THE APPLICATION OF ENZYME-LINKED IMMUNOSORBENT ASSAY IN DETECTION OF ANTI-PPD ANTIBODY IN SERUM AND OF MYCOBACTERIAL ANTIGEN IN SPUTUM OF PULMONARY TUBERCULOSIS PATIENTS

BY

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OD	Optical density
C	Culture on Lowenstein-Jensen's medium
C	Degree celcius
S	AFB staining by fluorochrome
X	Chest roentgenogram
+	Radiographic finding compatible with pulmonary tuberculosis
	Positive smear
	Positive culture for M. tuberculosis
-	Radiographic finding not compatible with pulmonary tuberculosis
	Negative smear
	Negative culture for M. tuberculosis or atypical mycobacterium
g	Gram
hr	Hour
i.e.	Id est (latin), that is
µg	Microgram
ng	Nanogram
mg	Milligram
ml	Millilitre
min	Minute
µ	Micron

INTRODUCTION

Even today, when the incidence of tuberculosis in the Western nations has markedly decreased and the mortality is less than ten per hundred thousand population, tuberculosis still remains one of the world's most prevalent infectious diseases. It is found at the present time, primarily in the developing areas of the world including Thailand (1).

The world-wide prevalence is estimated to be 15-20 million cases of active tuberculosis with an annual incidence of about 10 million and at least 3 million deaths each year (2-3). Of this total, 80% or more are in developing nations (4). As a cause of death among human beings, tuberculosis is still at the forefront of infectious diseases. Thus, on a world-wide basis, tuberculosis remains one of the important infectious diseases and of the leading causes of death for all infectious disease with which human beings have to cope.

Despite a century of research, there is still no simple and reliable diagnostic test for tuberculosis. Clinical and radiological signs are notoriously non-specific and culture technique, although providing a definitive diagnosis, is expensive and time-consuming and in some instance, it was too late when the diagnosis was made. Being an infectious disease, the diagnosis of tuberculosis is of importance both to individual patient and to community. Therefore, a sensitive and reliable test would be useful for screening contacts of infected individuals and for early detecting infectious cases in the

community ; especially, for patients with abnormal radiographic pulmonary shadow suggestive of active tuberculosis but negative smear, and for patients with non-pulmonary tuberculosis in whom bacteriological proof of diagnosis is difficult to obtain. Recent study in Thailand revealed only 18.9% of positive culture from a single sputum specimen in those who had radiographic abnormalities compatible with pulmonary tuberculosis (6). In 1983 there are approximately 679,000 pulmonary patients in Thailand and out of these 150,000 were infectious cases. Only 27% of all cases were detected and treated (7). Consequently, an additional rapid, sensitive and reliable diagnostic test is needed.

In the past, many investigators have used various methods to measure specific antibodies to mycobacterium antigen (8-12) but none of these are satisfactory for detecting active tuberculosis. The enzyme-linked immunosorbent assay (ELISA) recently developed (13) is one of the most efficient procedures for the detection of antibodies against certain antigens (14-16) as well as small amount of specific antigen in clinical specimen (17). The value for the diagnosis of clinical tuberculosis was clearly demonstrated by recent reports from many groups of investigators (18-27). In this study ELISA technique was chosen for measuring level of IgG antibody binding to purified protein derivative of tuberculin (PPD). Its usefulness for diagnosis of pulmonary tuberculosis patients was evaluated in active pulmonary tuberculosis who are smear or culture positive, patients with clinical, history and radiographic pulmonary shadow compatible with pulmonary tuberculosis but whose smear or culture are

negative. Also in the present study we set up another ELISA method to detect mycobacterial antigen in sputum of pulmonary tuberculosis patients. These two ELISA techniques, i.e., detection of IgG anti-PPD in serum and of mycobacterial antigens in sputum in patients with pulmonary tuberculosis especially in smear or culture negative cases would have a far greater diagnostic utility than is either one alone.

Discovery and history of tuberculosis

Tuberculosis has had various meanings through the centuries since it was first recognized, its clinical features and communicability were known before 1,000 B.C, such as "phthisis" in the Hippocratic age ; "phyma" in Greek ; "lung nodule" in christian age ; and "tuberculum" in latin. The word "tuberculoses" was first used in 1834, when diagnosis of the disease was based only on symptoms and pathology (28).

