# Chapter II

## Review of literature

### Tuberculosis, discovery and definition

Tuberculosis has been recognized as a widespread and grave clinical entity for many centuries, and its incidence was probably increased by the social consequences of the Industrial Revolution. However, its communicable nature was not recognized until Villemin produced a similar disease in rabbits in 1862, by injecting material from tuberculous lesions of man ; and in 1882 Koch discovered the tubercle bacillus , impressively fulfilling the criteria that he had developed for identifying the etiologic agent of an infectious disease. Since then,tuberculosis has been one of the most intensively studied infectious diseases. Not only has it been a major cause of death and prolonged disability, but also, until recent times, it usually struck people at the age of greatest vigor and promise (Davis, 1980).

Tuberculosis is a chronic infectious disease usually caused by *M. tuberculosis* and characterized by the formation of typical "tubercles" in various tissues of the body and associated with varying degree of necrosis called caseation. The disease attacks practically any organ such as nervous system particularly at meninges (tuberculous meningitis),gastrointestinal tract system or peritoneal tissue (tuberculous peritonitis or tuberculous ascites), lymph nodes, bones , etc. In addition to *M. tuberculosis*, tuberculosis can be caused by other mycobacteria such as *M. bovis*, *M. africanum* and *M. avium*. The disease caused by *M. bovis* has now

become very rare for years as a result of the widespread pasteurization of milk and the virtual elimination of tuberculosis in cattle. Tuberculosis caused by *M. africanum* has only been found in certain parts of Africa. *M.avium* , which was recognized as a distinct species prior to 1900, causes tuberculosis in chickens, pigeons and other birds but is rarely found in human. (Smith, 1981)

The mycobacteria

1. Classification of mycobacteria

The mycobacteria are considered as transitional form between eubacteria and actinomycetes : some of the latter , of the genus Nocardia, are weakly acid-fast, while some mycobacteria may exhibit branching. Accordingly, the mycobacteria has been classified in the order Actinomycetales, the family of Mycobacteriaceae and the genus of Mycobacterium.

Genus Mycobacterium , classified species based on the pathogenesis as ( Buchanan, 1974) :

a. Non-Rungon Group (table 1) : For example, *M. tuberculosis*, *M. leprae* , *M. bovis* , etc.

b. Rungon Group (table 2) : There are four groups such as group I,II,III and IV (based on growth rate and colonies characteristic). Another name of this group was "atypical mycobacteria".

Mycobacteria may also be classified according to only the growth rate as

a. Slow growers : Colonies are first seen on media usually more than or equal to two weeks of incubation. The organisms in this group are *M. tuberculosis* , mycobacteria in Rungon group I,II and III.

b. Rapid growers : Colonies are seen on media within only one week of incubation. The mycobacteria in this group are Rungon group IV.

2. *M. tuberculosis*

Tubercle bacilli in animal are typically slightly bent or curved slender rods, about 2 μm-4 μm long and 0.2 μm-0.5 μm wide. The rods may be of uniform width but more often appear beaded, with irregularly space, unstained vacuoles, or heavily stained knobs. In culture media, the cells may vary from coccoid to filamentous. Strains differ in their tendency to grow as discrete rods or as aggregated long stands, called serpentine cords or cord factor. The cells in infected tissue or sputum are longer than 3-4 μm and can be seen to be more curved than cells in culture and, of greater importance, cord formation is quite pronounced (Goren, 1972 and Youmans, 1979). They are non spore-forming, non motile and have no capsule or flagella. They are difficult to stain with the Gram stain but are considered strongly acid-fast as demonstrated in Ziehl-Neelzen or fluorochrome procedure. Unlike most other pathogenic bacteria, which are facultative aerobes or anaerobes, the tubercle bacillus is an obligate aerobe. The colonies are cream coloured, rough, raised thick with a nodular or wrinkled surface with an irregular thin margin.

The cell walls of mycobacteria contain peptidoglycan with diaminopimelate. The cells can be converted to spheroplasts by lysozyme. The walls have a remarkably high lipid content (up to 60%), much of which is attached to polysaccharide. These polysaccharides include glucan, mannan, arabinogalactan and arabinomannam. The glycolipid and protein are located in a firmly attached outer layer of the wall, and the external location of the lipid accounts for the hydrophobic character of the cells (Davis, 1980).

## 3. Growth characteristics and nutritional requirement

### a. Growth characteristics

The optimal temperature for growth is 35°-37° C and the optimal pH is 6.0 - 7.6 (Ratledge , 1976). The organism will grow best in 5-10% added $CO_2$ condition. The growth of *M. tuberculosis* is considerably slower than that of most other bacteria. Even under the optimal conditions, cell division requires 18-24 hours and colonies are first seen on media usually after 3-4 weeks (Robson, 1957 and Wayne, 1976).

### b. Nutritional requirement

#### 1) Carbon

Glycerol is the best available carbon source for growth of *M. tuberculosis*. It has been used as carbon source for this organism in several media preparation such as Lowenstein _ Jensen (L-J) medium, Middlebrook 7H10, 7H11 and 7H9 medium. In addition, glucose and pyruvate have been shown to be good carbon sources for mycobacterial growth (Redmont, 1957 and Mitchison, 1974).

Mycobacteria generally show a marked nutritional preference for lipids. Egg yolk has been a prominent constituent of the rich media used for diagnostic culture (L-J medium).

Though the tubercle bacillus is very sensitive to the inhibitory effect of long-chain fatty acids , it is stimulated by them at a very low concentration. A satisfactory concentration is maintained by the addition of serum albumin into the medium so the albumin would bind to the fatty acids at the sufficient affinity to maintain a low free concentration.

The examples of long-chain fatty acids are oleic acid and tween 80 (Lyon, 1974).

2) Nitrogen

Ammonium salts such as ammonium chloride ,ammonium phosphate , ammonium succinate and some amino acids are usually added to promote the initiation and improve the rate of growth of the organism (Redmond, 1957).

The amino acids used are asparagine (L-J medium, Kirchner medium) and glutamic acid (Ogawa medium, Middlebrook 7H10,7H11 and 7H9 medium).

It has been shown that L-asparagine controlled the ultilization of other amino acid by *M. tuberculosis* H37Ra in aerated, liquid synthetic media. In a mixture containing asparagine and either L-alanine or L-glutamic acid, amino acid ultilization is diphasic, with asparagine being ulilized first (Lyon, 1974).

Casein hydrolysate which contains a lot of trytophane is also used as nitrogen source (Mitchison, 1983).

Albumin , in Kirchner medium, comes from calf serum . In Middlebrook 7H11 and 7H9 medium , bovine albumin fraction V is added. This albumin is necessary for growth of INH-resistant *M. tuberculosis*.

In addition , catalase is also necessary for the growth of this organism. It was found that INH-resistant *M. tuberculosis* could not produce catalase enzyme (Cohn, 1946). Thus, the addition of catalase in the culture media is very useful as the growth promoter for *M. tuberculosis*, particularly the patient's strains which are often resistant to antituberculous agents.

3) Miscellaneous nutrients

Minerals that enhance growth of tubercle bacilli are zinc , phosphorus, magnesium and iron. Iron is important for glycerol ultilization of mycobacteria. These organisms could produce iron chelators (mycobactins), which compete with the host chelators (transferrins) and play a role in pathogenesis and drug resistance. Absorption of iron to mycobacterial cell requires oxochelin to dissolve ferric ion and requires mycobactin to transport this ion through cell wall. Thus, iron salts should be added to the media with serum containing transferrin, which could chelate iron in serum, in order to protect the lack of iron (Davis, 1980).

Biotin was either markedly stimulating or even absolutely required for the artificial cultivation of several strains of *M. tuberculosis* (Schaefer, 1955).

