# 2. Literature review

## 2.1 Mycobacterium Genus

### 2.1.1 Structure

*Mycobacteria* are a unique genus of organisms, due to the mycolic acid, which comprises approximately 60% of the cell wall. In structure, *Mycobacteria* are similar to gram-negative bacteria; however, they are not considered to be a type of gram-negative bacteria because mycolic acids in *Mycobacteria* replace lipo-polysaccarides of gram-negative bacteria. The mycolic acids give each organism a water resistant waxy coating, which is responsible for many unusual characteristic related not only to physical properties, but also to metabolism and pathogenicity (Tortora 1998a; Tortora1998b).

*Mycobacteria* grow in aerobic conditions and do not form spores (Tortora 1998b). They are slender rod shaped organisms and grow either curved or straight. The rods are 2-4 micrometers in length and 0.2-0.5 um in width (Murray 2003). *Mycobacteria* usually occur singly or in clusters, but occasionally they may exhibit filamentous growth, particularly on the surface of liquid media. It is this characteristic that has provided the genus name ("myco" meaning fungus) (Tortora 1998 c).

### 2.1.2 Species

#### 2.1.2.1 *Mycobacterium Tuberculosis*

*Mycobacterium Tuberculosis*, (*M.Tb.*) the organism responsible for Tuberculosis (TB), is one of the more notorious species in the *Mycobacterium* genus. The optimal growth temperature for this organism is $37^0$ C (Prescott 1999a) and it grows readily on simple substrate using glycerol as a carbon source and ammonia or amino acids as nitrogen sources (Murray 2003). Some types of media currently used for culturing *M.Tb.* include Lowenstein Jensen Agar (bioMerieux), *Mycobacteria* 7H22 Agar with OACD enrichment (Difco) and modified Sauton's medium.

Even on an appropriate medium, the growth of *M.Tb.* is very slow. The waxy cell wall decreases the entry of nutrients into the cell, and thus reduces the metabolic rate of the organism (Tortora 1998b). It takes 24 hours for a cell to divide and can take 2-3 weeks for visible cultures to appear on a solid medium (Young 1991).

*M.Tb.* was first isolated by Robert Koch in 1982 using his newly reported postulates (Prescott 1993b, Daniel 1997a). In spite of this, however, *M.Tb* is one of the few species that is an exception to Koch's postulates. The same organism is responsible for causing a disease that can be manifested in a number of different organs (for example Lungs, skin, bones and other internal organs), which, to an untrained eye, may appear to be a variety of completely unrelated diseases (Tortora 1998d).

#### 2.1.2.2 *Mycobacterium bovis*

*Mycobacterium bovis* as the name suggests, is an organism that causes TB in cattle, although it can also infects dogs, cats, pigs, parrots, badgers primates and humans (Murray 2003). This was a common occurrence before the days of pasteurized milk and screening of cattle for disease (Tortora 1998c).

#### 2.1.2.3 *Mycobacterium avium*

*Mycobacterium avium* causes TB in birds. Its optimal growth temperature is 41-45$^0$ C and its pathogenicity in other animals is mild to non-existent (Pelczar 1972). It is very uncommon for this organism to infect humans, except the late stages of HIV infection (Tortora 1998c).

#### 2.1.2.4 *Mycobacterium leprae*

*M. leprea* is another well-known species of the *Mycobacterium genus*. It is the organism that causes the disease most commonly known as leprosy, although the disease is also referred to as Hansen's disease, to avoid the fear associated with the world "leprosy". *M. leprae* has an optimum growth temperature of 30$^0$ C, which explains its preference for the extremities of the body. Its generation time has been estimated at a very long 12 days. It has never been cultivated in laboratory conditions and is, therefore, one of the few organisms that do not follow Koch's postulates (Tortora 1998e).

## 2.2 History: *Mycobacterium Tuberculosis*

### 2.2.1 Ancient History

TB has been recognized as one of the most ancient diseases which find a place in the ancient ayurvedic system practiced by Sushrutha, Charaka and others around 2500

BC. It is stated in the literature that the disease process can result from imbalance in the homeostatic, which can be due to variation in the Vata (combination of Earth and Air), Pitta (Fire) and Kapha (combination of Water and Earth). It has also been documented in the Vedas and Ayurvedic Samhitas as early as 2000 BC. There is archaeological evidence of spinal TB in Egyptian mummies dating back to 1000 BC. (Daniel 1997a).

24th March-1882 was a landmark day when Robert Koch isolated the causative organism for TB i.e. *M.Tb.* Throughout recorded history, the prevalence of TB has risen and fallen following the "herd immunity" concept, whereby those who become infected either die or develop immunity. Thus the disease runs rampant until it exhausts the host supply for some time, only to rise again at a later date (Daniel 1997b).

For many years, TB was thought to be a hereditary disease until Benjamin Marten suggested, in 1720, that "animacula" microorganisms were responsible for the disease. Quarantine laws were introduced, although not strictly enforced until the 1780s in Italy, and as late as the 1820s in England. The following years saw significant advances in science and technology that improved the quality of life and raised awareness of the infectious nature of diseases, including TB. In 1892 the first voluntary health agency, a Tuberculosis association, was organized in Philadelphia (Daniel 1997c)

### 2.2.2 Modern History

The concept that bacterium was responsible for TB was presented by Koch to a gathering of scientists in 1882 (Prescott 1999b). This news was spread quickly throughout the civilized world, sparking a fever of scientific research into the disease.

Four years after Koch's discovery that TB was a bacterial disease, German chemists, Franz Ritter von Soxhlet adapted the pasteurization techniques to the preservation of milk (Prescott 1999c). This resulted in the reduction of milk-transmissible diseases, including TB (*M. bovis*). Pasteurization of milk was introduced in the United States in 1889 (Prescott 1999c). By 1907, the mortality rate from TB had declined significantly

in all developed countries. The high public awareness to avoid TB infection led to expectations that the disease would be completely wiped out within a short time (Broker 1999). Developed countries established sanatoria to care for disease stricken consumptives and to reduce the spread of the disease (Pearn 1987).

Scientific research aimed at reducing the burden of TB and focused on prevention and treatment. A vaccine against TB, called BCG, was developed by Albert Calmette and Camille Guerin and was first administered in 1921 (Harboe 1996a). BCG vaccination is effective in reducing TB infection and only slight changes have been made to this vaccine since its developments (Pleczar 1972, Colditz 1994).

The first effective antibiotic for TB treatment was streptomycin (Tortora 1998c). The development of this antibiotic was announced by Selman Waksman in 1944 (Prescott 1999d). Other effective drugs for treating TB were also discovered in the 20 years followed. These treatments further reduced incidence and mortality. It was believed that TB was in the process of eradication in developed countries and funding for TB research diminished. Sanatoria were closed down and public health systems that related to TB were dismantled, with the focus on a greatly improved community care service (Brown 1993).

By the 1970s, the incidence of TB was so low that funding for the community care services was eliminated. Patients who did not complete therapy were not followed up and it was not long before drug-resistant TB mutants began to emerge. Global economic recession, military unrest, poverty, malnutrition and human migration, IV drug use and overcrowding contributed to an increased spread of TB (Mori 2000, Bloom 1992, Brown 1993, Yang 1998). In the 1980, the human immunodeficiency virus (HIV) began to emerge (Brown 1993). HIV and TB form an unusual partnership, with each disease spreading the progress of the other (Ginsberg 2000, WHO 2000).

The declining rate of incidence of TB, which had been 10% per year since 1950s, became completely reversed in 1980s. Between 1985 and 1992, the number of TB cases increased 18% in US (Bloom 1992). In April 1993, the World Health

Organization (WHO) showed its concern about the neglected state of TB control and declared TB to be a global emergency (WHO 2000).

Between 1992 and 1997 in the United States, the number of TB cases decrease among US- borne individuals by 38%, but the incidence among foreign-borne persons increased 6%. In Eastern Europe, TB incidence rates peaked around 2001 and with the exception of the African region, 2003 statistics indicate that worldwide incidence rate of TB are falling or stable (WHO report 2005).

## 2.3 Current Incidence

### 2.3.1 Worldwide

The following data show current status of TB in the world (WHO report 2009).

- 2 billion people or one third of the world's population are infected with TB.
- 1 person is infected with TB every second.
- TB kills 4400 people every day.
- 1.7 million People died from TB in 2008.
- There were 9.2 million new cases of TB in 2008.

TB is responsible for 5% of all worldwide deaths (Ginsberg 2000). 9.6% of adults between the ages of 15 and 59 die from TB, making it the most foremost cause of death from a single infectious agent (Raviglione 1995, Ginsberg 2000). Approximately one third of the world's population harbors the disease and is at risk of developing TB (Orme 1999a). In 2003, 8.81 million new cases were diagnosed, of which approximately 85% were pulmonary disease. 1.75 million, that is more than 3 people per minute, died as a result of TB infection. The prevalence of TB in India alone accounts for almost one quarter of the world's total TB infections (WHO report 2005). Between 2000 and 2020, it is expected that TB will infect nearly one billion people, 200 million of whom will develop active disease and 35 million will die (WHO 2000).

Of the 15 countries with the highest TB incidence rates per capita, 12 are from the African region, which also experience the highest rates of HIV/TB co-infection. The highest incidence of TB infection is in Swaziland where almost 1100 per 100,000

populations were diagnosed in 2003 (WHO report 2007). In spite of the high incidence in several African countries, the South-East Asia region accounts for 33% of global incidence (WHO report 2007).

### 2.3.2 India

India is classified along with the sub-Saharan African countries to be among those with a high burden and the least prospects of a favorable time trend of the disease as of now (Group IV countries). India is classified along with the sub-Saharan African countries to be among those with a high burden and the least prospects of a favorable time trend of the disease as of now (Group IV countries) (Chakraborty 2004).

WHO (2007) report indicates the following fact about prevalence of TB in India.

- India has 3.8 million TB patients at any time
- India records 18 lakh new cases of TB annually.
- Over 6 lakh Indians are unaware that they suffer from TB.
- India saw TB case detection rates increased by 10-12% during 2001-05.
- It fell to 5% in 2006.
- In India, TB infects 8 lakh people every year and when treated inappropriately (the administration of drugs is stopped prematurely or is not done properly), the patient may not only remain sick, but the bacteria that causes the illness may develop resistance to drugs ordinarily used to treat TB. MDR-TB is difficult to treat, as the drugs used are often toxic and can cause side effects. The people infected with this disease spread it readily to others. Treating MDR-TB patients is also expensive.
- Diabetes has been fuelling India's deadly TB epidemic. TB occurs more often among the people with diabetes; in India presently 35 million people are diabetic which will increase to 57 million by 2025.

## 2.4 Pathogenesis

### 2.4.1 Risk factors

Many social and health factors contribute to an increased risk of not only becoming infected with TB, but also subsequently developing the active disease. These factors include increase population mobility due to military unrest and political instability, conflict, natural disasters and human migration. Risks are also increased among

defined population subgroups, such as prisons, ethnic minorities, immigrants, indigenous persons and the elderly. In addition, economic recession leads to overcrowding, poor ventilation, poverty and malnutrition. The risk of developing TB is also increased among those with pathological diseases such as diabetes mellitus, renal failure, HIV and immuno-suppression; and among drug users, including IV drug use, alcohol abuse and smoking (Antic 2001, Mori 2000, WHO 2000, Fischer 1999, Prescott 1999b, Yang 1998, Plant 1995, Brown 1993, Frieden 1993, Bloom 1992).

All these risk factors contribute to disease risk in different ways. Population mobility not only increase the number of people that TB can be spread to, but it also poses significant problems in tracing contacts, which can result in delayed diagnosis of new cases, and in ensuring that treatments are completed. Military or political unrest is capable of destroying infrastructure that is put in place for the control of TB. Overcrowding, and defined population subgroups living in close proximity have increased risks simply through higher levels of contact with an infected individual. The risk of transmission increases as the amount of time spent with an infectious person increases (Braden 1995). Malnutrition increases the risk of developing active disease once infected.