The discovery of Mycobacterium tuberculosis and its identification as the causative agent of tuberculosis were first amounced by Robert Koch in 1882.

Definition of tuberculosis

Tuberculosis is an infectious disease usually caused by Mycobacterium tuberculosis and characterized by nodular, caseating granulomas (called tubercles) that fibrose, ulcerate or calcify. The disease remains localized to the lungs in most patients but may involve almost any part of the body, especially the meninges, kidneys, bones, and lymph nodes. It may also be disseminated (29). Disease caused by M. bovis and M. avium also was considered to be tuberculosis. Similar disease produced by other pathogenic mycobacteria in mammals, birds, fish and reptiles was likewise called tuberculosis.

Epidemiology

1. Incidence and prevalence

The prevalence of tuberculosis infection is estimated by tuberculin test surveys of sample populations. The incidence of tuberculous disease is obtained from data giving the number of new cases of tuberculosis as revealed by laboratory studies and clinical finding (29). Tuberculosis is among the main public health priorities of many countries (2, 30) including Thailand (1). The world-wide prevalence is estimated to be 15-20 million cases of active tuberculosis, with an annual incidence of about 10 million and at least 3 million deaths each year (2, 3). Tuberculosis ranks first as the cause of death among reportable infectious diseases. Except for pneumonia, tuberculosis is the leading cause of death for all infectious diseases (5). In Thailand, the epidemiological survey of tuberculosis in 1977 (31) revealed that the infection rate was 40.6% ; annual risk of tuberculosis infection, 4.9% ; the mortality rate, 1,400 per 100,000 populations, the mortality rate in 1983 was 11.0 per 100,000 populations, making tuberculosis the fourth leading cause of death in the country. The incidence of tuberculosis varies greatly from one country to another. From tuberculosis morbidity data in 1976/1977 the incidence was highest in Asia, i.e. 90.4 per 100,000 populations ; in some countries the rate was as high as 382 per 100,000. 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 (32). In the United States, it is estimated that only about 5-6 % of entire population are tuberculin

reactors.

Reported incidence of tuberculosis in Asia, 1976-1977 (33)

Rates per 100,000	Countries/Areas
<10	Afghanistan
11-25	Cyprus, Indonesia, Israel, Jordan, Nepal, Syrian, Arab Republic
26-50	Bangladesh, Iran, Turkey
51-75	Bahrain, India, Japan, Kuwait, Lao People's Democratic Republic
76-100	Burma, Malaysia, Pakistan, Sri Lanka, Thailand
>100	Singapore, Brunei, Hong Kong Iraq, Omen, Saudi Arabia, Vietnam, Public of Korea, Macau (382 per 100,000)

2. Source and modes of transmission

The sources of infection in tuberculosis are the sputum discharges from tuberculous foci into the atmosphere by aerosolization of pulmonary secretion by a diseased pulmonary patients via coughing, sneezing, speaking, and singing. The patient discharging tubercle bacilli in the sputum is by far the most important reservoir of infection. The bacilli have no natural existence outside the body and are transmitted from source to destination by some form of direct or indirect contact (34-35).

1) Direct contact : droplet infection is the commonest mode of spread. Aerosol droplets dry rapidly, leaving tiny droplet nuclei, those droplets in the range of 1-10, microns remain floating in the air for considerable period of time, depending upon environmental condition. The size of the infectious droplet in tuberculosis is about 5 to 10 μ ; such a particle can suspend indefinitely and can reach terminal bronchioles and alveoli when inhaled. It is thought that only one bacillus can cause disease. Particles greater than ten microns in size are largely trapped in the upper nasal passages or are expelled into the pharynx by the mucociliary mechanism of the lower respiratory tract and are harmlessly swallowed and digested.

In several classical studies, Riley (36-37) showed that droplet nuclei from smear-positive tuberculosis patients could infect guinea pig in the environment. The number of organisms in the air borne aerosol depends upon the number of bacilli excreted, expulsive force of the cough and the presence of cavitation in the lungs.

2) Indirect contact : The transfer of bacilli in to the mouth by contamination, the consumption of milk from infected cows, infection through the pulmonary route from handling contaminated fomites are the modes of transmismission. Infection can also occur when bacilli are introduced into or through the skin. This source of infection is occasionally seen among pathologists and laboratory workers (35, 38).