Jaquess (1981) showed that selenium in media containing L-cysteine HCl and iron in the form of ferric pyrophosphate or ferrous ammonium sulfate enhanced growth of *M. tuberculosis* but did not enhance growth as did media containing selenium alone. In contrast, Stauffer (1982) found that there was no significant enhancement of growth by the addition of sodium selenate to either Lowenstein-Jensen or Middlebrook 7H11 media. Selenate supplementation had no consistent growth stimulatory effect as compared to the nonsupplemented media.

4. Chemical structure

a. Lipid : The most chemical feature of the mycobacteria is their high lipid content. Various lipid fractions are defined by the conditions used for their extraction from dried organisms. The striking abundance of lipids in the cell wall (up to 60% of its dry weight) accounts for the hydrophobic

character of the organisms. Among the lipid extracted with neutral organic solvents are true waxes (esters of fatty acid with fatty alcohols) and glycolipids (mycosides : lipid-soluble compounds with covalently linked lipid and carbohydrate moieties).

1) Mycolic acid : While many different fatty acids are found in mycobacteria, the mycolic acids appear to be unique to the cell walls of these organisms, nocardiae and corynebacteria. These very large, saturated, $\alpha$-alkyl, $\beta$-hydroxyl fatty acids are found in both free and bound form (glycolipid) (Goren, 1972).

2) Cord factor : Cord factor in which acid fast bacilli are arranged in parallel chains is a mycoside that contain two molecules of mycolic acids which are esterified to the disacharide trehalose (tetrahalose-6,6 dimycolate). The cord factor can be found only in virulent strains. It was found that cord factor could inhibit the migration of polymorphonuclear leukocytes which caused chronic granulomatous formation. In addition, it could bind to mitochondrial membranes and caused the functional damage to respiration and oxidative phosphorylation (Bloch, 1950 and Kato, 1970). However, role of cord factor in the patho genesis of tuberculosis is unknown.

3) Sulfatide (or sulfolipid) : Sulfatide is a group of glycolipids similar to cord factor, which are multiacylated trehalose 2-sulfates. They have been shown to inhibit phagosome-lysosome fusion and, as such, seem to enhance survival of phagocytosed mycobacteria (Goren, 1976).

4) Wax D : Wax D is not a true wax but is a peptidoglycolipid (mycolic acid plus glycopeptides) in which 15-20 molecules of mycolic acids are esterified to a large polysaccharide which appear to be linked to the peptidoglycan of the cell wall. When emulsified with water and oil, wax D acts as an adjuvant to increase the antibody response to an antigen, and it is probably

the active component in Freund's complete adjuvant.

b. Polysaccharides : There are protein-free polysaccharides of four principal types, arabinogalactans, arabinomannans, mannans and glucans from both mycobacterial culture filtrates and cell wall extracts (Daniel, 1978). There is a high degree of chemical similarity or identity among polysaccharides isolated from various mycobacteria (Azuma,.1970 and Birnbaum, 1968).

c. Protein : *M. tuberculosis* processes a number of protein antigens that,by themselves, do not appear to be toxic or involved in virulence. It is probably that all cytoplasmic proteins of mycobacteria are antigenic in man and laboratory animals. They can elicit the formation of a variety of antibodies and also induce tuberculin sensitivity. Many antigens possess species specificity (Daniel, 1978).

5. Antigenic structure

Chromatographic fractionation of extracts, followed by immunodiffusion, has revealed that there are at least twenty antigens in *M. tuberculosis*. Some are species-specific, others are common to many mycobacteria and even nocardiae and corynebacteria. Strain or type-specific antigens are also identified by agglutination reactions and by skin testing in guinea pigs with partially purified protein (PPD) from diverse mycobacteria (Janicki, 1971).

Mycobacterial antigens are :

a. Lipid antigen : Phosphatidyl inositol mannosides (PIM) is glycolipid that can elicit antibody production and is also named serologically active glycolipids (SAG) (Khuller, 1971). A strain-specific phenolpthiocerol glycolipid has recently been reported in the Canetti strain of *M. tuberculosis* (Daffe, 1987). More recently, an analog of the phenolic glycolipid of *M. leprae* has also been found in *M. tuberculosis* H37Rv (Makonkaw-

keyoon, 1989).

b. Polysaccharide antigen : Arabinomannan has recently been shown that the native molecule is not a free polysaccharide but possesses a lipid tail so called lipoarabinomannan (LAM) (Hunter,1986). LAM and arabinogalactan have been shown to have antigenic property. They are capable of eliciting immediate skin reactions, serological reactions but incapable of eliciting delayed skin test reactions. Arabinomannan has also been shown to have immunosuppressive property (Daniel,1978).

c. Protein antigen :

1) Old Tuberculin (OT) : The crude culture filtrate antigens, designated as old tuberculin, was first prepared by Robert Koch in 1890 and subsequently used as a skin test reagent (OT is made from heated sterilized culture filtrate of tubercle bacilli and concentrating to one tenth of its original volume).

2) Purified protein derivative (PPD) : PPD consists of small molecular weight proteins, ranging form 2,000-9,000 daltons (Joklik, 1988). It is a protein fraction of OT, which has been wildely used as a standard antigen for the study of macrophage migration inhibition and blastogenesis in cultured lymphocytes (Schrek, 1963 and David, 1964).

3) US-Japan antigen : These antigens were isolated by simple one dimensional electrophoresis of unheated culture filtrate antigen prepared from *M. tuberculosis* H37Rv and a homologous polyvalent goat antiserum (Janicki, 1971). They were eleven antigens. The antigenic analysis showed that the antigen 1 and 2 are cell wall polysaccharides and antigen 4-11 are proteins. Antigen 5 , was found to be limited only to culture filtrates of *M. tuberculosis* and *M. bovis* (Daniel, 1978). However, subsequent data showed that antigen 5 still contained nonspecific epitopes (Olds,1987).

Pathogenesis

*M. tuberculosis* does not produce toxins and destructive enzymes. Thus, the consequences of inhaling tubercle bacilli depend on the virulence of the organisms and resistance of the host as well as the size of the inoculum. Since there is no immediate host response, the organism could grow and multiply until the development of an immune reaction. This organism is capable of prolonged survival in macrophages, probably due to the failure of fusion of tubercle entrapping phagosomes with lysosomes that contain microbicidins and hydrolytic enzymes that help to kill the bacillus and break down its components (Joklik, 1980 and Schlossberg, 1983). The initial infection in a tuberculin negative individual, most often produces a self limited lesion but sometimes the disease may progress. A few bacilli may survive for years in a dormant stage and the reactivation can occur whenever the host immunity has decreased as a results of malnutritions, diabetes mellitus, or immunosuppression. The primary infection is usually through inhalation of infectious droplet nuclei (1-5 $\mu$ in sizes) and is asymptomatic (Rosenthal, 1983). The lung is usually the first organ involved. The tubercle bacilli can produce infection in almost all tissues and organs in the body. The extrapulmonary tuberculosis is the dissemination from an initial pulmonary focus (Youmans, 1979 and Dannerberg, 1982). The inhaled bacilli reach the alveoli and normally are ingested by alveolar macrophages. In such macrophages, the bacilli will be destroyed or inhibited or else can multiply intracellularly. If they can multiply, the initial macrophages will die, the released organisms will again be ingested by other alveolar macrophages and by transformed macrophages emigrating from the pool of circulating monocytes. Thus, this causes the initial lesion which appears to be an exudative type, consisting of an acute or subacute inflammatory

reaction, with edema fluid , polymorphonuclear leukocytes and later monocytes around the tubercle bacilli. After then, the organism will be carried to the draining lymph node and, by way of lymph and blood, throughout the body of the host. Lymphocytes in lymph node become activated and released lymphokines, which will initiate the cell mediated immunity (CMI). After two to four weeks of infection, cellular immunity or delayed type hypersensitivity (DTH), which enable the host to increase the ability to destroy the bacilli by means of local accumulation and activation of macrophages, has developed. Activated macrophages are rich in lysosomal enzyme and produce relatively large amounts of reactive oxygen intermediates and other microbicidins (Dennenberg, 1989). At the same time, many of these macrophages are killed by the bacilli and their tuberculin-like products. The exudative lesion now has changed to the productive or granulomatous type which consists of multinucleated giant cells containing tubercle bacilli at the center and surrounded by lymphocytes, monocytes and proliferating fibroblasts. Such lesion is called a tubercle. Latter, caseation necrosis occured, in which the necrotic center of the tubercle remains semisolid rather than softening to form pus, presumably because the enzymes that usually liquefy dead cells and tissue are inhibited. Caseous lesion usually heal by fibrosis or calcification.