### 2.4.2 Natural Resistance

In spite of the risk factors mentioned, studies show that human have a natural resistance to acquiring TB (Schurr 1991). In mice, the gene that confers natural resistance is called the "intracellular pathogen resistance 1 (*Ipr 1*) gene" in the *sst1* (Super-susceptibility to TB 1) region of chromosome 1. The product encoded by *Ipr1* may be responsible for integrating macrophage mechanism with signals produced by intracellular pathogens, such as *M.Tb.* (Pan 2005). It remains to be seen whether natural resistance in humans is controlled by a gene equivalent to that in the murine genome.

### 2.4.3 Methods of Transmission

#### 2.4.3.1 Respiratory transmission

Respiratory mechanisms are by far the most cause of TB transmission. Bacteria are expelled in tiny droplets from people with active disease either by sneezing, singing,

talking or coughing (Bloom 1992, Loudon 1968 and Loudon 1967). These droplets can vary considerably in size and number. On average, one cough produces as many particles as about five minutes of loud continuous talking. The smaller droplets fall to the ground much slower than larger droplets and remain suspended in the air for longer time (Loudon 1967). These smaller droplets undergo evaporation and leave residue that contain the bacteria. This residue is called a "Droplet nucleus" and is so light that it may drift in air currents until it is either inhaled vented or destroyed (Wells 1948).

Due to waxy coating around the *M.Tb* cells, the bacteria are resistant to drying out and remain virulent, floating in the air currents, with the potential to cause infection (Tortora 1998c). They are however, sensitive to UV light, which will eventually kill the bacteria (Peccia 2004).

#### 2.4.3.2 Other means of transmission

Extra pulmonary TB is rarely considered contagious; however some cases have been reported. In 1992, a case was reported of *M.Tb* infection in an ulcer on the upper thigh of a patient. 18% of the persons present during dressing changes of that wound became newly infected with *M.Tb* (Frampton 1992). TB finger lesion has also been reported to occur as a result of a needle-stick injury obtained during the incision and draining of a patient's TB thigh abscess (Hutton 1990). Oral transmission is also possible but is far less effective than respiratory transmission of the organism (Bloom 1992).

#### 2.4.3.3 Inhalation of Bacteria by a new host

A single droplet nucleus contains one to three *M.Tb* cells. When inhaled, this is sufficient to begin the infection process (Wiegeshaus 1989). For this infection to occur, the droplet nucleus usually needs to reach the apical- sub- apical (A-SA) area of the lungs, a region where bacilli can survive in low numbers even after the development of an immune response. This zone is relatively poorly ventilated and multiple exposures to *M.Tb* are usually required before a primary infection can develop (Smith 1989).

## 2.5 Disease progression

### 2.5.1 Macrophage involvement

In the lungs, the infecting bacteria are engulfed by alveolar macrophages, which recognize the bacteria merely as foreign matter. At this point, the destruction of the bacteria depends upon the microbicidal power of macrophage, which reflects the interaction between the genetics of the host and the virulence of the bacterial strain. If the macrophage is able to destroy the bacteria, then the infection is arrested. If, however, the macrophage fails to destroy the bacteria, the infection progresses to the next stage (Danneberg 1991).

### 2.5.2 Tubercle formation

The macrophages, which are struggling to control the bacterial infection, release pro-inflammatory cytokines and chemokins to recruit monocytes and lymphocytes to control infection. The monocytes mature into multi-nucleated giant cells surrounded by T- lymphocytes, producing a glaucomatous lesion called tubercle, which walls off the infections. These microscopic tubercles form approximately two to three weeks after inhalation of the organism. The logarithmic growth of the bacteria ceases due to the cell death at the caseous center of the tubercle and equilibrium formed by the marginal cell division and destruction within the macrophages on the periphery of the lesion (Young 1998, Tortora 1998c, Andersen 1997, Dannenberg 1991).

This stationary phase lasts approximately 8 weeks, by which time the host's immune system has switched from the cell-mediated immunity (CMI), which failed to stop intracellular growth of the bacteria, to delayed-type hypersensitivity (DTH) response, which sacrifices the host's own tissues (Dannenberg 1991). At this time tubercle may begin to progress and become calcified, thus showing up clearly on radiological testing (Tortora 1998c). In spite of the calcification on the lesions, the bacteria can survive for many years in dormant state in the center of tubercle, with potential for re-activation of disease (Andersen 1997).

In some hosts, the macrophage fail to effectively wall off the lesion and the necrotic center of the tubercle, including dead macrophage and bacilli, continues to enlarge

and becomes macroscopically visible within four to five weeks. The host may then manifest the infection as symptomatic active disease.

### 2.5.3 Reactivation

*M.TB* bacteria held in the caseous tubercles in the lungs of an infected individual may exist in static form for many years without the individual experiencing any symptoms or, in fact, even being un-aware that they have been infected. At some stage, or triggering factor, such as immuno-suppression, causes reactivation of the tubercle and the progression to active disease (Andersen 1997, Ginsberg 1998). It is possible for some lesions to progress to an active disease state whilst other tubercles in the same lung may actually regress (Dannenberg 1991). Progression to active disease over the lifetime of an individual only occurs in 10% of those infected with *M.TB* (Ginsberg 2000).

### 2.5.4 Active Disease

Active disease may follow on from infection, either immediately or at a later time following reactivation. Hydrolytic enzymes produced by the activated macrophages, cause liquefaction of the tubercle (Harboe 1996a), providing a liquid environment that enables the bacteria to proliferate outside of the host macrophages for the first time (Dannenberg 1991). The fast growing bacteria produce toxins that attack the host tissues and cause necrosis and rupture of nearby bronchi resulting in cavity formation. The cavity provides an oxygen rich environment that further favors proliferation of the bacilli (Dannenberg 1991, Tortora 1998c).

Liquid from the TB lesions, containing large numbers of bacteria, provide a perfect vehicles for aerosol formation when air is expelled from the host lung. In this manner, the organisms can be transported to the other regions of the lung and also other individuals (Tortora 1998c, Dannenberg 1991). The more progressed the disease, the greater the number of bacteria expressed into the air and the greater the chances of infecting other individuals (Prescott 1999b).

Cavity formation in the host lung also provides a direct method of entry for the bacilli into the circulatory and lymphatic systems of the infected individual (Tortora 1998c).

Thus, secondary infections may occur throughout the body, a disease state referred to as extra-pulmonary or military TB (Prescott 1998b, Ginsberg 1998).

The clinical symptoms of active pulmonary TB include chest pain, coughing, shortness of breath, fever, night sweats, fatigue and weight loss (Murray 2003). The prognosis for treated TB sufferers is reasonably positive with 73% cure rate of registered TB sufferers worldwide (WHO reports 2005).

## 2.6 Diagnosis

### 2.6.1 Microscopy

Smears can be made from cultures or directly from samples and then stained for microscopic examination. *Mycobacteria* stain positively by Gram stain, making them difficult to distinguish by this traditional method. Because of their waxy coating, *mycobacteria* can, however, be stained for microscopy by using an acid-fast staining technique. In this technique, carbol fuchsin is applied to a fixed smear, and heated to enhance cell wall penetration and retention of the dye. Acid alcohol is used as a de-coloriser. The carbol fuchsin is retained by *mycobacteria* because it is more soluble in the waxy cell wall than in the acid alcohol. Methylene blue is used as counter-stain (Tortora 1998f). Under the microscope, *mycobacteria* stained by this method appear as pink rods on a blue background.

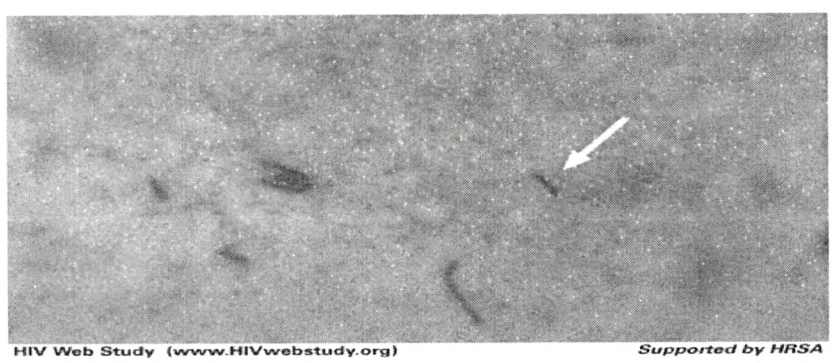



Figure 2.1: Ziehl Nielsen Stain of *M.Tb* showing the typical "clumping" of the bacteria. The acid- fast organisms appear pink and the background staining is blue.

Another microscopic technique for examining *mycobacteria* is fluorescence microscopy. The fluorochrome auramine O, is strongly absorbed by *M.tb* and when examined under microscope using ultra-violet light, it appears bright yellow against a dark background (Tortora 1998g).

Smears are used as a diagnostic tool as they quickly provide results with a relatively high specificity. The disadvantage of relying on smears for is that the bacteria appear in a smear only when the disease has progressed to a point where the patient is highly infectious. In addition, this method of diagnosis requires well trained staff and sufficiently maintained laboratory equipment (Zumla 1998, O'Brien 1993).

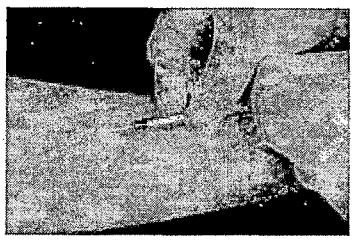
### 2.6.2 Culture

Tubercle bacilli can be isolated from sputum, bronchial aspirates, gastric contents, spinal fluid, urine and tissue samples of persons infected with the microorganisms (Murray 2003). The bacteria can be grown on solid or liquid media; however diagnosis from culture alone is very slow. It takes 24 hours for a cell to divide and can take 2-3 weeks for visible cultures to appear on a solid medium (Young 1991). In spite of the time taken to culture *M. tb,* culture is considered the "gold standard" to which all other identification methods are compared (Gillespie 1997).

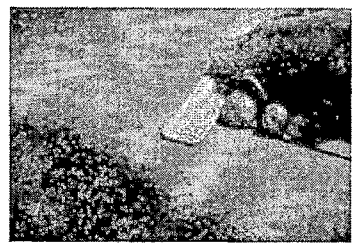
Automated culture systems are available, such as the bioMĕrieux BacT/Alert® system, which employs colorimetric technology, and the Becton Dickensen BACTEC™ MGIT™ 960 system, which uses fluorometric technology, for rapid detection of bacterial growth (Pffyfer 1997). Automated systems however, are more costly than basic culture methods in terms of requiring skilled training and operation. As a result of the difficulty in culture and identification of *Mycobacterium* isolates, centralized laboratories that specialize in identification of these organisms are beneficial (Foulds 1998, Zumla 1998).

### 2.6.3 Tuberculin skin test

The tuberculin skin test, called a "Mantoux test", is the traditional method of diagnosing *M.Tb* infected individuals.



The Mantoux skin test consists of an intradermal injection of exactly one tenth of a milliliter (mL) of PPD tuberculin.



The size of induration is measured 48-72 hours later. Erythema (redness) should not be measured.

Figure 2.2 shows the tuberculin skin test

The tuberculin skin test, called a "Mantoux test", is the traditional method of diagnosing *M.Tb* infected individuals. It was a technique initially used by an Austrian physician, Clemens Freserr Baron von Pirquet in 1907, and refined by Montoux in 1908. The technique used "tuberculin" which was described by Robert Koch in 1890 (Daniel 1997a). Tuberculin, which is actually a purified protein derivative (PPD) of *M.Tb* cultures, is intra-dermally injected into the forearm. A delayed type hypersensitivity response (DTH) results in an inflamed area within 2-3 days, the size of which is relative to the levels of the immune response that the body has against the PPD. Please refer Figure 2 (WHO 2004).