Tuberculosis is clearly an airborne disease due to

droplet nuclei infection in the majority of patients (39). The close contacts of a smear-positive are at the maximum risk of being infected. However, the disease is not a highly infectious as some of the viral infections. In some studies the infection rate was different in contacts of smear-negative, culture-positive patients than in the community, but prolonged contact with such a person can be a danger. It appears that exposure generally must be close and substained, heavily laden with droplet nuclei, and the prospective host unprotected previously activated immune mechanisms.

3. Risk factors for infection

Among the natural tuberculin reactors the case rate was 29 in 100,000 per year, but the risk was 30 times more in persons with abnormal chest radiographs (40). The risk was only 2 times greater in person with nothing more than calcified residuals of a primary infections. Various factors may influence the risk of developing tuberculosis in an individual or a population, depending upon the point of time with regard to the tuberculosis "epidemic wave". (41-43).

The age and sex variables are influenced by the timing of epidemic wave. At this time, the age distribution of TB cases over past 20 years has shift from more cases in the first decade of life than in the last to almost no first-decade cases and over 50% of cases over the age of 65 (45-46). Other factors associated with the risk of developing tuberculosis are living in urban or crowded living condition (47), socioeconomic status (48), alcoholism, Diabetes mellitus and certain type of

histocompatibility (49).

4. Host resistance to tuberculosis infection

External barriers to infection include skin and mucous membranes, ciliary movement in the upper respiratory tract, mucociliary stream of the lower respiratory tract, acidity of the stomach, peristalsis, and lysozyme in secretions (50).

Internal defense mechanisms include inflammation, phagocytosis, opsonizing activity of natural antibodies, complement, and bacteriocidal agents but none of these is very effective in preventing the growth and multiplication of virulent tubercle bacilli (34).

Resistance to attack by tubercle bacilli : three factors influencing resistance to attack (initial multiplication) of these bacilli are

a. The trapping of bacilli in the lung. This mechanism is in part, dependent on the ability of the alveolar macrophages (AM) to ingest them, AM from certain inbred resistant rabbits are able to ingest twice as many bacilli as those from certain inbred susceptible rabbits (51-52). The alveolar macrophages are rich in lysosomes and digestive enzymes.

b. The initial inactivation of tubercle bacilli. Only a few out of many inhaled bacilli produce lesions, most are destroyed or inactivated, presumably by the alveolar macrophages that initially ingested them (53-54).

c. Resistance to the progress (continued multiplication). If an inhaled tubercle bacillus that is ingested by alveolar macrophage is not destroyed, the progress of

tuberculosis is controlled by blood-borne macrophages (i.e., monocytes that enter the tissue from the blood stream). It involves lymphocytes, their lymphokines, and activation of these cells.

Variation in host resistance ; There is no significant natural defense mechanism against tuberculosis, though degree of susceptibility to tuberculosis varies widely among various species. Even within a given specie of animal, different strains may show considerable variation in susceptibility (55-58). The resistance in man varies more strikingly with tuberculosis than with most infectious disease.

1) Genetic factors : Recently a subset of tuberculous patients was found (53). They were anergic when skin tested with tuberculin and had adherent suppressor mononuclear cells in their circulation. They probably had a genetically-determined predisposition to develop such suppressor cells because HLA-linked differences in host response to both virulent tubercle bacilli and BCG are known to exist (59-61).

2) Physiologic Factors : Epidemiologic evidence on the frequency of tuberculosis has been shown that the following factors associate with the susceptibility of tuberculosis (62-63, 54):-

- malnutrition
- overcrowding
- stress
- hormonal factor
- age : newborn infants and old age are highly

susceptible to tuberculosis, perhaps because of incompetent immunity.

- Miscellaneous factors : debilitating disease, patients with diabetes, silicosis, cancer, and those on corticosteroid or other immunosuppressive drugs are particularly susceptible to tuberculosis.

5. Prevention and control

Theoretically, tuberculosis could be prevented and eradicated if every active case could be diagnosed, isolated and treated. New drugs have speeded up the process but not initiate it, nor can they alone eliminate tuberculosis (64). There are two relatively effective methods for preventing clinical tuberculosis :

1) Isoniazid prophylaxis (35, 65)

INH prophylaxis is recommended for all household contacts of a patients with an infectious stage and for immunocompromised patients. Chemotherapy with isoniazid for 1 year has been shown to reduce the risk of the evolution of a dormant infection into tuberculous disease by approximately 75%.