The secondary infection is usually due to reactivation of long dormant foci remaining from the primary infection and rarely by bacilli newly inhaled from the environment. Secondary tuberculosis is characterized by more chronic tissue lesion and a predominantly "productive" type of tissue response (the formation of tubercle, caseation and fibrosis). By the time the disease is recognized, liquefaction of the caseous lesion usually has occured and a cavity has provided a favorable site for the rapid proliferation of the bacilli.

Epidemiology

The incidence of tuberculosis may be estimated by tuberculin test surveys as well as from reports of active cases or of deaths. The frequency is higher in improverished social groups that leave under crowded condition, compared with the more affluent and with those who live in sparsely populated regions. (Braude, 1981).

The world-wide prevalence is estimated to be 15-20 million cases of active tuberculosis and at least 3 million deaths each year. Tuberculosis ranks first as the cause of death among reportable infections (Immunological research in tuberculosis,1982). In developing countries, as many as 25% of children between ages 5 and 6 years and 75% or higher of those age 40 years and over, react positively to tuberculin (prevalence rate),over 90% of Thai population above 30 years old are tuberculin positive (Sunakorn, 1969).

Tuberculosis is among the main public health priorities of many countries including Thailand. In Thailand, the epidermiologic surveys of tuberculosis in 1991 (from 1 January to 15 July) for six and a half month period showed that the patients with tuberculosis were totally 6,983 cases with the morbidity rate 12.4 per 100,000 populations and mortality rate 1.3%. Among these cases they were pulmonary tuberculosis 6,696 cases (95.9%), tuberculous meningitis 142 cases (2.03%) and others tuberculous system 145 cases (2.07%). Morbidity rate were high in adult group with the ages of 35 years or over. The incidence in male was twice as much higher than in female (Division of epidemiology,1991).

In addition, the mortality rate of tuberculosis from 1986 to 1989 were shown to be 9.8,10.2,8.2 and 7.6 per 100,000 populations, accordingly. This made tuberculosis to be the fourth leading

cause of death in Thailand (Konjanart, 1987 and table 1).

However, the epidemiologic study has indicated the slow decrease of incidence of tuberculosis in Thailand. One of the reason is that a number of effective anti-tuberculous drugs has been developed during the present decade.

## Chemotherapy and drug resistance

### 1. Chemotherapy

Before the specific therapy has become available, with the discovery of streptomycin (STM) in 1945, the treatment of tuberculosis was limited to rest. Unfortunately, the value of STM was restricted by its toxicity to the eighth cranial nerve on prolonged administration, as well as by the frequent emergence of resistance to tubercle bacilli during therapy. The real revolution in tuberculosis therapy came with the introduction of isoniazid (INH) , which is more effective, because it is bactericidal agent and has an effect on intracellular as well as extracellular tubercle bacilli. Moreover, it is remarkably free from toxicity, inexpensive, and easy to take (i.e., small doses, given orally). The rapid response to INH and the low toxicity have made ambulatory treatment feasible. Rifampicin (RMP), which has been introduced later, is also very effective, relatively nontoxic, and absorbed very well when given by mouth. The combination of RMP and INH may prove to be the most effective regimen (primary drugs). The p-aminosalicylate (PAS), used as a companion to INH, has been largely replaced by ethambutol(EMB). Other useful drugs include kanamycin, capreomycin (injected), cycloserine, ethionamide and pyrazinamide (given orally); they are generally used only in the presence of allergy or resistance to the primary drugs.

In general, patients must be treated with a combination of available drugs, since each drug was active against the resistant mutants to other drugs. They are many anti-tuberculous drugs available today. The standard regimen is 9-month daily course of therapy with INH,RMP and EMB. (Grosset,1989). However, the short - course chemotherapy of 6 months' duration was found to be as effective as the standard 9-month regimen. The drug combination of INH, RMP,PZA and either EMB or STM, has now become routinely practice in many countries (Farga, 1983, Algerian Working Group, 1984 and Fox, 1985)

STM has bactericidal effect against *M.tuberculosis* but is not effective against organisms in macrophage. PAS has only bacteriostatic action. It interferes with the conversion of p-aminobenzoate to folic acid. It is also relatively ineffective against bacteria localized within macrophages, presumably because of the presence of metabolites that reverse its action. RMP also has bactericidal action against tubercle bacilli, including those in macrophages. INH acts by interfering with mycolic acid synthesis, which explains why it is effective only against mycobacteria.

2. Drug resistance

Drug - resistant mutants in patients with tuberculosis is favored by several features of the disease. The multiplication of bacteria in the lesions, especially in the walls of cavities ; during the required long periods of therapy ; and the limited degree of host resistance provide the rare mutant cell with a good opportunity to proliferate before being eliminated by host defenses. Hence, specifically resistant strains often appear in patients treated with any one of the drugs used singly. The

rationale for preventing the emergence of resistant strains is the use of the combined therapy.

The problems of chemotherapy include the poor compliance of the patients and the occurence of drug-resistant mycobacteria after treatment with standard regimen, which implies the unsuccessful therapy. The poor compliance is related to the duration of treatment, the number of drug used , the frequency of administration of each drug and the toxicity of the drugs (Reichman, 1987) Therefore, the new chemotherapy is needed for improving the compliance and attacking the resistant mycobacteria.

Immune response

In 1982, Robert Koch was the first scientist who described the reinfection phenomenon that still bears his name as the Koch phenomenon. The primary dermal infection site was associated with a slowly progressive, localized granulomatous response with extensive lymph node involvement. The secondary infection site developed an early localized indurative response that peaked at 72 hours, followed by rapid healing. Koch went on to show that a similar local swelling response could be induced in the skin of tuberculous individuals by the intradermal injection of heat-killed tubercle bacilli or tuberculin (Collin, 1982).

1. Cell mediated immunity (CMI)

CMI usually develops within 2-4 weeks after infection. The development of CMI requires cooperation of two cell types, specific lymphocytes which act as specific inducers, and mononuclear phagocytes, which serve as nonspecific effector cells.

The role of T cells is to recruit mononuclear phagocytes for the formation of granulomatous lesions and activate the phagocytes to enhance the bactericidal activity within the lesions. The expression of immunity ultimately depends on the performance of nonspecific effector cells, the activated macrophages. The activation of macrophages is mediated by lymphokines released by immunocompetent T cells when they contact processed antigens of the tubercle bacillus. There are many types of lymphokines such as migration inhibitory factor (MIF), chemotactic factor, mitogenic factor, etc. MIF inhibits the migration of macrophages and lymphocytes from the sites of infection. Chemotactic factor causes the accumulation of macrophages to the same area while mitogenic factor causes the multiplication of the macrophages and lymphocytes. The most important lymphokine is the macrophage activating factor (MAF) which activates the normal macrophage to become activated macrophage which is very effective in the phagocytosis of tubercle bacilli. In addition, activated macrophages can be directed against a number of unrelated microbial species, viruses and tumor cells (Edwards, 1986,).