This method of diagnosis, however, is only effective in countries where TB is not endemic and where few people have received BCG vaccinations (Ginsberg 1998). Exposure to TB infected individuals may result in sero-conversions to one or more antigens in PPD. Also, BCG vaccines can expose individuals to antigens that are present in PPD. In both of these circumstances, a positive Montoux test does not necessarily indicate infection by *M.Tb*. In addition, 20-30% of patients with active TB infections are negative for the tuberculin test (Bonay 1999).

### 2.6.4 Use of Secreted proteins

Proteins that are secreted from actively growing *M.Tb* have been examined, with variable success, for diagnostic purposes with the hope of developing an "easy-to-use" method of detecting infection by the organism due to the immunological response of the host (Lagrange 1999, Saunders 1999, Ginsberg 1998).

ESAT-6, a 9.9 kDa protein and culture filtrate protein 10 (CFP-10), are both found in culture filtrates of infectious strains of *M.Tb* and *M. bovis* but not in *M. bovis* BCG (Harboe 1996b). These two antigens have been incorporated into two commercially available diagnostic tests used for detecting TB infection. One is T-SPOT$^{TM}$ TB assay (Oxford immunotec), an enzyme-linked immunospot (ELISPOT) test and another is the QuantiFeron TB Gold assay (Cellestis). This assay measures plasma IFN-γ levels using enzyme-linked immunosorbent assay (ELISA) following centrifugation of whole blood that has been incubated in tubes coated with ESAT-6 or CFP-10 (Cellestis 2005, Brock 2004). This method is simpler and less labor involved, more accurate for the diagnosis of TB (Ferrara 2004, Brock 2004).

### 2.6.5 Radiological Methods

Chest X-rays are the traditional diagnostic tools for TB. They provide immediate results without having to wait for several weeks for culture results. There are, however, numerous disadvantages with relying on X-rays for the diagnosis of TB. Firstly, the technique has very poor specificity, with many other chest conditions resulting in similar radiographic abnormalities (Foulds 1998). This can lead to over-diagnosis of TB and un-necessary prescription of antibiotics against TB (O'Brien 1993). Sensitivity is also poor, with the easy stages of infection failing to appear on X-rays to determine whether the patient is part of an outbreak, or simply suffering reactivation of an old infection (Kline 1995). For these reasons, X-rays should only be used as diagnostic tool for TB in conjunction with microscopy and cultures.

### 2.6.6 Molecular biology methods

In the early 1990s, molecular biology techniques used in research began to be applied as laboratory diagnostic tools (Kulaga 1999). Initially, Southern blotting was used for detection of *M.Tb* DNA restriction fragments. PCR techniques were developed that detected a fragment of IS 6110, an insertion sequence specific to *M.Tb*. other insertion sequences specific to other *mycobacterial* species have also been identifies (Saunder 1999, Gillespie 1997, Hellyer 1996, Kent 1995). The current role of PCR is limited and its sensitivity and specificity is low. PCR techniques are expensive and complicated and require specialized equipment and dedicated space. Although this

technique may play a small role in current diagnostic methods, the recent developments of new simpler methods, such as those using secreted proteins, have attracted more attention (Lodha 2004).

### 2.6.7 Susceptibility testing

The Becton Dickensen BACTEC TM MGIT TM 960 system, an authomated *mycobacterial* culture system, can also be used for susceptibility testing of *M.Tb*. This system provides relatively rapid results to the common antibiotic used for treatment of TB, including streptomycin, isoniazed, rifampicin, ethambutol and pyrazinamide (Yajko 1995, Franzblau 1998).

## 2.7 Treatment

*Mycobacterium TB* has been present in the human population since antiquity-fragments of the spinal column from Egyptian mummies from 2400 B.C. show definite pathological signs of tubercular decay.

After the infectious nature of TB was demonstrated in 1865 by French scientist, Jean-Antoine Villemin, the treatment of TB sufferers was largely focused on prevention of the spread of disease. Sanatoriums and hospitals were built to specifically house and confine the patients. It was not until the 1940s and 1950s that antibiotics were introduced and refined into disease-fighting drugs (Daniel 1997a).

In 1882, Robert Koch discovered a staining technique that enabled him to see *Mycobacterium TB*. What excited the world was not so much the scientific brilliance of Koch's discovery, but the accompanying certainty that now the fight against humanity's deadliest enemy could really begin.

The chemotherapy of infectious diseases, using sulfonamide and penicillin, had been underway for several years, but these molecules were ineffective against *Mycobacterium TB*. Since 1914, Selman A. Waksman had been systematically screening soil bacteria and fungi, and at the University of California in 1939 had discovered the marked inhibitory effect of certain fungi, especially actinomycete, on bacterial growth. In 1940, he and his team were able to isolate an effective anti-TB

antibiotic, actinomycin; however, this proved to be too toxic for use in humans or animals.

Success came in 1943. In test animals, streptomycin, purified from *Streptomyces griseus*, combined maximal inhibition of *M.Tb* with relatively low toxicity. On November 20, 1944, the antibiotic was administered for the first time to a critically ill TB patient. The effect was almost immediately impressive. His advanced disease was visibly arrested, the bacteria disappeared from his sputum, and he made a rapid recovery. The new drug had side effects - especially on the inner ear - but the fact remained, *M.Tb* was no longer a bacteriological exception, it could be assailed and beaten into retreat within the human body.

A rapid succession of anti-TB drugs appeared in the following years. These were important because with streptomycin mono-therapy, resistant mutants began to appear with a few months, endangering the success of antibiotic therapy. However, it was soon demonstrated that this problem could be overcome with the combination of two or three drugs.

Following streptomycin, *p*-aminosalicylic acid (1949), isoniazid (1952), pyrazinamide (1954), cycloserine (1955), ethambutol (1962) and rifampin (rifampicin; 1963) were introduced as anti-TB agents. Aminoglycosides such as capreomycin, viomycin, kanamycin and amikacin, and the newer quinolones (e.g. ofloxacin and ciprofloxacin) are only used in drug resistance situations. Combinations of a *B*-lactam antibiotic with a *B*-lactamase inhibitor enhance treatment effectiveness, but the newer drugs, including the macrolides, have not received much clinical testing.

### 2.7.1 Antibiotics

Current treatments of TB involve the use of a cocktail of chemotherapeutic agents. The preferred treatment regimen is an initial 8 weeks of daily doses of isonizid, rifampicin, pyrazinamide and ethambutol. This is followed up by either daily or twice weekly doses of isoniazid and rifampicin for further 18 weeks, giving a total treatment time of approximately 6 months as a minimum (WHO 2003).
Figure 2.3 shows mechanism of various antibiotics used for treatment of TB.

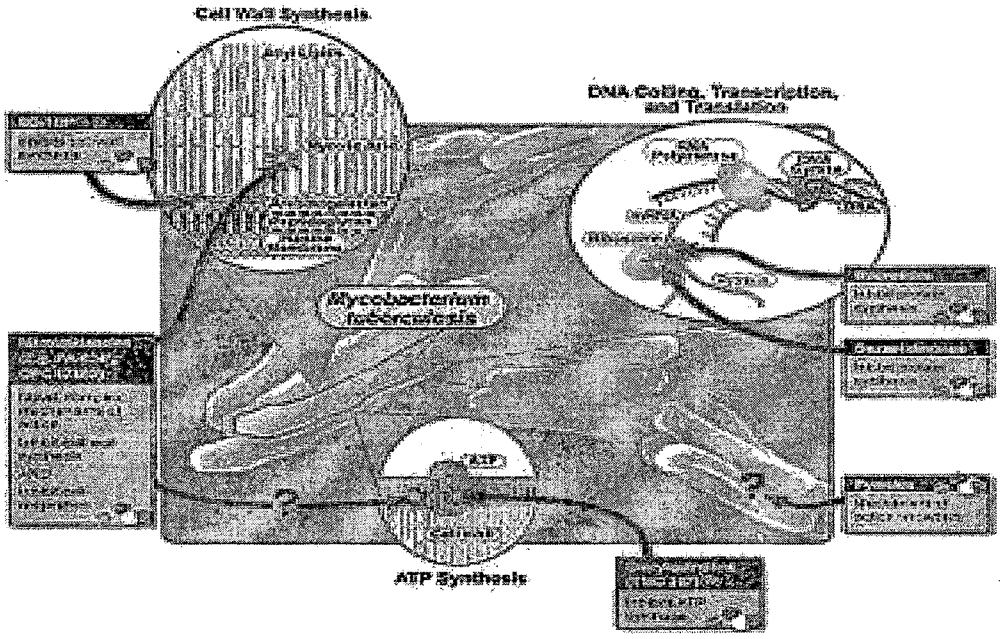



Figure 2.3: Mechanism of various antibiotics

Antibiotics are not only used for the treatment of TB, but are also prescribed as preventive therapy for latent TB infections. Isoniazid is the drug of choice for prophylaxis and is given orally twice weekly for 6-9 months. Alternatively, rifampicin may be given daily for 4 months. Previously, a combination of rifampicin and pyrazinamide has also been available for prophylaxis. This combination, however, is now, strongly discouraged due to the risks of severe liver injury and death due to result of the treatment (Valway 1998).

### 2.7.1.1 First line antibiotic therapy for TB

The first line chemotherapeutic agents used for the treatment of TB includes: Streptomycin

- Isoniazid
- Rifampicin
- Ethambutol
- Pyrazinamide

### 2.7.1.2 Second line chemotherapeutic agents

There are six classes of second-line drugs (SLDs) used for the treatment of TB. A drug may be classed as second-line instead of first-line for one of two possible

reasons: it may be less effective than the first-line drugs (e.g., *p*-aminosalicylic acid); or, it may have toxic side-effects (e.g., cycloserine); or it may be unavailable in many developing countries (e.g., fluoroquinolones):

- aminoglycosides: e.g., amikacin (AK), kanamycin;
- polypeptides: e.g., capreomycin, viomycin, enviomycin;
- fluoroquinilones: e.g., ciprofloxacin (CIP), levofloxacin, moxifloxacin (MXF);
- thiomides: e.g. ethionamie, prothionamide
- cycloserine (the only antibiotic in its class);
- *p*-aminosalicylic acid (PAS or P).

#### 2.7.1.3 Other drugs that may be useful, but are not on the WHO list of SLDs:

- rifabutin;
- macrolides: e.g., clarithromycin (CLR);
- linezolid (LZD);
- thioacetazone (T);
- thioridazine;
- arginine;
- vitamin D;
- R 207910.

These drugs may be considered "third-line drugs" and are listed here either because they are not very effective (e.g., clarithromycin) or because their efficacy has not been proven (e.g., linezolid, R207910). Rifabutin is effective, but is not included on the WHO list because for most developing countries, it is impractically expensive.

### 2.7.2 Compliances

Due to prolonged treatment and the side effects experienced, compliance with chemotherapeutic agents is one of the greatest hurdles in the treatment of TB. Whilst relatively short lapses in therapy have no substantial effect on the rate of recovery, patients who default on greater than 2 months of treatment experience a 10-fold increased risk of poor outcomes of therapy (Burman 1997).

### 2.7.3 Multi-drug Resistance

Drug resistant TB has been reported since the early days of the introduction of chemotherapy, but recently multi-drug resistant TB (MDR-TB) has been an area of growing concern and is posing a threat to control of TB. A review of 63 surveys conducted between 1985 and 1994 suggested that primary and acquired MDR-TB was between 0-10.8% and 0-48% respectively. However, the qualities of these studies were variable due to the lack of proper representative and size of population sampled, as well as lack of standardized laboratory methods. Current estimates report, the prevalence of primary and acquired multi-drug resistance in India as 3.4% and 25% respectively. It must be emphasized that optimal treatment of MDR-TB alone will not curb the epidemic. Efforts must be focused on the effective use of first line drugs in every new patient so as to prevent the ultimate emergence of multi-drug resistance. The use of reserve drugs to cure MDR-TB and to reduce further transmission should be considered, but only as part of well structured programs of TB control.