2) BCG prophylaxis (35, 64-66)

Vaccination with BCG has significant value in the control of tuberculosis, especially in area which case rates are high and tuberculosis mortality is centered on the very young. The vaccine is made from a bovine tubercle bacilli strain that was made avirulent over a period of years by Calmette and Guerin. The vaccine is harmless when properly prepared and administered but gives only a partial protection.

The vaccination of human being with BCG has a draw

back because it renders tuberculin-negative people to tuberculin-positive thus destroys the diagnostic usefulness of tuberculin test. BCG has been grown all over the world and is very difficult to standardize. For these and other reasons, controversy exists as to the overall efficacy of BCG vaccine.

Microbiology of M. tuberculosis

1. General characteristics

a) Morphology and morphogenesis

M. tuberculosis is a slender, straight, or slightly curved rod with rounded ends, ranging in size from 0.3-0.6 by 1-4 μm. Occurring singly and in occasional threads, branching is seen occasionally in old cultures and in smears from caseous lymph nodes. The bacilli are acid-fast, nonmotile, nonsporogenous, and nonencapsulated. They are difficult to stain with the gram stain but are usually considered to be gram positive and strongly acid-fast as demonstrated by Ziehl-Neelsen or fluorochrome procedure. The procedure is based on a lipid barrier principle, an increased hydrophobicity of the surface layer follows the complexing of dye with mycolic acid residues that are present in the cell wall. This prevents exit of carbolfuchsin that has become trapped in the interior of the cell (67). Growth trends to be in serpentine, cordlike masses in which the bacilli show a parallel orientation, 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 (68-69, 34-35).

Colonies of avirulent forms are less compact. On most

solid media, colonies are rough, raised thick, with a nodular or wrinkled surface and an irregular thin margin ; may become slightly pigmented.

Acid-fastness is the capacity of biological materials to form acid-stable complexes with certain arylmethane dye. Such materials contained dye complexes that are not decolorized following exposure to acidic ethanol or mineral acids. The property of acid-fastness is shared by the mycobacteria an few other microorganisms, some Nocardia strains (70), spores of certain fungis (71), the spores of Bacillus cereus, human sperm (72), the hooklets of Taenia echinococcus (73), the embryophores of Taenia saginata (74), Corynebacteria, certain inclusions found in the lung of lipid pneumonia. The capacity for acid-fastness of various Mycobacteria can be removed with alkaline ethanol (75).

b) Physiochemical properties

Constituents of tubercle bacilli: The constituents listed below are found largely in the cell walls.

LIPID : Mycobacteria are rich in lipids (up to 60%) (68) which are largely bound to proteins and polysaccharides, probably responsible for most of the cellular tissue reaction to tubercle bacilli, formation of caseation necrosis, and acid-fastness (76-77, 68). Analysis of the lipids by gas chromatography reveals species-specific patterns that aid in classification (78). A "cord factor" or "serpentine cords" in which acid fast bacilli are arranged in parallel chains (34-35, 68-69) is composed of trehalose-6, 6 -dimycolate which can be

extracted from virulent strains. It inhibits migration of leukocytes, causes chronic granuloma , and can serve as an immunologic "adjuvant".

PROTEINS : mycobacterial proteins contain a complexity of antigens. It is probable that all cytoplasmic proteins of Mycobacteria are antigenic in man and laboratory animals. They can induce tuberculin sensitivity and also elicit the formation of a variety of antibodies. Many such antigens probably possess species specificity. Hence, so many of the standard techniques have been applied to the purification of mycobacterial proteins. (77, 79).

POLYSACCHARIDES : mycobacterial polysaccharides have been reviewed by Stacey (80) and have been isolated by many physicochemical techniques (79). The attention has been directed primarily at cell wall components. Mycobacterial cell walls are rich in peptidoglycan. There are protein-free polysaccharides of four principal types, arabinogalactans, arabinomannans, mannans, and glucans from both mycobacterial culture filtrates and cell wall extracts (81-83). There is a high degree of chemical similarity or identity among polysaccharides isolated from various Mycobacteria (82, 84). In fact, Corynebacteria and Nocardia have been shown to contain polysaccharides similar to those found in Mycobacteria (82, 84-85).