2. Humoral immunity (HI)

The primary humoral immune response to tuberculosis appears to be an initial rise in IgM followed by a rise and persistance of IgG (Grange, 1984) The role of antibodies in immunity to tuberculosis is not clear. Serum antibodies do not account for this immunity. Thus, though the tubercle bacillus enhances formation of antibodies to a wide variety of immunogens, antibodies to proteins and polysaccharides of the tubercle bacillus itself are generally found only in low titers in tuberculous

individuals, and the levels observed have no prognostic value. Moreover, these antibodies are not bactericidal *in vitro* , even in the presence of complement ; and though they promote phagocytosis of tubercle bacilli *in vitro*, the organisms multiply within the phagocytes. Finally, increased resistance to infection cannot be transfered passively with serum, but it can be transfered with viable lymphoid cells. This may cause the delayed-type hypersensitivity to tuberculin. This implies the role of CMI in host defense against tubercle bacilli but not the role of HI. Thus, humoral antibodies may be useful only for clinical staging or serodiagnosis of infected persons.

3. Complement and immune complexes

Total hemolytic complement in serum is increased in patients with tuberculosis (Reiger, 1979). In addition, circulating immune complexs (CIC) which are immunoglobulins (IgA, IgG, IgM) and mycobacterial antigen (Bhattacharya, 1986) have been found in serum of these patients (May, 1983 and Samuel, 1984). The CIC concentration is increased in patients with poor prognosis (Brostoff, 1981). The increased CIC concentration may also have a role in the granuloma formation (Spector, 1969).

## Diagnosis

1. Clinical and history feature

Tuberculosis can involve in almost every organ system. Its clinical symptoms are divided into two categories, constitutional and local symptoms. The constitutional symptom most frequently seen is fever. Characteristically, the fever develops in the late afternoon and may not be the dominant symptom. Other

sign such as weakness, lost appetite and weight loss may also be present (Holden, 1971).

The local symptoms are divided into three categories as followed :

a. Respiratory symptom : Respiratory symptom occurs in patients with pulmonary tuberculosis who usually have pleural effusion. These symptoms include cough, dyspnea and chest pain.

b. Gastrointestinal (GI) symptom : GI symptom in patients with tuberculous peritonitis mainly is ascites. Diarrhea, abdominal pain, nausea and vomiting are often found.

c. Nervous system symptom : The symptom in patients with tuberculous meningitis usually are headache, drowsiness, stiffness-neck or stiffness-back and paralyse.

The example of patient history include underlying disease, smoking, alcoholism, life style, contacted tubercle bacilli or tuberculosis, etc.

Clinical and history feature are useful for the diagnosis by physicians. However, similar clinical and history feature may also be found in patients infected with other infectious agents such as atypical mycobacteria, some pathogenic bacteria or fungi and patients with carcinoma.

2. Chest X-ray examination

It can be possible that the abnormal changes in the chest X-ray examination in patients with pulmonary tuberculosis is similar to those patients with other diseases such as lung carcinoma, etc.

The most common characteristic in chest X-ray of pulmonary tuberculosis is the presence of a parenchymal infiltration and enlargement of hilar or mediastinal lymph nodes with or without cavitation. These changes are characteristic of a granulo-

matous infection of the lung and are not limited to tuberculous infection but are also seen in coccidioidomycosis, histoplasmosis and other non-mycobacterial pneumonia (Reichman, 1975 and Khan, 1977). Another common radiographic manifestation of tuberculosis is the appearance of a pleural effusion which represents an extension of the initial parenchymal tuberculous focus to the pleural surface. The effusion is the result of netincreases influx of fluid into pleural space by the hypersensitivity reaction to "tuberculoprotein".

Although radiographic abnormality should be interpreted as consist with tuberculosis, the diagnosis should be confirmed by clinical evaluation, particularly skin test in non-endemic area and bacteriological studies to establish the presence of tuberculosis (Wehrle, 1981).

3. Tuberculin skin test

Delayed-type hypersensitivity to tuberculin is highly specific for the tubercle bacilli and closely related mycobacteria. Reactivity appears about one month after infection in man and persists for many years, often for life ; hence the frequency of reactors in the population increases cumulatively with age. A positive test thus reveals previous mycobacterial infection ; it does not establish the presence of active disease.

The persistence of hypersensitivity probably depends on persistence of bacilli in dormant foci, and reactivity may disappear following chemotherapy of recent infection.

Advantage of tuberculin test : The tuberculin test can provide valuable information regarding :

a. The prevalence of tuberculosis in the community.

b. The prevalence of opportunistic mycobacterial infection.

c. The effectiveness of control measures against tuberculosis.

d. The differential diagnosis of tuberculosis.

e. The differential diagnosis and assessment of conditions which may influence type IV reactivity.

f. Those who may require BCG vaccination.

g. Those who may require chemophylaxis.

(Caphin, 1980).

Disadvantage of tuberculin test : Tuberculin skin test can be found both false negative result and false positive result.

False negative result can occur : in patients with : (Comstock, 1975)

a. Overwhelming disease.

b. Associated lymphoproliferative disease

c. Malnutrition

d. Circulating adherent suppressor mononuclear cells ; these patients frequently have an increased number of peripheral blood monocytes.

e. An acute viral illness such as exanthemata or influenza , live virus vaccination, during the 6-8 weeks interval following first attack of *M. tuberculosis*.

f. Prolonged corticosteroid therapy.

g. The sarcoidosis syndrome associated with tuberculous disease.

h. Using the out-dated tuberculin, inaccurate technique, etc.

False positive results may be attributed to a number of technical or biologic cause including : (Pepys,1955 and Chaparas, 1978).

a. Some sensitive individuals may develop local ulceration and necrosis formation.

b. The patients have already had BCG vaccination.

c. The poor technique of injection used.

d. Tuberculin is not consequently specific because it is a biological product from *M. tuberculosis* which shares antigens with other atypical mycobacteria.

Two types of tuberculin are in use, OT and PPD. There are several testing procedures (Caphin, 1980) such as The Von Pirquest test, The Vollmer test, The multiple-puncture test and Mantoux test. The last test is intradermal tuberculin skin test. It is the standard initial procedure for the detection of tuberculous infection. The test is read 48-72 hours after the injection. The reading consists of the measurement of the greatest diameter of induration in millimeters, the tuberculin specific material injected and its strength.

Laboratory diagnosis

The definifive diagnosis of tuberculosis is to demonstrate the tubercle bacilli in the clinical specimens. Therefore , the laboratory tests are very useful. These tests include :

1. Direct microscopy :

Direct microscopy of acid-fact bacilli (AFB) is the most rapid and simple procedure for the detection of tubercle bacilli in clinical specimens.

Two types of staining method commonly used are carbol-fuchsin and fluorochrome.

a. Carbol-fuchsin method : This method can be performed using either Ziehl-Neelsen or Kinyoun technique . In Ziehl-Neelsen technique , heating is required to promote the complete formation

between dye and mycolic acid whereas the increased amounts of basic fuchsin by phenol is used in Kinyoun or cold technique (see the formulas of dyes in appendix part). Carbol-fuchsin binds with mycolic acid to form fuchsin-mycolic acid complex, which tolerates to the decolorization with acid alcohol. After counterstaining with methylene blue, tubercle bacilli appear as red rod against the blue background observed under oil immersion len of the microscope.

b. Fluorochrome method : The commonly used staining reagents are auramine O and rhodamine B. After decolorization and counterstaining with potassium permanganate, the bacilli appear as bright yellow rods against the dark background observed under fluorescent microscope. Modifications of the auramine fluorochrome stain include the use of rhodamine, giving a golden appearance to the cells ; or the use of acridine orange as a counterstain, resulting in a red to orange background (Pollock, 1977).

