical practice.

The objectives of the study are to determine efficiency of routine diagnostics like microscopy and radiological examination used in the Revised National Tuberculosis Control Programme (RNTCP) for diagnosis of pulmonary tuberculosis in Pune Municipal Corporation (PMC) area.

To determine the comparative efficiency and cost effectiveness of PCR using MPB64 primers versus other routine diagnostics in the detection of *M. tuberculosis*.