Cultural and growth characteristics : Mycobacteria are obligate aerobes and derive energy from oxidation of many simple carbon compounds. Growth is also enhanced by an increased CO_2 tension. It is a very slow-growing organism even under the

most known optimal growth conditions. Its doubling time is 18-24 hours. (86-87). The optimal temperature for growth is 37°C, and optimal pH is a range of 6.0 to 7.6 (88).

Resistance ; Tubercle bacilli are highly resistant to drying. The environment in which bacilli are found is an important factor in their viability such as in cultures maintained at 37 C for 12 years, in sputum when exposed to direct sunlight for 20-30 hours, or in sputum when protected from direct sunlight for 6-8 months they still are viable and virulent. Tubercle bacillus is generally more resistant than other bacteria to chemical agent, such as dyes, acids, and alkalines because of the hydrophobic nature of the cell surface and their clumped growth (77). Hence, there is no single satisfactory disinfectant for M. tuberculosis.

Genetics : Mutations in this organisms occur at very low frequencies. Spontaneous mutation can be detected by looking at alternations in colony morphology ; the human H37 strain was dissociated into the rough virulent (Rv) and rough avirulent (Ra) variants. Although both variants are rough, their colony morphology are different and characteristic (34-35).

The virulent factors of tubercle bacillus may be the cord factor (trehalose dimycolate-a potent toxic glycolipid), the sulfatides, and other acidic lipids. This important area has recently been reviewed by Goren (98).

c) Antigenic structures (35)

Many constituents of mycobacterial cell, cell wall (polysaccharides, proteins and peptides) and cytoplasmic

membrane have antigenic potential (90). Some of these are species-specific. Other are shared by many species and contribute to the cross reactivity, even to Norcardiae and Corynbacteria (91-93). Strain-or- type specific antigens are also identified by agglutination reactions and by skin test with partially purified proteins (PPD).

Because of the complexity of the antigenicity of mycobacteria, chromatographic fractionation of extracts, followed by immunodiffusion has revealed at least 20 antigens in M. tuberculosis. Eleven major antigens can be identified from a reference unheated culture filtrate from M. tuberculosis H37Rv. These have been compared and related to the 5 fractions obtained by Seibert with electrophoretic and immunoelectrophoretic separation for Seibert's protein and polysaccharide fractions (94) :- Seibert's protein A, B, C, D and Seibert's polysaccharide I and II fraction.

Seibert's protein A and b are capable of eliciting skin reactions and are more potent than PPD. Seibert's polysaccharide I andII have been shown to be arabinogalactan and arabinomannan, derived from the cell wall of Mycobacterium. The antigenic determinant is an arabinose sidechain and is probably shared by all species of Mycobacteria, Nocardia and Corynbacteria. The arabinomannan is probably responsible for the surface antigen specificity which permits specific seroagglutination of many mycobacterial strain.

The best characterized protein antigen of M. tuberculosis is a tuberculin-active protein with a molecular weight of 9,700 obtained from acetone powders of heated cells.

This protein exhibits a higher tuberculin activity than PPD.

d) Immunology properties

Tuberculoimmunity, infection with virulent or attenuated tubercle bacilli will develop a markedly increased resistance to reinfection. It is characterized by intracellular bacteriostasis, rather than by intracellular killing. This process has been ascribed to a cytophilic antibody, to delayed hypersensitivity, or to hyperactive macrophages.

No endotoxins or exotoxin have been discovered from tubercle bacillus. Consequently, when human beings are infected there is no immediate host response. This characteristic allows the organism to grow more or less unimpeded. However, a variety of constituents of the tubercle bacillus, derived from mycobacterial cell wall, will induce host defense mechanism by the activation of macrophages and/or to the induction of a significant granulomatous response. This immunity is relatively nonspecific, but able to cope with heterologous microorganism and not mediated by an antibody. Study so far, could not demonstrate the protective role of antibody in TB immunity. There is no relationship between amount of antibody formed and immunity to tuberculosis (34, 95).