The advantage of fluorochrome staining is that this staining method is more sensitive than carbolfuchsin technique. Fluorochrome-stained smears can be scanned using a 25 x objective (Carbolfulsin-stained smears is used a 40 or 100 x objective) thus, a significantly larger area of the smear can be scanned per unit time ; an oil immersion objective is required for viewing smear stained with carbolfuchsin. A sharp contrast between the brighly colored mycobacteria and the dark - background offers a distinct advantage in scaning the fluorochrome - stained slide.

The disadvantage of fluorochrome staining is that it is an indiscriminate staining of non viable and viable organism, so mycobacteria killed by chemotherapy may be also stained. The questionable fluorochrome results may be confirmed by carbolfuchsin method.

Although the acid-fast staining is available for the diagnosis of pulmonary tuberculosis but it is rarely useful for the diagnosis of extrapulmonary tuberculosis or for the diagnosis of pulmonary tuberculosis with minimal amount of tubercle bacilli. At least $10^4$-$10^5$ bacilli per millilitre of specimens are required for the detection of the organism by acid fast staining (Toman, 1979). The sensitivity of acid-fast staining is variable while the specificity is usually high. Boyed et al.(1975) showed that the sensitivity of the staining was 45% and specificity was 99.5% while Lipsky et al.(1984) showed that the sensitivity is 33.0% and the specificity was 99.8%. Similar to the study by Burdash et al. (1976) which showed that the fluorochrome staining method had the sensitivity ranged from 22% to 43% and the specificity ranged from 99.3% to 99.9%.

It can be concluded that the acid fast staining method is quite specific for acid fast bacilli. Eventhough it was also found that this staining method can also stained some Norcardia (Beaman, 1973), head of human sperm (Berg, 1954), the hooklets of *Taenia echinococcus* (Brundelet, 1973) and the embryophares of *Taenia saginate* (Pawlowski, 1972). However, besides the direct microscopy, additional laboratory tests should be necessary for the diagnosis of tuberculosis.

2. Culture

The culture of specimens for tubercle bacilli yields a more positive result than that by microscopy. Cultivation of clinical specimens is the only way to obtain pure isolates of *M. tuberculosis* for subsequent identification, confirmation and antimicrobial agent susceptibility tests. The cultural procedures are as followed :

a. Specimens collection

When pulmonary tuberculosis is suspected, specimens should be a deep respiratory expectorated sputum. Containers of specimens should be sterilized before collection. If the specimens have some materials which can produce coagulation, the containers should be filled with anticoaglulant such as heparin, sodium citrate, etc. These specimens are pleural effusion, ascites, blood, etc.

b. Process before inoculation

The purpose of this step is to homogenize, liquefy and decontaminate the specimens, thus liberating bacilli that are entrapped in mucous and cell debris, and eliminating other bacteria insofar as possible. The process can be accomplished by digestion of the specimens with alkali, acid, enzyme or mucolytic agent, then followed by chemical treatment.

The commonly agents used for this process are : (Lennette, 1980)

- N-acetyl-L-cysteine (NALC) plus 2% NaOH
- Dithiothreitol plus 2% NaOH
- 13% Trisodium phosphate plus benzalkonium chloride (Zephiran)
- 1% Cetyl-pyridium chloride plus 2% NaCl
- 4% NaOH
- 3% Lauryl sulfate plus 1% NaOH
- 4% Sulfuric acid
- 5% Oxalic acid

The processed specimen is then concentrated by centrifugation and the sediment obtained will be to inoculate into the culture media.

c. Cultures preparation

In order to isolate the tubercle bacilli, enriched media should be used. The example of these enriched culture media are as followed : (The formula of each media was shown in the appendix)

1) Solid media : There are two types of solid media used : egg based and agar based media

a) Egg based media : Egg based media which contain egg yolk, are coagulated by heating (80-90°, 30 min). The example of these types of media are the Lowenstein-Jensen (L-J) medium, most commonly used, Ogawa medium, Gudohs' P.D. medium, etc.

The mycobacterial laboratory , Siriraj Hospital, Bangkok, Thailand, used L-J medium for isolation of mycobacteria. From the data in 1990 showed that the most positive culture for TB (960 out of 1,024 or 93.75%) was from sputum specimens which often contain higher amount of tubercle bacilli than other specimens (table 3). The positive culture from pleural fluid and CSF were 8 out of 313 or 2.55% and 4 out of 234 or 1.71%, respectively. No positive culture from ascitic fluid, gastric washing and urine.

Comparative study of the WHO culture method (L-J medium) and Ogawa's culture method (Ogawa medium) for isolation of tubercle bacilli from sputum was shown that there were 33.3% positive with the L-J method and 37.5% were positive with the Ogawa's method. In addition, the comparative study of L-J method and the new method with modified Ogawa medium (Kudoh medium) was shown that 16.9% and 17.9% were positive culture, respectively. (Kudoh, 1974). Long after that, Kudoh medium was then modified to Gudohs' P.D. medium. This medium was compared to Ogawa medium on routine mycobacterial laboratory. The result was found that 82.24% and 84.13% were positive culture on Ogawa medium culture and Gudohs'

P.D. medium culture, consequently. The growth rate in Gudohs' P.D. medium was better than Ogawa medium, 60.83% and 50.66% were positive culture at three weeks of incubation, respectively (Pumprueg, 1990).

b) Agar based media : Middlebrook 7H10 and 7H11 media contain agar, could enhance the visualization of characteristic colonies of *M. tuberculosis*. The formula of Middlebrook 7H11 medium is very similar to that of the 7H10 except that pancreatic digest of casein USP (1g/litre)is also added.

Nuchprayoon (1980) investigated the growth of *M. tuberculosis* in 7H10 agar medium containing two different kinds of the enrichment, the OADC (oleic - albumin - dextrose - catalase) and the bovine serum. These media were inoculated with 94 strains of *M. tuberculosis* isolated from patients. Growth in both media were assessed weekly for 4 weeks of incubation. Of the 66 paired cultures (excluding contaminations), 59 and 57 of which contained bovine serum and OADC respectively demonstrated growth by the end of fourth week of incubation. This experiment suggested that bovine serum which was cheaper and easier to prepare in the laboratory could be substituted for OADC as an enrichment in 7H10 medium.

Mitchison (1973) compared selective 7H11 medium (Middlebrokk 7H11 medium plus antibiotics ; see appendix) with L-J medium for isolation of tubercle bacilli from 490 tissue specimens. He showed that 7H11 medium was the more efficient culture medium by demonstrating the 2.4% positive cultures on selective 7H11 medium as compare to 1.8% positive cultures on L-J medium.

2) Liquid media : There are several formula of liquid media. For example : Sula medium , Kirchner medium, Middlebrook 7H9 medium, etc. The formula that have been reported to be useful for diagnosis of tuberculosis are as followed :

a) Sula medium : The comparative efficacy study of Sula medium and L-J medium in primary isolation of 41 strains of *M. tuberculosis* showed 36 positive cultures in Sula medium and 34 positive cultures in L-J medium. There was no difference in the number of contamination (Sula, 1960).

b) Kirchner medium : In 1932 , Kirchner medium was first used for isolation of mycobacteria. Kirchner medium was then modified by the addition of 0.5 g/litre casein hydrolysate and the substitution of 10% calf serum for horse serum. This medium was called modified Kirchner medium. It was then made selective by the addition antibiotics and was called selective Kirchner medium. In 1973, Mitchison performed the experiment which showed that selective Kirchner medium increased the yield of positive cultures as compared to that abtained by using L-J and selective 7H11 media (Mitchison,1974).

In 1983, Allen et al examined the specimens obtained from the operation of patients with spinal tuberculosis for *M. tuberculosis*. The media used were L-J, selective 7H11 and selective Kirchner media. Cultures in Kirchner media were never found to be contaminated and were twice more positive cultures than those in the L-J media. Nine from fifty-two patients were found to be culture positive using the selective Kirchner media. In addition, the number of positive cultures in Kirchner medium were also more than those in selective 7H11 medium. In the same year, Mitchison used the same set of media as in Allen's study in the culture of specimen other than sputum for mycobacteria. The results showed that the use of L-J medium alone would yield only 64% positive culture. The use of selective Kirchner medium increased the yield of positive to 98%. Selective 7H11 medium was less contaminated than selective Kirchner medium but selective Kirchner media showed higher positive yield than selective 7H11 medium.