Today, with the greatly expanded efforts to strengthen TB prevention and control programs worldwide, there is growing concern about the currently reported and potential future rates of drug-resistant TB, and more importantly, the emergence of strains resistant to Isoniazid and Rifampicin, defined as multi-drug resistant TB, or "MDR-TB". Drug resistance develops either due to infection with a resistant strain, or as a result of inadequate treatment, such as when a patient is exposed to a single drug, or because of selective drug intake, use of inappropriate non-standardized treatment regimens, irregular drug supply, poor drug quality, or rarely, erratic absorption of the medications (WHO 2009, Mahmoudi and Iseman 1993). Drug regimen required for these treatments costs up to $250,000 per patient, thus making MDR-TB an untreatable in developing countries.

### 2.7.4 Directly Observed Therapy

The WHO recommended strategy Directly Observed Treatment – Short course (DOTS) is the main weapon in the battle against the global TB epidemic. DOTS is a dynamic systematic strategy with 5 components: sustained political and administrative commitment, access to quality assured diagnosis by sputum-smear microscopy, standardized short-course chemotherapy for all cases of TB under proper

case-management conditions, including direct observation of treatment, systems for the maintenance of uninterrupted supply of quality assured drugs, and a recording and reporting system for enabling assessment of treatment outcome. There is abundant evidence that, when all the recommended procedures are in place, chemotherapy under DOTS can achieve cure rates of 90% or more, and prevent the emergence of resistance to first-line drugs.

The first component of DOTS is sustained political commitment. It deals with the perception of TB as preventable disease and the development of organized strategies at all levels of government to combat TB and alleviate the poverty and social issues that are associated with high TB prevalence. Government need to provide financial and human resources for making control of TB a part of their national health strategy (WHO 2000).

The second component of DOTS discusses case detection by sputum smear microscopy. Access to quality health care and rapid detection of symptomatic patients is priority. Particular attention must be paid to high-risk categories, such as HIV-infected and institutionalized persons (WHO 2000).
Standardized treatment and proper case management are the third component of DOTS. Ensuring that standardized treatments are administered involves educating the health-care providers regarding the standard drug regimes. Provision of support and care of patient is essential for motivation and adherence to treatments. This may require establishing community- or workplace- based direct observation therapy. WHO recommends direct observation of therapy whenever rifampicin is administered for control of TB (WHO 2000).

Un-interrupted supply of TB treatments, as the fourth components of DOTS, covers the reliability and quality of available chemotherapeutic agents and their distribution to required destinations. It is also suggested that fixed dose combinations (FDCs) of the numerous drugs required during initial TB therapy be provided in a single tablet to assist patients with complicated nature of treatments, thus reducing the risk of development of multi-drug-resistant strains (WHO 2000).

Finally DOTS incorporates the establishment of a recording and reporting system of detection, treatments and outcomes of each TB patients. This enables surveillance and monitoring programs and encourages communication and support on all levels from local health care to international strategies (WHO 2000).

In 2003, 182 countries, covering 77% of the world-wide population, were implementing the DOTS strategy. Between 1995 and 2003, DOTS programs treated 17.1 million TB patients and 8.6 million smear positive patients. DOTS programs resulted in a case detection rate of 45% in 2003, which was considerably higher than previous years. The 5% decrease in prevalence since 1990 has also been attributed to DOTS (WHO 2005).

**DOTS Plus** is a case management strategy under the aegis of DOTS to manage MDR-TB using second line drugs and infection control measures. As per definition, it is clear that DOTS is pre-requisite to DOTS-Plus and hence can be considered only when the situation where effective DOTS is being implemented. Overall goals for DOTS-Plus strategy are to reduce morbidity and mortality from MDR-TB and to cut the chain of transmission.

## 2.8 BCG Vaccine

### 2.8.1 History

The history of BCG is tied to that of small pox. Jean Antoine Vilemin first recognized bovine TB in 1854. Robert Koch was the first who distinguished *M. bovis* from *M. Tb.* by culturing *M. bovis* isolate from a cow for a period of 13 years and a total of 231 passages, Calmette, a physician, and Gu´erin, a veterinarian, created an attenuated variant of *M. bovis*, bacillus Calmette-Gu´erin (BCG). BCG was first tested in infants in 1921 as an oral vaccine. New methods of administration were later introduced, such as intradermal, multiple puncture, and scarification. Since 1974, BCG vaccination has been included in the WHO Expanded Program on Immunization, resulting in more than three billion doses injected worldwide (approximately 100 million immunizations in children each year).

No other widely used vaccine is as controversial as BCG. Its effects in large randomized, controlled, and case-control studies have been widely disparate, from excellent protection against TB to no protection (Smith 2000).

### 2.8.2 Variable efficacy

The most controversial aspect of BCG is the variable efficacy found in different clinical trials that appears to depend on geography. Clinical trials conducted in the UK have consistently shown a protective effect of 60 to 80%, but trials conducted elsewhere have shown no protective effect, and efficacy appears to fall the closer one gets to the equator. The first large scale trial evaluating the efficacy of BCG was conducted from 1956 to 1963 and involved almost 60,000 school children who received BCG at the age of 14 or 15; this study showed an efficacy of 84% up to 5 years after immunization. However, a US Public Health Service trial of BCG in Georgia and Alabama published in 1966 showed an efficacy of only 14%, and did much to convince the US that it did not want to implement mass immunization with BCG. A further trial conducted in South India and published in 1979 (the "Chingleput trial"), showed no protective effect. BCG seems to have its greatest effect in preventing military TB or TB meningitis, for which reason, it is still extensively used even in countries where efficacy against pulmonary TB is negligible (Smith 2000).

### 2.8.3 Uses

The main use of BCG is for vaccination against TB. It is recommended that the BCG vaccination be given intradermally by a nurse skilled in the technique. Having had a previous BCG vaccination is a cause of a false positive Mantoux, although a very high-grade reading is usually due to active disease.

The age and frequency that BCG is given has always varied from country to country.

**WHO BCG policy:** The WHO recommends that BCG be given to all children born in countries highly endemic for TB because it protects against miliary TB and TB meningitis.

**United States** The US has never used mass immunization of BCG, relying instead on the detection and treatment of latent TB.

(TST) may be negative (Hanson 2001). A TST as small as 5 mm in diameter is sufficient to indicate TB infection in a HIV positive patient.

### 2.9.3 Vaccination

Vaccination with current BCG vaccine is not recommended in any immuno-compromised persons, including those infected with HIV. Although BCG is an avirulent strain of *Mycobacteria*, it is still a live organism and diminished immune system of HIV patients can be incapable of containing it (Elhay 1997).

### 2.9.4 Treatment and recovery

Treatment of TB is also complicated as a result of HIV infection and it is a fine line between treating the TB and exacerbating HIV (Kirschner 1999). DOTS should always be used to avoid the added complications of drug-resistant organisms. In addition, Rifampin has known to interact with anti-retroviral agents and an alternative to this may be needed (CDC fact sheet #250120, 2005).

## 2.10 Alternate to BCG vaccine

### 2.10.1 The need for an alternative TB vaccine

BCG (Bacille- Calmette-Guerine), an existing vaccine for TB, which provides an excellent protection against disease to the children. However, it shows variable efficacy in protecting Adult- Pulmonary TB. Current scenario of disease supports the need for the development of an alternative vaccine which can control active disease or BCG-booster vaccine which enhances protection efficiency of BCG vaccine in adult. The emergence of multidrug-resistant (MDR)-TB and extensively drug-resistant (XDR)-TB along with HIV-TB co-infection is a real threat to achieve TB control and elimination (Giri 2008).

The purpose of vaccination against TB is to boost the Cell Mediated immune response, thus preventing the immune progression to DTH response, which sacrifices host tissues in its attempt to combat disease. In TB, the protective immune response are the same as those involved in the pathology underlying the disease and stimulating immune protection without worsening the disease is a fine line (Doherty 2005). The understanding of the immune responses to infection is paramount to discoveries leading to successful vaccine development.

The initial identification of individual proteins in culture filtrate that were strongly immunogenic sparked a desire to more rationally categorize antigens. The evaluation of antigenic properties of antigen from culture filtrate was based on random selection followed by testing. This protein represents a heterogeneous group of molecules. However, it was gradually recognized that several of the important antigens were related and belonged families of genes. The ability of bio-informatics analysis of whole genome to group genes is different gene families coupled with the recognition that such related genes are often coordinately regulated and co-expressed in response to the same stimuli. This was a random selection and testing of protein molecules and antigenic testing along with bioinformatics tools correlates gene families For TB, three families have emerged stronger and they are:

The **Ag-85 complex (A-C)** consists of a family of 30-32 KDa proteins that act as mycolyl transferases. Because of this key role in cell wall biosynthesis and extension prior to fission, they are made in copious amounts, particularly when the bacterial culture is in log phase. These proteins have been reported to be leading vaccine candidates by number of workers (Girard 2005, Yadav 2001, Brookes 2001 and Orme 2006).

Ag 85 has been widely studied in mouse primarily as a DNA vaccine (Ulmer 1997) and some have shown promising results using purified proteins (Olesan 2001). Sinha et al reported that Ag-85 purified from the avirulent strain H 37Ra could protect well ad Yadav et al reported a variety of delivery vehicle such as liposome and micro spheres. Liposomes and microspheres carrying antigens have shown promising results in protection against the disease in mice.

Boosting immunity of mice in middle age was found when they are immunized with Ag-85A after BCG vaccination when young restored waning immunity to *M.Tb* challenge in old mice (Brookes 2001).

**ESAT-6** (Early Secretary Antigen Target) are 9.8 KDa proteins which are strongly recognized by T-cells from *M.Tb.* infected individuals or in animal models of TB. This protein has been found to be immuno-dominant, inducing IFN-γ production by T-cells from infected mice. ESAT-6 induces protection either as a subunit vaccine or as a DNA vaccine. Brandt et al reported that its protective efficiency was as good as that of the BCG (Brandt 2000).

Third strong immuno dominant protein is **TB 10.4**, which has been recognized in BCG-vaccinated donors. Dietrich et al reported that vaccination with this protein against TB comparable to that induced by Ag-85B-ESAT-6 and BCG and better than either of individual proteins (Dietrich 2005).

The main advantage of these proteins are in-vivo stability as these are produced by the body against the *M.Tb* infection.

#### 2.10.2.4 Fusion Proteins

Compared to mixtures of proteins extracted from cultures to cell lysates, the fusion protein approach offers at least two substantial advantages:

1. it reduces the number of recombinant protein expression and purification steps,
2. it has amplified immune response compared to individual proteins or cocktail proteins.

For example, ESAT-6 has low inherent immuogenicity. Delivery system containing single ESAT-6 requires strong adjuvants like DDA or MPL to enhance their immunogenicity, but fusion of ESAT-6 with Ag-85 amplify immune response (Olsen 2001). Moreover, fusion of two proteins of different families will have more numbers of epitopes which bring strong immunity at lower dose than individual protein and immunological memory also reported to found stable and stronger.

## 2.11 Immunity

The ability to defend against infectious agents is defined as "immunity".

Immunology is the study of all aspects of host defense.

The term immune response or immune reactions refer to the production of cells and soluble factors that defend the body against potentially dangerous biological and chemical agents which in term are referred to as immunogens or antigens.

An **immune system** is a collection of mechanisms within an organism that protects against disease by identifying and killing pathogens and tumor cells. It detects a wide variety of agents, from viruses to parasitic worms, and needs to distinguish them from the organism's own healthy cells and tissues in order to function properly.

One of the greatest achievements of the 20th century is prevention of numerous fatal, infectious diseases through the administration of vaccines. Vaccination against

smallpox, polio, diphtheria, pertusis, tetanus, measles and other pathogens has reduced mortality more than any other disease intervention (Plotkin 1997).

| Components of the immune system | |
|---|---|
| **Innate immune system** | **Adaptive immune system** |
| Response is non-specific | Pathogen and antigen specific response |
| Exposure leads to immediate maximal | Lag time between exposure and maximal response |
| Cell-mediated and humoral components | Cell-mediated and humoral components |
| No immunological memory | Exposure leads to immunological memory |

Table 2.2: Component of Immune system

#### 2.11.1.1 Innate immune system

The innate immune system comprises the cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner. This means that the cells of the innate system recognize, and respond to, pathogens in a generic way, but unlike the adaptive immune response, it does not confer long-lasting or protective immunity to the host. Innate immune systems provide immediate defense against infection, and are found in all classes of plant and animal life.