M. tuberculosis is capable of prolonged survival within 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.

The defense mechanisms, that protect against

tuberculosis are related to cell mediated immunity (96-97) which usually develop within approximately four to eight weeks of the initial infection. At that time a positive tuberculin, delayed type hypersensitivity develops. Unfortunately, the defense mechanisms lead to destruction of host tissue in addition to killing tubercle bacilli, resulting in caseation necrosis. Failure to react to PPD is termed "anergy" and occurs in patient with miliary tuberculosis, pulmonary tuberculosis while acutely ill and malnutrition (98-99). Anergy is mediated by suppressor monocytes resulted from the sequestration of specifically reactive lymphocytes in tuberculous tissue. Bhatnagar and Lenzini have reported that patients with tuberculin test anergy have high levels of circulating antibody to mycobacterial antigens. It has now been shown that immunity to tuberculous infection can be transferred to nonimmune host by peritoneal exudate cells or thoracic duct lymphoid cells 100-101).

2. Taxonomy and classification of mycobacteria

The causative agent of human tuberculosis is usually Mycobacterium tuberculosis, occasionally M. bovis, and rarely M. avium. The human tubercle bacillus is a group of Mycobacteria that contains species ranging from saprophytes to obligate parasites. The following classification is based upon Bergey's Manual of Determinative Bacteriology (69) and a detail is taken from (76, 102-103).

ORDER I Actinomycetales :- comprise eight families

Family I Actinomycetaceae.

Family II Mycobacteriaceae.

Family III Frankiaceae.

Family IV Actinoplanaceae.

Family V Dermatophilaceae.

Family VI Nocardiaceae.

Family VII Streptomycetaceae.

Family VIII Micromonosporaceae.

Family II Mycobacteriaceae :- Contains a single genus, Mycobacterium.

Genus I Mycobacterium :- Classified species as do not belong to Runyon Group as shown in Table 1 or belong to Runyon group as shown in Table 2.

The International Working Group of Mycobacterial Taxonomy have now 40 named species of mycobacteria.

Table 1 *Mycobacteria'* species that do not belong to Runyon group.

Characters	Species	Year of discovery	Pathogenicity	Synonyms
In vitro not growing	*M. leprae*	1847	P	Hansen's bacillus 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

* = in the immunodeficiency patient

Table 2 Runyon's speciation of the atypical mycobacteria

Characters	Species	Year of Discovery	Pathogenicity	Synonyms
Runyon Group I	*M. kansasii*	1955	F	yellow bacillus
Phatochromogens	*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	For F	*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 Rapid growers	*M. smegmatis*	1885	S	smegma bacillus *M. butyricum*, *M. lacticola*
	M. phlei	1895	S	*M. moeller*
	M. thamnopheos	1929	F	

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. Farafortuitum*
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 = Pathagen

F = Facultative pathogen

S = Saprophyte

3. Identification of the species of Mycobacteria

The identification of M. tuberculosis has traditionally been based on the following criteria.

1. Growth rate : M. tuberculosis may be detected 12 days after inoculation on egg-based media or on a nonselective medium in petri dishes, some strains may require 4 to 6 weeks before detectable colonies are apparent ; however, the average recovery time is 21 days.

2. Colonial morphology and pigmentation : M. tuberculosis colonies are rough "cauliflower" like and light buff colored.

3. Niacin production : Niacin is formed as a metabolic by product of all Mycobacteria species. Most species possess an enzyme that converts free niacin to niacin ribonucleotide ; but M. tuberculosis lacks this enzyme, and niacin accumulates as a water-soluble substance in the medium 104).

4. Nitrate reduction : M. tuberculosis possesses the enzyme nitroreductase, an enzyme that can reduce sodium nitrate into sodium nitrite.

5. Catalase production : Most Mycobacteria produce enzyme heat-labile catalase (105). The quantity of catalase activity can be detect by adding Tween-80 peroxide solution to an actively growing culture. M. tuberculosis produces a bubble column measuring less than 50 mm.

6. Thiophene-2-carboxylic acid hydrazine susceptibility : Susceptible to TCH is determined by the

presence of growth on the antibiotic-free 7H11 agar and the absence of growth on the TCH medium. M. bovis is susceptible to TCH, whereas M. tuberculosis exhibits resistance (106).