In 1984, Sparham et al performed the comparative study in the isolation of mycobacteria by using selective Kirchner medium and L-J medium. They found that selective Kirchner medium was much more superior than conventional medium (L-J medium).

c) Fluid medium : In 1984, Leelarasamee, and Trakulsomboon used this medium for isolation of tubercle bacilli from CSF which was the clinical specimen that contained a very low number of the organism. The results showed one positive culture from totally 25 specimen cultures.

d) Middlebrook 7H9 medium : Martin (1989) found three cases of mycobacteria infection by using 7H9 medium while these cases were all failed in solid media (L-J and selective 7H11 media). He then suggested the use of liquid media in addition to egg-based or agar-based media, especially for isolation of mycobacteria from specimens with small numbers of bacilli.

e) Transport medium : A single step method for the digestion and decontamination of sputum in order to culture the tubercle bacilli was described by Vasanthakumari(1990). This method employed a transport medium. Sputum was collected directly using this medium. The method was found to be superior to several other commonly used techniques and it also avoided the problem of lengthy, multistep procedures with associated risks of contamination from the atmosphere, costly reagents, etc. The single step method was superior to the NALC-NaOH method with respect to contamination rate, through not significantly to culture results.

3. Guinea-pig inoculation (GPI) : Guinea-pig is very susceptible to *M. tuberculosis* , so GPI is useful for diagnosis of tuberculosis except that the animal experiment is costly. Fortunately, the improved culture methods for *M. tuberculosis* have very greatly reduced the need for guinea-pig inoculation (Morris, 1983 and Martin, 1989)

4. Serological tests

The development of many serological techniques for diagnosis of tuberculosis is really necessary because there are a lot of problems concerning in isolation and identification of the tubercle bacilli such as the low sensitivity of acid-fast staining, the long time of culture method and the difficulty in obtaining sputum from patient with less extensive disease or extrapulmonary disease , and from children, who characteristically do not produce sputum.

The serological techniques involve the demonstration of antibodies to mycobacterial antigens, the mycobacterial antigens and the immune complexes in various clinical specimens (Dhand, 1986 and Leelarasamee, 1989). The examples of the serological tests are :

a. Precipitation and gel diffusion test

This method has been used for the detection of antibodies in serum against *M. tuberculosis* antigens. The ability of antigens and antibodies to form insoluble complexes has been widely exploited in serological investigations. The antigen and antibody were allowed to diffuse towards each other in a gel to form a precipitin line.

Diffusion in gel techniques were first used for diagnosis of tuberculosis by Parlett et al (1958). They used the culture filtrates of *M. tuberculosis* H37Rv as antigen and found the positive results of anti - culture filtrate of *M. tuberculosis* H37Rv in sera of 49 out of 52 tuberculous patients sera (sensitivity = 94%) and only 2 in 38 healthy control subjects (specificity

= 95%). However, these promising initial results could not be repeated in subsequent studies.

Schubert and Brasher (1969) performed the similar study and found the positive results in 61 of 140 tuberculous patients sera (sensitivity = 44%) and 41 of 242 nontuberculous patients sera (specificity = 71%).

Kaplan and Chase (1980) used culture filtrate of *M. tuberculosis* H37Rv as the antigen in microimmunodiffusion test for the detection of specific antibodies in sera and found the positive results in 33 of 52 active tuberculous patients (sensitivity = 63.5%).

In contrast , Burrell et al.(1956) Glenchur and Kettel (1965) suggested that sputum microscopy was a more valuable test than serology.

The differences in the efficacy study in various studis may be due to the false interpetation of non-specific precipitation (Grange, 1982).

b. Agglutination test

Arloing (1904) was the first who showed that liquid homogenous cultures of tubercle bacilli could be agglutinated by the serum of tuberculosis patients.

The experiences accumulated from the agglutination test performed during the past 90 years show that this test is unreliable and has not increased diagnostic yield in tuberculosis. For example, Mitchison et al.(1977) found false negative up to 30% and false positive up to 63% and Williams et al. (1978) found false negative up to 29% and false positive up to 52%.

c. Hemagglutination test

Middlebrook and Dobos were the first who demonstrated the hemagglutinating antibodies to tuberculin in the serum of patients with active tuberculosis by using normal erythrocytes incubated with extracts of tubercle bacilli (Middlebrook, 1948). Subsequently developed hemagglutination technique was done by Boyden (1951) and Cole (1955). However, none of these tests has been proved to be useful in the diagnosis of active tuberculosis, either beause of their lack of sensitivity or false positive results obtained with the serum of nontuberculous patients (Cole, 1955).

Turcotte et al. (1963 and 1966) found that $\gamma_2$-globulin fraction of serum, obtained from tuberculous patients had high hemagglutination titer. But their subsequent study with this fraction showed that only 81 of 145 patients (sensitivity = 56%) with active tuberculosis and 5 of 190 (specificity = 97%) nontuberculous patients gave positive results.

Jagannath and Sangupta (1951), using aldehyde treated ergthrocytes sensitized with *M. tuberculosis* cell extracts or PPD to detect serum antibodies, found the sensitivity of only 54-58% in 71 tuberculous patients.

d. Complement fixation test

Middlebrook, using the hemolytic modification of the Middlebrook-Dubos hemagglutination test, found that sensitivity and specificity of the test were 88.89% and 60%, respectively (Middlebrook, 1950).

Maillard and Gagliardo (1983) showed that the sensitivity of the test was 94% and specificity was 95%. Subsequently, Schubert and Brasher (1967) found less attrative results, the

sensitivity of the test was 45% while the specificity was 79%.

e. Fluorescent antibody tests

Antibodies bound to an antigen are demonstrated by the addition of an antiglobulin conjugated to a fluorescent dye. The antigen may be either an insoluble one, such as a whole bacterium, or a soluble one bound to a suitable solid surface. The latter test is referred to as the soluble antigen fluorescent antibody (SAFA) test which has been developed as a test for the diagnosis of tuberculosis by Toussaint et al. (1969)

Naussau and Merrick (1970) used whole cells of *M. tuberculosis* H37Rv as an antigen and assessed the fluorescence visually by the use of an ultraviolet microscopy. They found that the sensitivity and specificity of the test were 96% and 97.5%, respectively.

Bhardwaj (1981) used saline extract of *M. tuberculosis* H37Rv as the antigen in SAFA test for the diagnosis of disseminated and extrapulmonary tuberculosis and found the sensitivity of the test were 29% in disseminated tuberculosis and 50% in extrapulmonary tuberculosis.

Chawla et al.(1986), with the same test, found the sensitivity and specificity were 83% and 92%, respectively.

f. Radioimmunoassay (RIA)

This highly sensitive technique is based on the binding of a radiolabelled antibody to an antigen. In the Farr technique (Grange,1984),labelled antigen combines with antibody which is then precipitated by the addition of a protein-precipitating agent. The radioactivity of the co-precipitated antigen is measured. If the antigen itself is a protein, the labelled antigen-

antibody complex may be precipitated by an antiimmunoglobulin serum.

Naussau et al.(1975) developed a solid phase RIA with culture filtrate of *M. tuberculosis* H37Rv as the antigen for the detection of antibody in sera of tuberculosis patients. The solid phase RIA for measuring level of antibody specific to sonicated *M. tuberculosis* H37Rv antigen in CSF was carried out by Samuel et al.(1983) who found the sensitivity and specificity of the test were 83% and 100%, respectively.