Functions of Innate immunity are as follows:

- Recruiting immune cells to sites of infection and inflammation, through the production of chemical factors, including specialized chemical mediators, called cytokins.
- Activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes.
- The identification and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells.
- Activation of the adaptive immune system through a process known as antigen presentation.

### 2.11.1.2 Adaptive immune system

The adaptive immune system is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogenic challenges. The adaptive immune system is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogenic challenges.

The adaptive immune response provides the immune system with the ability to recognize and remember specific pathogens (to generate immunity), and to mount stronger attacks each time the pathogen is encountered. It is adaptive immunity because the body's immune system prepares itself for future challenges.

The major functions of the adaptive immune system include:

- The recognition of specific "non-self" antigens in the presence of "self", during the process of antigen presentation.
- The generation of responses that are tailored to maximally eliminate specific pathogens or pathogen infected cells.
- The development of immunological memory, in which each pathogen is "remembered" by a signature antigen. These memory cells can be called upon to quickly eliminate a pathogen should subsequent infections occur.

## 2.11.2 Immunological Reaction

Specific acquired immunity against infection is primarily a property of a group of serum glycoprotein called antibodies. These antibodies have been produced by a subpopulation of white blood cells in the immune system called lymphocytes. These are small round cells, 6 to 7 μm in diameter, with a high nuclear-to-cytoplasmic ratio in their resting stage, and they are capable of expanding greatly in volume and activity in response to an antigen. This process is called lymphocytic activation.

Antibodies are termed "immunoglobulin" (abbreviated Ig) since they are globular proteins with immune function, the bulk of which greatly separate in gamma region on electrophoresis. Figure 2.4 shows basic structure of Immunoglobulin.

All antibody molecules have two basic functions: (1) antigen binding and (2) participation in effectors functions depending upon the physical properties of the antibody. These properties can be separated by cleavage of the antibody molecule with a proteolytic enzyme, papain. This produces two antigen-binding fragments (named Fab or antigen binding fragment) and a single crystallizable fragment (termed

Fc or crystallizable fragment) which possesses the physical properties of the molecule. Only the Fc fragment has been shown to fix complement and bind to Fc receptor, and Fab does not interact.

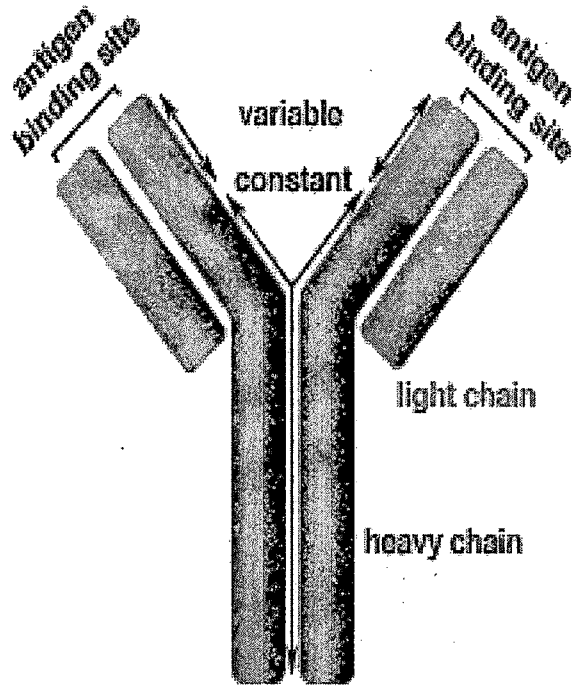



Figure 2.4: Basic Structure of Immunoglobulin

Subsequent biochemical analysis demonstrated that every monomeric antibody molecule consisted of two pairs of polypeptide chains linked to each other by disulfide bonds. The larger pair composed of two heavy chains, each chain having a molecular weight of 50,000 Daltons. The smaller pair is composed of two light chains, each with a molecular weight of 25,000 Daltons.

Amino acid analysis of the antibody molecule revealed that the antigen binding site is flanked by a variable amino acid sequence portion of the chians. while remiander has relatively constant amino acid sequence. Thus the amino acid sequence of the constant regions determines the common biological and physical properties of immunoglobulin, and the variable region determines its individual specificity. In actual fact, there are five separate classes of immunoglobulins: immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin E (IgE) and immunoglobulin D (IgD).

| Immunoglobulin | Heavy Chain Designation | Molecular Weight | Physical State | J Chain | Subclasses |
|---|---|---|---|---|---|
| IgG | γ | 150,000 | Monomer | - | 4 |
| IgM | M | 900,000 | Pentamer | + | 1 |
| IgA | A | 160,000 (serum) | Monomer | - | 2 |
| | | (160,000)n Serum | Polymer | + | |
| | | 390,000 Secretions | Dimer | + | |
| IgD | Δ | 180,000 | Monomer | - | 1 |
| IgE | E | 185,000 | Monomer | - | 1 |

Table 2.3: Classes of Immunoglobulin

(--): Indicate Absence, (+): Indicates Presence

| Immuno-globulin | Site Found | Complement Fixation | Crosses Placenta | Function |
|---|---|---|---|---|
| IgG | Internal Body fluids, particularly extravascular | + | + | Major line of defence against infection during the first few weeks of a baby's life; neutralizes bacterial toxins; binds to microorganism to enhance their phagocytosis and lysis |
| IgM | Largely confined to bloodstream | +++ | - | Efficient agglutination and cytolytic agent; effective first line defence in cases of bacteremia. |
| IgA | Serum, external body secretions | - | - | Protects mucosal surfaces from invasion by pathogenic microbes |

| | | | | |
|---|---|---|---|---|
| IgD | Serum, on lymphocytes surface of newborn | - | - | Regulator for the synthesis of other immunoglobulins; fetal antigen receptor |
| IgE | Serum | - | - | Responsible for severe acute and occasionally fatal allergic reactions; combats parasitic infections. |

Table 2.4: Biological properties of Different Classes of Immunoglobulin

### 2.11.3 Antigen Presentation

When *M.TB* bacteria ingested by the macrophage, they excrete, metabolites that prevent the fusion of the phagosome and the lysosomes, thus preventing contact with destructive lysosomal contents (Prescott 1999b). The bacteria multiply within the host cell and the macrophage processes and presents bacterial antigens as MHC (Major Histocompatibility Complex) class II antigens (Andersen 1995), which focuses on the presentation of exogenous or vacuolar antigens (Harding 1996).

MHC class II antigens have been associated with CD4+ (Cluster of Differentiation surface markers type 4) T-lymphocyets activation (Kaufmann 1999). It is now known that some MHC class I presentation and CD8+ T-cell activity may also associated with TB infections (Kaufmann 1999; Feng 2000). In addition, a small group of cells, called γδ T-cells, are capable of recognizing antigens in the absence of traditional presentation molecules (Chien 1996).

Infected Macrophage also secrete interleukin-12 (IL-12), a cytokine that activates natural killer (NK) cells to produce interferon- γ (IFN- γ) in turn reactivates the macrophage to kill the phagocytes organisms (Tsuyuguchu 1996).

A summary of each vaccine platform and its mechanism of action is provided in Figure 2.5 (Ingolotti 2010).

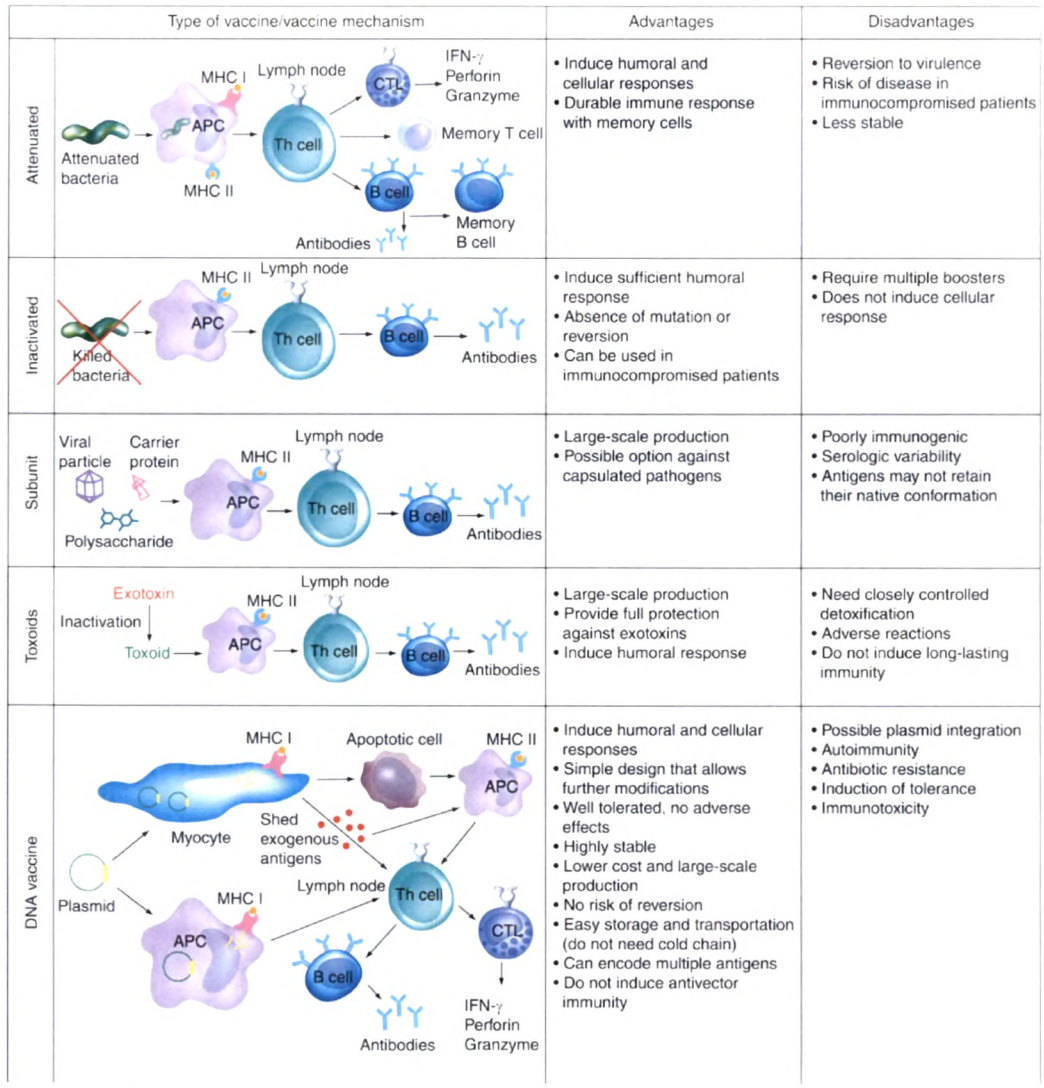



Figure 2.5: Vaccine mechanism.

## 2.11.4 T-lymphocytes types

### 2.11.4.1 CD4+ cells and immunological memory

CD4+ cells express αβ T cells receptors on their surface. They are often referred to as T-helper (Th) cells and recognize antigens associated with MHC class II molecules (Roitt 1991, Roitt 1993). They are activated by antigen presenting cells when only small numbers of T-cells secretes large numbers of cytokins that assist in granuloma formation and inhibition of bacterial growth (Orme 1993a).

CD4+ cells can further be divided into Th-1 and Th-2 cells types according to the cytokins they produce (Mosmann 1986). These specific cytokins inhibit activity by the other Th-cell-type and direct certain pathways of immune responses (Barnes 1996), such as activating macrophages to control or eliminate intracellular organisms using IFN- γ (Flynn 2004).