Pathogenesis and Pathology

After being inhaled, tubercle bacilli lodge in the lung and produce an inflammatory response ; they are then ingested by macrophages, where they survive destruction. Then the baccilli may multiply and being carried to regional lymph nodes and often to other parts of the body. About two weeks rather a delayed-type hypersensitivity to the tubercle bacilli develops. At the sites where the bacilli have lodged, macrophages gather around the bacilli and some coalesce to form large multinucleated cells (Langhans giant cells). Lymphocytes then collect around these multinucleated cells and tend to wall them off from surrounding tissue. This inflammatory focus, called a "granuloma", is the characteristic response of the body to the invasion of organisms and other foreign substances that resist digestion. The granulomas of tuberculosis are called "tubercles". The mycobacteria cease to multiply in the tubercles, but a few may remain viable for many years. The blood supply in areas of large collections of tubercles may be so poor that the tissue cells die and autolyse. If this involves a bronchus, the dead material may discharge into the airway, causing a cavity and spreading the bacilli to other parts of the lung. Indeed, a persistent lung cavity may remain with prolonged shedding of bacteria into the bronchus (34-35, 38, 96, 107).

Tuberculosis is a chronic necrotizing infection, that shows a wide spectrum of severity varying from individuals who are in perfectly good health (apparent in a dormant state and a tendency to reactivate many years after initial infection) to those with chronic relapsing illness and including those with an overwhelming fulminant fatal disease.

M. tuberculosis produces neither exotoxins nor endotoxins. Consequently, when human beings are infected there is no immediate host response. This characteristic allows the organism to grow more or less unimpeded until the development of an immune reaction. M. tuberculosis 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 (35, 38).

There are three types of tuberculosis lesions

1. The inflammatory or exudative lesions consisting of vasodilatation, edema, fibrinous exudate, and an influx of all elements of the leukocytic series.

2. The productive lesions consisting of aggregations of macrophages or histiocytes that undergo a metaplasia to form epithelioid cells. Sometimes the cell membranes are lost and Langhans' multinucleated giant cell are formed. When the cells are arranged in a small sphere they are termed tubercles.

3. Caseation necrosis are the lesions containing a homogeneous, amorphous mass resembling cheese, it occurs in the center of exudative lesion or productive lesions.

Over a period of time the sclerosis leads to an

obliteration of the epithelioid tissue. Calcification occurs in lesions where sclerosis has left a central necrotic residue.

The massive numbers of bacilli in a cavity lead to 3 significant clinical problems ; spreading to other portion of the lung ; the major source of an infectious risk ; and an increased chance of developing a drug resistance. Canetti states that cavities have relatively large total bacterial population of 10^{7} - 10^{9} bacilli, a caseous mass or nodule has small population of 10^{2} -10^{5} bacilli (108-109).

Primary tuberculosis is the infection that takes place in a person that has had no previous experience with this organism. The lung is usually the first organ involved. M. tuberculosis can produce infection in almost every tissue and organ in the body, extrapulmonary tuberculosis is the dissemination from an initial pulmonary focus (28, 34, 96).

Secondary (reinfection, adult) tuberculosis is a disease that occurs in persons who have been previously infected ; therefore, it occurs in those persons who are tuberculin-positive, and it occurs inspite of the presence of acquired cellular immunity. Secondary tuberculosis may come about as a result of recrudescence of an old infection (endogenous) or by reinfection from an active case (exogenous). The initial lesion of reinfection tuberculosis is the occurrence of necrosis which is the results of the destructive nature of the inflammatory reaction of tuberculin hypersensitivity. The lesion is circumscribed and localized, because of cellular immunity that, operating in all of the adjacent tissue (28, 34, 96, 107).

Clinical feature

1. Clinical presentation of pulmonary tuberculosis.

a) Symptoms and signs

Pulmonary tuberculosis, frequently develops without any striking clinical evidence of disease. Since the primary pulmonary focus is usually subpleural, rupture into the pleural space may result with the development of a tuberculous pleurisy with effusion (110). Local spreading to the hilar lymph nodes is common. It is this hematogenous dissemination of the organisms that results in the pulmonary and extrapulmonary foci that are responsible for the major clinical manifestations of tuberculosis. However, since the disease has a wide spectrum of manifestations ranging from skin positivity with negative X-rays to far advanced tuberculosis, a variety of clinical presentations also may occur. Ordinarily, until the disease is moderately or far advanced, as shown by changes on the roentgenogram, symptoms are minimal and often attributable to other causes, such as excessive smoking, hard work, debilitating, non-mycobacterial pulmonary disease (38).