Kadival et al.(1982) developed a competitive RIA, using $^{125}$I-labelled sonicated *M. tuberculosis* antigen and anti-BCG antibody. The assay was sensitive to detect 1 x $10^2$ organisms/ml or 1 ng/ml of sonicate antigen. Sputum was assayed and the antigen levels determined in 39 specimens with either smear or culture positive were higher than those 62 specimens with both smear and culture negative. However, Samuel et al.(1983) found no reliable result when they performed semilar study with CSF.

Radioimmunoassay, although is a powerful research tool,is unlikely to find much routine application in the study of tuberculosis outside major research centres on account of the cost of the equipment and reagents (Grange, 1984).

### g. Enzyme-linked immunosorbent assay (ELISA)

The ELISA uses an antiglobulin conjugated to an enzyme to detect and quantitate antibodies bound to antigen adsorbed to a solid phase. The conjugated enzyme serves as an indicator by converting a colourless substrate into a coloured product which can be assayed optically. The test was first applied to tuberculosis by Nassau et al. who used a concentrated culture filtrate of *M. tuberculosis* H37Rv as an antigen. A serum dilution of 1:100 gave discrimination between patients and controls. They showed the sensitivities of

the test to be 84% and 67% in pulmonary tuberculosis and extrapulmonary tuberculosis, respectively (Nassau,et al., 1976). The use of a more purified antigen was suggested to improve the sensitivity of the method. A similar result was obtained when PPD was used in place of whole cell free extract. In this study, significant levels of antibody were detected in 80% of AFB positive of tuberculosis, 66% of AFB negative tuberculosis cases and 4% of control subjects (Gandon,1980). Similar results were reported by Grange et al.(1980) who used an ultrasonicated of BCG as antigen. They quantitated the antibodies in the IgG,IgA and IgM classes and found significant levels of antibody to be 75.2,31.4 and 11.8%, respectively, of sera from 153 cases of tuberculosis. Reggiardo et al.(1980) used ELISA to quantitate antibodies in tuberculosis patients to three serologically active glycolipids, A,B and C , extracted from purified BCG. The results showed that glycolipid A was the most discriminative antigen among the three glycolipids.

In 1982, Viljanen et al. developed ELISA for determination of Ig M, IgG, IgA antibodies against PPD. The mean level of three immunoglobulin classes were found to correlate with the extent of the tuberculous infection. However, the concentration of IgM antibody was lower than those of IgG and IgA antibodies which confirmed the study of Grange et al.(1980) Stroebel et al. (1982) used highly purified antigen "antigen 6" in ELISA and found that the test was highly sensitive (94%) and specific (100%) for diagnosis of extrapulmonary tuberculosis. Benjamin et al. (1984) performed the ELISA using three different antigens : a highly purified antigen "antigen 5", PPD and antigen 6. The last overlapping ELISA values between the diseases and controls was observed when using "antigen".

Enzyme immunoassays have several advantages over RIA such as the preparation of reagents are easier and cheaper and the reagents are stable for months, the equipment is relatively inexpensive and there is no radiation hazard (Grange, 1984). Daniel et al.(1985) compared antigen 5 and PPD for the detection of antibodies in sera by ELISA and found that the sensitivity in PPD test was higher than antigen 5 test but the specificity was lower.

In 1986, Kadival et al. developed a double antibody sanwich ELISA using anti-BCG antibody for the detection of mycobacterial antigens in CSF. They found the sensitivity and specificity of the test to be 65.79% and 100%, respectively. Using the similar ELISA technique, Yanez et al.(1986) found the similar results which were 57.58% sensitivity and 91.43%, specificity.

Trakulsomboon (1986) developed an indirect ELISA for immunoglobulin G antibody detection and a modified double antibody sanwich method for *M. tuberculosis* antigen detection in order to assess 3 groups of pulmonary tuberculosis patients. It was found that 48% of the patients (proven by chest roentgenogram and positive sputum culture) were positive for antibody and 92% for antigen while 50% for antibody and 95% for antigen in pulmonary tuberculosis patients proven by chest roentgenogram and acid fast staining, and 36.1% for antibody, 50% for antigen in those with only positive chest roentgenogram. In comparison with non mycobacterial pulmonary patients or healthy control group 30% were antibody positive but only 5% of the patients or 8% of healthy subjects were antigen positive.

In 1987, Banchuin et al. applied this technique to assay anti-PPD antibody in pleural fluids of 25 tuberculous patients and 28 cancerous patient control. They were unable to find the evidence of local antibody production. The anti-PPD IgG levels in pleural fluids were not different from those in sera. In addition, anti-PPD IgG levels in both pleural fluids and sera

of tuberculous patients were also similar to those of controls.

In 1988, this technique was also used for the detection of mycobacterial antigens in pleural fluids, by Dhannd et al. They found that 12 of 15 tuberculous patients, 1 of 9 patients with miscellaneous diseases and all of 12 cancer patients gave positive results. False positive reactions found in patients with lung cancer may limit the usefulness of this test. Mycobacterial antigen detection in CSF, pleural and ascitic fluids by a competitive ELISA was performed by Ramkisson et al.(1988) The antigen was found in CSF of all 10 patients with tuberculosis and in 1 of 94 controls. All 10 ascitic fluids obtained from tuberculous patients were positive, whereas all 10 specimens obtained from non-tuberculous patients were negative. Antigen was also found in all 5 pleural fluids obtained from tuberculous patients. However, no pleural fluid from the control group was included in this study. Watt et al.(1988) used combination of two ELISA methods to examine CSF specimens. Anti-BCG antibody was used in a double antibody sandwich ELISA for the detection of antigen and BCG was used to an indirect ELISA for detection of IgG and IgM antibody. The antigen was detected with sensitivity of 39% and specificity of 98%. Antibodies were detected with sensitivity of 24% and specificity of 98%.

Wongwajana (1990) found that the detection of mycobacterial antigen in sputum by double antibody sandwich ELISA (anti-BCG system) could be used efficiently for the diagnosis of pulmonary tuberculosis. Though the determination of antibodies both anti-PPD and anti-PM antibodies, to mycobacterial antigens, in CSF, serum and pleural fluid showed that the sensitivity of the tests in all specimen was low, but the specificity of the test for CSF was quite specific.

h. Hybridization with deoxyribonucleic acid (DNA) probe

DNA probes, developed as tools for molecular biology and genetic engineering reseach, are unique DNA sequences labelled with either radioisotopes, enzyme or chemical agent. They can specifically hybridize with complementary single-stranded DNA or RNA. Shoemaker et al.(1985) used total genomic DNA isolated from *M. tuberculosis* as DNA probes for identification of *M. tuberculosis* in clinical isolates. They found that total genomic DNA probes hybridized not only with DNA from *M. tuberculosis* but also with DNAs from other mycobacterial species. DNA from bacteria other than mycobacteria were not hybridized with this total DNA probes. Pao et al.(1988) used cloned DNA fragments as the DNA probes for the detection of mycobacterial DNA sequences. They found that the mycobacterial DNA sequences could be detected in 30.4% of various types of uncultured clinical specimens, whereas traditional culture method showed only 19% positivity rate for the same specimens. The overall sensitivity and specificity of this DNA probes technique were 90.5% and 83.8%, respectively.

i. Radiometric method

The BACTEC system, a radiometric culture system, had been developed for shortening the culturing time of mycobacteria. The growth can be recognized by detecting production of $^{14}CO_2$ from metabolizing of $^{14}C$ labelled palmitic acid present in BACTEC Middlebrook broth medium, in an automatic ion chamber system (BACTEC(R), Johnston Laboratories). The BACTEC system may detect the early growth of mycobacteria in 2 to 12 days (Collins, 1985), and now is the most rapid method for culturing mycobacteria. However, its cost and isotopic material requirement make

this system unsuitable for using in the developing countries. Detection of *M. tuberculosis* from smear-positive specimens by the BACTEC system required an average of 7 days compared with 18 days by conventional system. From smear-negative specimens, the BACTEC detected *M. tuberculosis* in an average of 20 days versus 28 days by conventional procedure (Park, 1984).