Th-1 cells secrete IFN- γ and IL-2 and are responsible for primary antibody reponses and strong cellular immuinity against intracellular pathogens (Mosmann 1986; Orme 1993b, Ferrick 1995). It is this cell subset that develops into long-lived "memory" pheotype once direct exposure to the antigen ceases (Orme 1998b, Andersen 1995).

Very high level of IFN- γ have been measured in memory-immune mice within the first 24 hours following infection with M.TB, suggesting that they are rapidly recruited from the circulating pool to the site of infection. Although CD8 cells are also initially recriuted to the site, it is the CD4 cells that continue to proliferate (Andersen 1995).

Th-2 cells produce IL-4 and IL-5, which lead to antibody production in B cells and the development of humoral responses (Mosmann 1986; Ferrick 1995, Barnes 1996). These cells play very little role in fighting TB infection and are present simply as regulatory cells (Orme 1993b; Tsuyuguchi 1996).

#### 2.11.4.2 CD-8+ cells and cytotoxic response

For many years, it was thought that CD8+ cells did not play an important role in controlling TB infections. Now, however, it appears that this category of cells does assist in controlling this disease within the host to some degree (Feng 2000; Flynn 2004). Like CD4+ cells, CD8+ cells fall into the category of cells expressing αβ T-cell receptor on their surface. CD8+ cells are often referred to as cytotoxic T-cells (Tc) and recognize antigens associated with MHC class I molecules (Roitt 1991; Roitt 1993). It has been suggested that this type of antigen presentation may be due to the ingested bacilli having access to the macrophage cytoplasma via pores in the vacuole membrane (Mazzaccaro 1996; Teitelbaum 1999).

Cytokine secreted by CD8+ cells include IFN- γ and tumor-necrosis-factor-α (TNF-α), which enables them to activate macrophage (Flynn 2004). These cells are

associated with formation of granulomas successful in restricting cellular infiltrates are responsible for macrophage lysis within the granuloma (Andersen 1997, Ladel 1995b). CD8 T-cells in humans can contribute to bacterial death within macrophage by releasing granulysin, which is capable of entering the macrophage and is directly toxic to *M.Tb* (Stenger 1998).

#### 2.11.4.3 DN αβ T-cells

Cells that express αβ T-cells receptors, but are negative for CD4 and CD8 surface markers are referred to as Double Negative (DN) αβ T-cells (Roitt 1991, Roitt 1993). These cells are present in very low numbers and have shown to recognise *M. Tb* mycolic acids in association with CD1 molecules rather than MHC (Andersen 1997, Beckman 1994).

#### 2.11.4.4 DN γδ T-cells

T-cells that express γδ, instead of αβ T-cells receptors, are usually CD4+ and CD8+ negative and are often simply referred to as γδ T-cells. They constitute 5% of T-cells in lymphoid organs and 1-5% of circulating T-lymphocyets (Orme 1993a). Studies in mice show that DN population in the spleens of infected mice is predominantly γδ T-cells, whereas the pulmonary DN population is largely αβ T-cells (Phyu 1999). γδ T-cells are capable of recognizing protein antigens directly, without assistance of any antigen processing molecules. It is therefore possible that the types of proteins stimulating these cells are completely different from those involved in αβ T-cell activation (Chien 1996).

The exact role of this cell type, however, is unclear. It has been shown that γδ T-cell lines experience rapid expansion within 4 days following stimulation and that the immune response is directed towards a Th-1 type response, suggesting contribution toward cell-mediated immunity (Garcia 1997). In addition, when systemic immune response fail, mucosal γδ T-cells regulate the maintenance of IgA responses (Fujihashi 1996), and show Th-2-like activity (Wen 1998). It has been suggested that γδ T-cells are able to mediate a protective immune response in the absence of αβ cells (Chien 1996). Further study is shown that rather than being protective, the γδ cells control the migration of macrophage to the site of infection (Orme 1999a).

### 2.11.5 Cytokins

Many cytokins are involved in the immune responses to *M. Tb.* infection. Interleukins, such as IL-1, IL-6 and Il-14, transforming growth factor (TGF-β) and various chemokines recruit monocytes and lymphocytes to the infected area and regulate the extent of the immune response (Mosmann 1989; Andersen 1997; Strober 1998). Other cytokins that play a more direct role in combating TB infections are discussed as below.

#### 2.11.5.1 Interferon- γ (IFN-γ)

IFN-γ is secreted by CD4, CD8 and γδ T-cells and levels of IFN-γ peak around 2 weeks following infection (Flynn 2004; Ferrick 1995, Ogha 1990, Andersen 1995). Γδ T-cells are the most efficient at producing IFN- γ and CD8 cells are least effective, being unable to produce the cytokin in the absence of CD4 cells (Ladel 1995b; Tsukaguchi 1999). Production of IFN- γ is dependent on antigen presentation and co-stimulators provided by monocyets (Tsukaguchi 1999).

- Mice with a genetic disruption disabling them from producing IFN-γ suffer severe caseous mecroses, large abscesses and wide spread dissemination of *M.Tb* following infection, resulting in rapid death. These mice are inadequately able to activate both infected macrophage and arriving monocytes (Flynn 1993, Cooper 1993). Due to the ability of IFN- γ to control infection, this cytokine can be used to monitor emergence of protective T-cells (Orme 1992).

#### 2.11.5.2 Interleukin-12 (IL-12)

IL-12 is an important cytokin produced by macrophage and B cells that stimulates IFN- γ production and enhances the cytotoxic activity of CD4, Cd8 and natural killer cells (Flynn 1996). The presence of IL-12 improves the systemic and mucosal Th-1 cytokin responses and the similar cytokin responses to γδ T-cells to nonpeptide antigens (Garcia 1997, Williams 1999, Arulanandam 1999). This presence can be artificially created by oral or intra-nasal administration of IL-12 (Marinaro 1997, Arulanandam 1999). These anti-tuberculous effects, however, have not been observed in the absence of IFN- γ (Flynn 1995).

#### 2.11.5.3 Tumor necrosis Factor (TNF-α)

TNF-α is produced by macrophages, CD4 T-cells, γδ T-cells and monocytes (Tsukaguchi 1999, Flynn 2004). It contributes to both pathogenesis and protection against TB infection. TNF –α can cause severe tissue necrosis that aid progression of the disease by releasing otherwise aids protection against TB by activating macrophage and influenciing cell migration to infected regions. It affects chemokines and their receptors and adhesion molecules, thereby contributing to the formation of granulomas (Flynn 2004, Cooper 1993).

## 2.12 Mucosal immunity and TB

The mucosal immune system represents highly compartmentalized immunological system and essentially functions independently from the systemic immune system. Consistent with high degree of compartmentalization, the mucosal system is populated by phenotypically and functionally distinct B- cells, T-cells and accessory cell sub-populations compared with those systemic lymphoid tissues (Gallichan 1996, Belyakov 1999).

M, Tb normally enters the host via the respiratory mucosal surface after inhalation of infectious droplets received from an individual with an active disease. Intracellular pathogen *Mycobacterium TB* is an acid fast and obligate aerobes bacillus with a replication time of approximately 12 hours, prefer tissues site with high pO values such as lung and hence TB infection usually initiate in the lungs. The mucosal immune system protects the mucous membranes that line the respiratory system, digestive system, urogenital tracts, eye conjunctiva, inner ear and the ducts of exocrine glands (Mowat 1997, Holmgren 2005). The main functions of this system include protection of mucous membranes from pathogen infection and colonization and conversely, to prevent uptake and potentially harmful immune responses to proteins derived from ingested food, airborne particles and commensal microorganism (Holmgren 2005).

The immune system of mucosal surface is distinct in many ways from that operating at internal sites. Comparisons are shown in table 2.4.

| Systemic Immunity (Internal) | Mucosal Immunity (External) |
|---|---|
| • IgG and IgM produced | • IgA produced |
| • Injectable inoculation | • Injection not necessary |
| • Systemic immunity only | • Local as well as systemic immunity |
| • Boosts mucosal response | • Enhanced by parenteral priming |
| • Respond to small amount of antigen | • More antigen needed to induce response |
| • Sensitive to complex microbial antigen | • Mucosal adhesion is critical property |

Table 2.5: Characteristic of systemic and Mucosal Immunity

The following advantages are associated with mucosal immunization:

1. The efficacy of currently available vaccines can be enhanced by vaccination procedure to achieve both mucosal as well as systemic immunity.
2. Safety and minimization of adverse effects may be increased by vaccination strategies involving mucosal immunization.
3. Delivery of vaccines by oral or other mucosal routes reduces the need for personnel and equipment required for injections and would make possible home administration. This advantage could bring benefits to developing countries and also increase acceptability of and access to vaccination in industrial nations.
4. Mucosal administration might increase vaccine effectiveness in the elderly, particularly in TB when effectiveness of BCG vaccines is waning with age and varies between 0 to 80%. To boost the immunity against TB, the mucosal immunization would be a better choice along with BCG as a prime boost vaccination strategy.
5. If the mucosa associated lymphoid in humans' remains immunologically vigorous at a time when systemic immunity declines, it may be possible to exploit this vaccination of older persons.
6. As per immunization schedule, vaccination should be at earliest in infants, but persistence of maternal antibodies can interfere with parenterally administered vaccines. Mucosal immunization might be safe and successful in infants under 6 months of age.

7. Mucosal immunization may facilitate eradication of some disease caused by pathogens which persist in the environment through asymptomatic colonization of mucosal surfaces.
8. As many new vaccines are being developed, immunization schedule will become more complex unless many of these vaccines are combined. However, the use of combined vaccines will be limited by the amount of material which can be injected parenterally into infants. Greater volumes of vaccines can be administered by mucosal routes. Further administration of some vaccines by mucosal routes could reduce the no of vaccines to be administered parenterally.

### 2.12.1 DDAB- Adjuvant for mucosal TB vaccine

Dimethyldioctadecylammonium bromide (DDAB) is a synthetic lipophilic quaternary amine. DDAB is a well-known adjuvant used for the development of TB vaccine. DDAB has also been reported to stimulate both serum antibody response and cell-mediated immunity against the co-administered antigen. DDAB has already been used intramuscularly widely in the human population with no toxic effects reported. Recently, it has been demonstrated that DDAB induces both mucosal and systemic immune responses when intranasally co-administered with protein antigens [Ref]. It's been reported that intranasal administration of DDAB with Ag85 complex proteins induced significantly higher protection than antigen alone-treated mice (Giri 2008, Unpublished Observations). One could postulate that the mucosal adjuvant property of DDAB comes partially from its ability to increase the amount of antigens crossing the epithelium that covers the nasal cavity. DDAB is a depot-forming agent and acts as a carrier of antigen by direct binding of antigen or modification at the oil/water interface. Another nonexclusive explanation could be that DDAB generates inflammatory stimuli, thereby promoting the uptake of antigen. Indeed, the systemic adjuvant properties of DDAB have been ascribed to its capacity to stimulate an inflammatory response (synthesis of IL-1 and interferons) and the recruitment of activated mononuclear cells.

## 2.13 Intra- Nasal route for TB vaccination

Nasal Mucosa is an important arm of the mucosal immune system since it is often a first point of contact for inhaled antigens (like *MTb*) and as a result intra-nasal immunization has emerged as possibly the most effective route for vaccination It is important to note that nasal vaccine is superior to oral vaccine in inducing specific immunoglobulin IgA and IgG in the upper respiratory tract (Davis 2000).

Moreover, Compartmentalization within the mucosal immune system places constraints on the choice of vaccination route for inducing effective immune responses at the desired sites. Oral immunization may induce substantial antibody responses in the small intestine (strongest in the proximal segment); it is relatively in-efficient at evoking an IgA antibody response in the distal organ segments of the large intestine, respiratory mucosa, tonsils or female genital tract mucosa (Quiding 1991, Kozlowski 1997, Holmgren 2005).

Conversely, rectal immunization evokes strong local antibody responses in the rectum but little if any responses in the small intestine and in the proximal colons (Eriksson 1998, Kozlowski 1997, Jertborn 2001). Nasal and tonsilar immunization in humans results in antibody responses in the upper airway mucosa and regional secretions (Saliva, Nasal) without evoking immune responses in the gut (Johansson 2004, Johansson 2001).