By combinding radiometric and probe methodology, most isolates of *M. tuberculosis* , *M. avium* and *M. intracellulare* in the study performed by Ellner (1988) could be detected and identified within 4 weeks while it required 9 to 11 weeks with conventional media and biochemical identification.

Comparison of improved BACTEC and L-J medium of culture of mycobacteria from clinical specimens. The results showed that 91% were positive culture (for mycobacteria) with BACTEC and 73% with L-J medium. The number of positive culture for *M. tuberculosis* complex were 95% with BACTEC and 87% with L-J medium. One disadvantage of this techniques was that the contamination rate was significantly higher (5%) than in L-J medium (3.3 %). The mean isolation time with BACTEC was 15.5 days, and 25.6 days with L-J (Anargyros, 1990).

j. Polymerase chain reaction (PCR)

The recently developed polymerase chain reaction (PCR) permits the *in vitro* amplification of DNA segments. This technique has been shown to increase the level of detection of mycobacteria enormously. By extracting DNA from clinical samples, amplifying a pathogen-specfic DNA sequence and detecting the amplified sequence with a labelled oligonucleotide probe, it is theoretically possible to detect a single microoganism in clinical sample. By this technique abount $10^3$ *M. tuberculosis* were detecta-

ble in sputum. In addition, it is possible to detect *M. tuberculosis* cells in clinical material such as pleural fluid, brochial washings and biopsies using PCR technique (Hermans-Peter, 1990). Patel et al.(1990) demonstrated the use of PCR in the developmental method for the specific identification of *M. tuberculosis*. They proved that a combination of amplification and subsequent hybridization with labelled PCR products was the most sensitive and specific method for the identification of *M. tuberculosis*.

The PCR technique was compared with conventional bacteriological method and an ELISA technique for the detection of antibodies in CSF for the diagnosis of tuberculous meningitis (TBM). PCR was found to be the most sensitive technique, it detected 15 (75%) of 20 cases of highly probable TBM (base on clinical feature), 4 (57%) of 7 probable cases, and 3 (43%) of 7 possible cases. ELISA detect 11 (55%) of the highly probable cases and 2 each of the probable and possible cases. Culture was positive in only 4 of the highly probable cases. Among the control group, 6 subjects tested early in the study were PCR positive. Second DNA preparation from their stored CSF samples were all PCR negative, suggesting that the false-positive results were due to the cross-contamination. The eighteen PCR-positive TBM samples retested were all still PCR positive. The antibody ELISA was positive in 3 controls despite the use of a high cutoff value (Shankar, 1991).

Table 1 Classification of non-Runyon group

| Characters | Species | Year of discovery | Patho-genicity | Synonyms |
|---|---|---|---|---|
| *In vitro* not growing | *M. leprae* | 1847 | P | Hansen's bacillus<br>Leprosy bacillus |
| Slow growing | *M.lepraemurium* | 1903 | P | Rat leprosy bacillus |
| | *M.paratuberculosis* | 1895 | P | *M. johnei.* |
| | *M.ulcerans* | 1948 | P | *M. buruli.* |
| *M.tuberculosis* complex | *M.tuberculosis* | 1883 | P | *M.tuberculosis* typhus humanus |
| | *M.microti* | 1937 | P | *M.tuberculosis* var murines |
| | BCG | 1924 | P* | |
| | *M.africanum* | 1969 | P | |
| | *M. bovis* | 1896 | P | *M.tuberculosis* typhus boviness |

P = Pathogen

P* = in the immunodeficiency patient

Table 2 Classification of Runyon group

| Characters | Species | Year of discovery | Pathogenicity | Synonyms |
|---|---|---|---|---|
| Runyon Group I | *M. kansasii* | 1955 | F | yellow bacillus |
| Photochromogens | *M. marinum* | 1926 | F | *M. balnei* |
| | *M. simiae* | 1965 | P | *M. habana* |
| Runyon Group II | | | | |
| Scotochromogens | *M. scrofulaceum* | 1959 | F | *M. marinum* |
| | *M. gordonae* | 1962 | F | *M. aquae* |
| | *M. szulgai* | 1972 | F,P | *M. aquae* |
| Runyon Group III | *M. avium* | 1891 | P | *M. tuberculosis* avium |
| Non-chromogens | *M. intracellulare* | 1949 | F | Batty bacillus |
| | *M. xenopi* | 1959 | F | *M. littorale* |
| | *M. terrae* | 1966 | S | Radish bacillus |
| | *M. nonchromogenicum* | 1965 | S | |
| | *M. novum* | 1967 | S | |
| | *M. triviale* | 1970 | F | |
| | *M. gastri* | 1966 | S | "J" group |
| Runyon Group IV | *M. smegmatis* | 1885 | S | smegma bacillus |
| Rapid growers | | | | *M. butyricu,,* |
| | | | | *M. lacticola* |
| | *M. phlei* | 1895 | S | *M. moellei* |
| | *M. thamnopheos* | 1929 | F | |

Table 2 (continue)

| Characters | Species | Year of discovery | Patho genicity | Synonyms |
|---|---|---|---|---|
| | *M. fortuitum* | 1938 | F | *M. mimatti*,*M.ranal* |
| | | | | *M. giae*, |
| | | | | *M. peregrinum* |
| | *M. chelonei* | 1953 | F | *M. abscessus* |
| | | | | *M. runyonii* |
| | | | | *M. borstelense* |
| | *M. salmoniphilus* | 1960 | S | |
| | *M. flavescens* | 1962 | S | *M. acapulcensis* |
| | *M. vaccae* | 1964 | S | |
| | *M. diernhoferi* | 1965 | S | *M. parafortuitum* |
| | *M. aurum* | 1966 | S | |
| | *M. thermoresistibile* | 1966 | S | |
| | *M. chitae* | 1967 | S | |
| | *M. rhodesiae* | 1971 | S | |
| | *M. obuense* | 1971 | S | |
| | *M. duvalii* | 1971 | S | |
| | *M. gilveum* | 1971 | S | |
| | *M. aichiense* | 1973 | S | |

P = Pathogen

F = Facultative pathogen

S = Saprophyte

Table 3 Number of positive AFB staining, positive culture and identify result from various specimens, data from mycobacterial laboratory, Siriraj Hospital, Bangkok, Thailand (1990)

| Specimens | Number of specimens | AFB+ | Culture+ | AFB+ Culture+ | AFB- Culture+ | AFB+ Culture- | Identify | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | TB | non-TB |
| Sputum | 8383 | 937 | 1016 | 652 | 362 | 201 | 960 | 15 |
| Pleural fluid | 313 | 1 | 8 | 2 | 7 | 1 | 8 | 0 |
| Ascitic fluid | 112 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CSF | 234 | 0 | 6 | 0 | 6 | 0 | 4 | 0 |
| Bronchial washing | 39 | 10 | 24 | 6 | 18 | 4 | 23 | 1 |
| Gastric washing | 44 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Pus | 219 | 8 | 27 | 4 | 21 | 0 | 18 | 4 |
| Urine | 35 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tissue | 75 | 1 | 5 | 0 | 4 | 0 | 2 | 1 |
| Other | 177 | 5 | 12 | 2 | 7 | 0 | 9 | 1 |
| Total | 9,988 | 962 | 1,098 | 666 | 425 | 206 | 1,024 | 22 |

$AFB^+$ = positive acid fast staining $AFB^-$ = negative acid fast staining

$Culture^+$ = positive culture on L-J medium $Culture^-$ = negative culture on L-J medium

TB = tuberculosis non-TB = non - tuberculosis