This compartmentalization within mucosal immune system places constraints on the choice of vaccination route for inducing effective immune response at the desired sites. Considering the fact that *M. Tb.* infection occurs via the respiratory tract affect respiratory tract primarily, 75% cases of TB is of pulmonary TB and Extra-pulmonary infection spreads from lung, the intra-nasal route would be the ideal route of vaccination for the development of a mucosal TB vaccine (Giri 2008).

The intra nasal route of administration offers following advantages (Partidos 2000):

- ✓ Much lower doses of antigen is required as there is no significant dilution of vaccine formulation in nasal fluid.
- ✓ No exposure to low pH
- ✓ No exposure to secreted digestive enzymes
- ✓ Nose is easily accessible, highly vascularized and contains numerous microvilli covering the nasal epithelium providing a large absorption area

- ✓ Moreover, intra nasal immunization provides both systemic and mucosal immunity
- ✓ Delivery of Dose without needles; hence needle stick side effects can be minimized.

## 2.14 Nano Particles for nasal delivery

In last decade, there is significant development in vaccine and polymer science, and with that it is possible to design formulation with antigen which target immune system efficiently when delivered through Nasal route. Most promising vaccine carrier candidates includes Poly Lactide (PLA), Ply Glycolide (PGA) and their derivatives (PLGA). Nano-particles (in the range of 200-500 nm) fabricated from biodegradable polymers (like PLA, PLGA), are found more suitable than micro-particles for surface presentation at APCs as well as encapsulation of antigens for localized or targeted delivery of antigens (Vajdy 2001, van der Lubben 2001, Singh 2002 and Alpar 2005).

Mucus penetration of nanoparticles prepared from PLA, PLGA can be facilitated by modifying surface chemistry by coating them with low molecular weight hydrophilic polymer like Poly Ethylene Glycol (Suh 2005). PEGylation helps for mucus penetration (Lai 2009).

There are several techniques for synthesis of nanoparticles, which are explained in brief as follows:

### 2.14.1 Techniques for preparation of nanoparticles

**(1) Nanoparticles prepared by polymerization process:**

Two types of polymerization processes have been adopted to prepare polymeric nanoparticles

**Dispersion polymerization**: Dispersion polymerization starts with monomer, an initiator, solvent in which the formed polymer is insoluble, and a polymeric stabilizer. Polymer forms in the continuous phase and precipitates into a new particle phase, which is stabilized by the polymeric stabilizer. Small particles are formed by aggregation of growing polymer chains precipitating from the continuous phase as these chins exceed a critical chain length. Coalescence of these precursor particles with themselves and with their aggregates results in the formation of stable colloidal

particles, which occurs when sufficient stabilizer covers the particles (Aftabrouchad and Doelker, 1992).

**Emulsion polymerization**: In this technique, the monomer is emulsified in non-solvent containing surfactant, which leads to the formation of monomer swollen micelles and stabilized monomer droplets. The polymerization is performed in the presence of initiator. Emulsion polymerization may be performed using either organic or aqueous media as continuous phase. Poly (methyl methacrylate), poly (alkyl cyanoacrylate), acrylic copolymer, polystyrene, poly(vinyl pyridine) and polyacrolen nanoparticles are prepared by emulsion polymerization technique (Herrmann and Bodmeier, 1998).

**(2) Nanoparticles prepared from preformed polymers**: Several techniques have been suggested to prepare biodegradable polymeric nanoparticles from preformed polymers such as poly (D,L-lactide) (PLA), poly (D,L-glycolide) (PLG) and poly (D,L-lactide-co-glycolide) (PLGA (Tamber 2005). The basic methodologies of the commonly used preparation methods are as follows:

**Single Emulsion-evaporation:**

This is one of the most frequently used methods. The preformed polymer and drug are dissolved in a water-immiscible organic solvent, which is then emulsified in an aqueous solution containing stabilizer. The emulsification is brought about by subsequent exposure to a high energy source such as high pressure homogenizer. The organic phase is evaporated under reduced pressure resulting into formation of nanoparticles, which are then collected by ultracentrifugation, washed and lyophilized for storage (Chung 2002). Figure 2.6 shows schematic presentation for single emulsion- evaporation techniques.

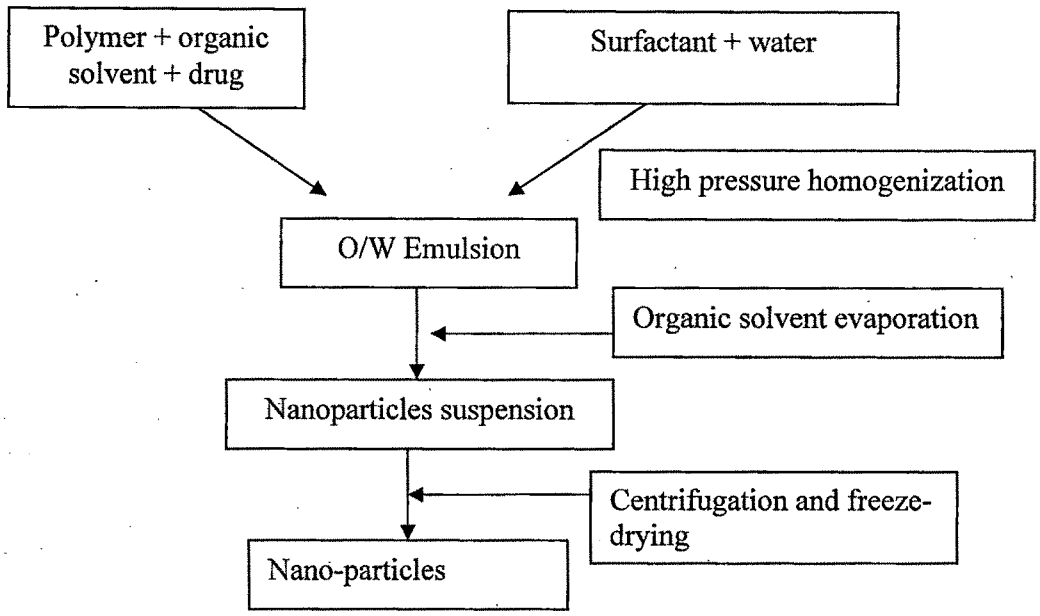



Figure 2.6: Schematic presentation: Single Emulsion- evaporation

**Double-emulsion evaporation**: This procedure is used to encapsulate hydrophilic drugs and proteins (Maa 1997). Figure 2.7 represents schematic diagram for double emulsion method.

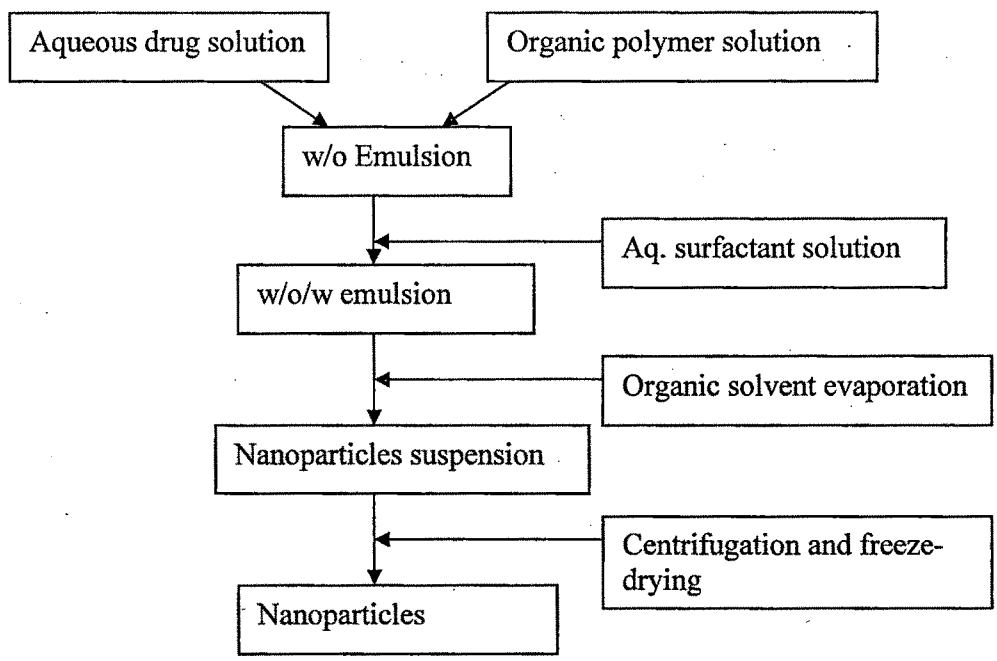



Figure 2.7: Schematic presentation: Double Emulsion- evaporation

**Salting –out**: This technique involves the addition of polymer and drug solution in a slightly water-miscible solvent such as acetone to an aqueous solution containing the salting out agent and a colloidal stabilizer under vigorous mechanical stirring. When this o/w emulsion is diluted with a sufficient volume of water, it induces the formation of nanoparticles by enhancing the diffusion of acetone into the aqueous phase (Freitas 2004). Figure 2.8 indicates schematic presenations for the salting out method.

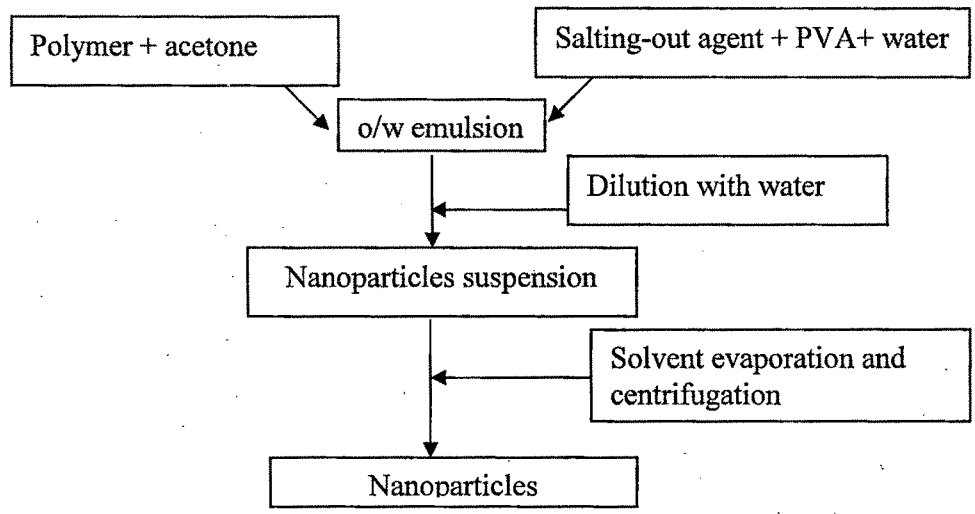



Figure 2.8: Schematic presentation: Salting out techniques

**Emulsification-diffusion**: This method is derived from the salting-out procedure. It involves adding of a polymer solution, in partially water miscible solvent (such as ethyl acetate, benzyl alcohol, propylene carbonate) presaturated with water, to an aqueous solution containing stabilizer under vigorous stirring. The subsequent addition of water to the system destabilizes the equilibrium between the two phases and causes the solvent to diffuse into the external phase, resulting in reduction of the interfacial tension and in nanoparticle formation (Jeyanthi1997).

**Nano-Precipitation:** This method is usually employed to incorporate lipophilic drugs into the carriers based on the interfacial deposition of a polymer following displacement of a semi-polar solvent miscible with water from a lipophilic solution (Leelarasamee 1998).

Figure 2.9 shows schematic presentation for nano-precipitation method.

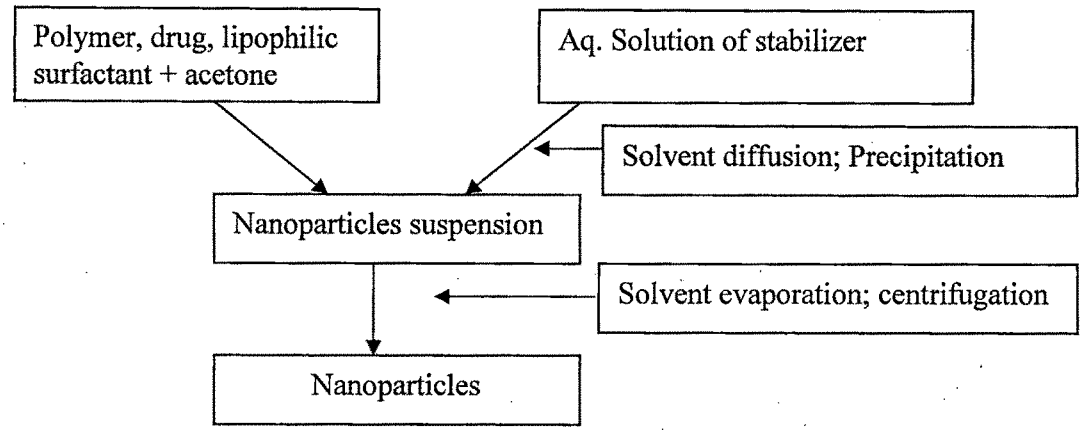



Figure 2.9: Schematic presentation: Nano Precipitation techniques

### 2.14.2 Techniques for characterization of Nanoparticles

Characterization of the nanoparticle carrier systems to thoroughly understand the properties is essential before putting them to pharmaceutical application. After preparation, nanoparticles are characterized at two levels. The physicochemical characterization consists of the evaluation of the particle size, size distribution, and surface properties (composition, charge, hydrophobicity) of the nanoparticles. The biopharmaceutical characterization includes measurements of drug encapsulation, in vitro drug release rates, and in vivo studies revealing biodistribution, bioavailability, and efficacy of the drug (Muramatsu 1995).

There are many sensitive techniques for characterizing nanoparticles, depending upon the parameter being looked at; laser light scattering (LLS) or photon correlation spectroscopy (PCS) for particle size and size distribution; scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) for morphological properties; X-ray photoelectron spectroscopy (XPS), Fourier transform infrared spectroscopy (FTIR),a and nuclear magnetic resonance spectroscopy (NMR) for surface chemistry; and differential scanning calorimetry (DSC) for thermal properties (as shown in Fig 2.10 below). Parameters such as density, molecular weight, and crystallinity affect release and degradation properties, where as surface charge, hydrophilicity, and hydrophobicity significantly influence interaction with the biological environment (Sansdrap 1993).

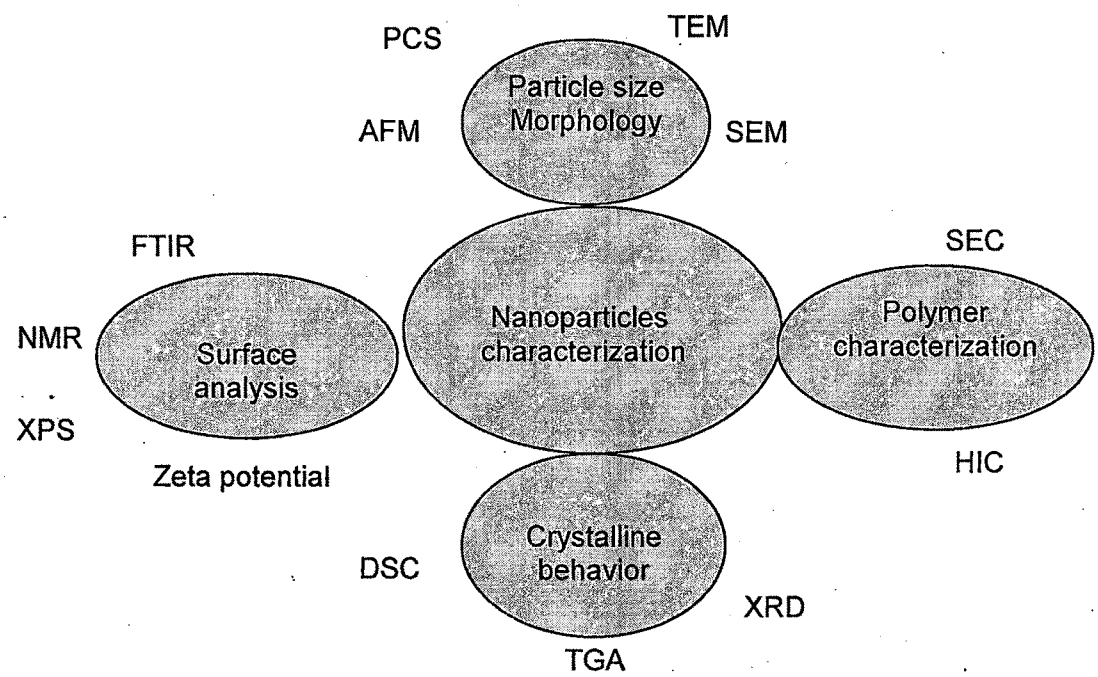



Figure..2.10 Various techniques to characterize nanoparticles.

**Particle size and Morphology**: Nanoparticle size is critical not only in determining its release and degradation behaviour (Dunne 2000) but also in determining the efficacy of the therapeutic agent by affecting tissue penetration or even intracellular uptake. Particle size can be determined by Photon correlation spectroscopy, Scanning electron microscopy, Transmission electron microscopy, Atomic force microscopy (Zhu 1990).

**Molecular weight:** Polymer molecular weight influences the nanoparticle size, encapsulation efficiency and degradation rate of the polymer, hence affecting the release rate of the therapeutic agent. A low molecular weight polymer can be used to prepare small-sized nanoparticles but at the expense of reduced encapsulation efficiency (Sepassi 2007). The average molecular weights and polydispersity of the polymers are found by size exclusion chromatography (Bootz 2005).

**Crystallinity**: The physical state of both the drug and the polymer are determined because this will have an influence on the in vitro and in vivo release characteristics

of the drug. The crystalline behaviour of polymeric nanoparticles is studied using X-ray diffraction and thermo-analytical methods such as differential scanning calorimetry (Bunjes 2007) (DSC) and differential thermal analysis (DTA) (Oh 1999). DSC and X-ray diffraction techniques are often combined to get useful information on the structural characteristics of both drugs and polymers.

**Surface charge:** Zeta potential is measure of the surface charge of the nanoparticles. The zeta potential value can influence particle stability and mucoadhesion as well as intracellular trafficking of nanoparticles as a function of pH. High zeta potential values, either positive or negative, should be achieved in order ensures stability and avoid aggregation of the particles. The extent of surface hydrophilicity can then be predicted from the values of zeta potential (Soppimath 2001). Surface charge is generally detrmined by well-known electrophoresis method with the help of zetasizer (Panagi 2001).

### 2.14.3 Stability of Nano-particles

Freeze-drying is one of the well established methods for the preservation of unstable molecules over long periods of time (Corveleyn 1996, Diminsky 1999). Most studies have shown a good preservation of the physicochemical properties of the particles when the cryoprotectant was employed in a sufficient concentration.

## 2.15 Animal Testing

Most of the vaccine candidates are first evaluated in mice model and the promising ones are subsequently taken up for evaluation in guinea pig model. But this strategy could be harmful in those potential candidates which are failed to show promising results in mice due to certain animal model limitations. Though guinea pig resembles more to the human in TB immuno-pathology, it has several limitations which preclude its use as first order screening model.

Mouse model of *M.Tb* infection was the first to demonstrate the roles of T cells and mononuclear phagocytes, the involvement of various T cells subset, importance of CD4+ T cells (Muller 1987, Caruso 1999), interleukin- 12 (Copper 1997, de Jong 1998) and tumor necrosis factor α (Bean 1999, Flynn 1995, Mohan 2001), while Guinea pigs are very sensitive to infection with *M. Tb* and well characterized (Mc Murray 1994).

Other than these two models, Rabbit, Cattle and Non-human primates can be used for screening candidates for Tb vaccine, not at preliminary level but definitely to get more insightful information at later stages of the studies (Orme 2005a).

### 2.15.1 Mice

The mouse model has been used extensively in the field of TB research, not only for vaccine testing, but also to examine the immune responses to TB infection. A considerable quantity of discoveries has led to increased knowledge regarding differentiation of T-cell sub-sets, cytokines, the function of macrophages and antigen presentation (Orme 2005b). Many of the discoveries relating to immunity that have been studied in mouse models have been as a result of gene disruption in the animals (Harboe 1996a, Chien 1996, Flynn 1995, Flynn 1993). In fact, more is known about TB in mice than humans (Malin 1996).

Mice are the cheapest animals to house, readily available, easy to handle (McMurray 2000). The main disadvantage of using mice in TB studies is that the pathology of the disease in this model does not correlate well with that in humans. Similarly, mice are not as susceptible to disease; they do not develop DTH skin test responses following vaccination (McMurray 2000).

The two strains of mice that have been used in most studies examining TB and immunity are C57BL/6 and BALB/c. It has been found that C57BL/6 mice have much higher levels of Th1 type responses and less readily succumb to infection (Wakeham 2000, Flynn 1996, Flynn 1995).

### 2.15.2 Guinea Pig

Compare to the mouse model, guinea pigs exhibit disease pathology that more closely compares to TB in humans (Elhay 1997). They are quite susceptible to infection following inhalation of the organism and develop the necrotic lesions seen in untreated humans (Harboe 1996a, Orme 1999a). Additionally, they exhibit strong DTH skin test responses (McMurray 2000) and remarkable success in vaccine-initiated protection has been observed in these animals (Brandt 2004). Guinea Pigs, however, are very expensive to keep under level III bio-safety condition (Orme

1999a) and specific immunologic reagents are rare (McMurray 2000). In spite of these disadvantages, the guinea pig is considered the gold standard for testing vaccine candidate against TB (Orme 2001).

### 2.15.3 Primates

Following success in guinea pigs, assessment of vaccine candidates in larger animals prior to human clinical trials has been advocated. Some believe the cow to be the best mode of human TB (Hewinson 2003). Equally, the primate model has also been promoted (Langermans 2005; Reed 2003). Primate models of TB vaccine trails have included Rhesus monkeys and more frequently, the cynomolgus macaque (Orme 2005b).

An alternative to using large animals for examination of vaccine candidates prior to clinical trials is a new method of in vitro testing BCG *lux* (Kampamann 2004). This new method of in vitro testing uses a BC strains that carry *lux* A and *lux* B genes from *Vibrio harveyi*, which enables luminescent detection that correlates with *mycbacterial* growth.

### 2.15.4 Human Clinical trails

In the human model particularly, there are defined steps and regulations for evaluating vaccine candidates to ensure that the vaccine administered is pure, non-toxic and effective (Ginsberg 2000, Brennan 2005). Other issues that must be addressed prior to clinical trials are: trial site (certain populations experience higher latent infection and active TB rates), logistic (include resources and trained staff), standardization of protocols (include vaccine administration and measurement of immune response), ethic approvals, definition of end-point, medical care and counseling and data management (Ginsberg 2000, Melles 2005).

There are three phase of clinical trials, each of which must experience success before progression to the next phase. Phase I, involving between 10 and 60 volunteers, establishes the safety of the vaccine in humans and determines the maximal dose that ca be tolerated. Phase II is a larger study involving 200-300 participants. In addition to observing any side-effects, it characterizes the immune responses to the vaccine and examines variable doses and schedules (Ginsberg 2000).

The scope of testing is broadened in phase III trials with 7000 to 300,000 subjects participating, and a trial period of 10 to 30 years may be required. Phase III trails can include three different designs. Pre-infection vaccine trials can be performed in children or young adult who have not previously been infected with mycobacteria. Complete prevention of infection is the obvious advantageous outcome; however these trials may be required to continue for 20-30 yrs of adequate evaluation. Alternately, post-infection vaccine trials can provide indications of efficacy with 5-10 years and a long-term follow up of naïve subjects (Ginsberg 2000).

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**Reviewer's comments**

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