Symptoms : may be divided into two categories, constitutional and pulmonary, the constitutional symptom most frequently seen is fever, characteristically, the fever develops in the late afternoon and may not be dominant symptoms. With defervescence, usually during sleep, sweating occurs. Others sign of toxemia, such as malaise, irritability, weakness, unusual fatigue, headache, and weight loss, may be present. With the

development of caseation necrosis and concomitant liquefaction of the caseation, the patient will notice coaugh and sputum, with mild hemoptysis. Chest pain may be localized and pleuritic. Shortness of breath, usually indicates extensive disease, occurs late in the course of the disease (38, 111-112).

Signs : chest examination is ordinarily of minimal help early in the disease, the principal finding over areas of infiltration is one of fine rales detected on deep inspiration followed by full expiration and a hard, terminal cough. The sign is found particularly at the apexes of the lungs, where reactivation disease has its onset in a large majority of patients. More extensive findings are present corresponding to the areas of involvement and the type of pathology. Allergic manifestations, erythema nodosum and the phlyctenular conjunctivitis, may occur (38, 111-112).

b) Laboratory examination

Routine laboratory examinations are rarely helpful in suggesting the diagnosis (113). The white blood count is often normal, and counts over 20,000 per cubic millimeter would suggest another infectious process ; however, a "left shift" in the differential white blood count may be seen occasionally in miliary tuberculosis, and can occur in advanced disease, but it does not occur in tuberculosis confined to the chest. These changes are neither specific nor useful. Other non-specific tests that may be elevated in active tuberculosis include the sedimentation rate, alpha-2 globulins, and the gammaglobulins. A positive delayed hypersensitivity reaction to tuberculin

indicated only the occurrence of a prior primary infection (114).

Smear-negative pulmonary tuberculosis (115). There are many patients in whom the diagnosis of pulmonary tuberculosis is evident from the symptoms and chest X-ray, yet sputum tests are repeatedly negative. Such a discrepancy can occur because laboratory techniques are relatively insensitive and in a sample of sputum which contains a small number of bacilli they may escape detection both by microscopy and culture.

Progressive (reactivation) tuberculosis usually develops after a period of dormancy and arises from the sites of hematogenous dissemination. In the majority of patients the disease stays dormant either indefinitely or for many years and, when a breakdown occurs, it may be secondary to a decrease in body immunity or another factors that increase susceptibility to tuberculosis (116).

Diagnosis of pulmonary tuberculosis

1. Clinical and history (see page 27)

2. Chest X-ray examination

Various abnormal changes on chest X-ray examination are frequently not detected by physical chest examination and since pulmonary tuberculosis may exist in a far advanced form in the absence of signs or symptoms, it is mandatory to obtain a standard 14x17 inch chest X-ray film as the basic minimum procedure in a tuberculosis control program. The abnormal changes in the lung can be consistent with or suggestive of tuberculous disease, it must be emphasized that these

abnormalities can be caused by other diseases.

The most common presentation in chest X-ray of pulmonary tuberculosis is the presence of a parenchymal infiltrate representing extention of the original site of infection via bronchial and lymphatic channels and radiographic enlargement of hilar or mediastinal lymph nodes or both. These changes are characteristic of a granulomatous infection of the lung and are not limited to tuberculous infection but are also seen in coccidioidomycosis, histoplasmosis and other non mycobacterial pneumonia (116-118).

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 net increased influx of fluid into pleural space by the hypersensitivity reaction to "tuberculoprotein".

The cavities that develop in tuberculosis are characterized by a moderately thick wall, a smooth inner surface, and the lack of an air fluid level. Radiographically it will appear as multiple small acinar shadows.

Although the presence of upper lobe infiltrations with or without cavitation is associated with tuberculous disease, it must be emphasized that these changes can be produced by other disease processes and that any type of infectious or granulomatous disease may be radiologically identical to tuberculosis, especially systemic fungal infections (histoplasmosis, coccidioidomycosis, and blastomycosis), bacteria

(Pseudomonas pseudomallei), atypical mycobacteria (mainly M. kansasii and M. intracellulare), the pulmonary abnormality in ankylosing spondylitis, aspiration pneumonia, and carcinoma of the lung. Lobar consolidation caused by tuberculosis pneumonia will frequently describe to other infective agents and tuberculous cavities will be attributed to aspiration pneumonia with pyogenic lung abscess (119). Therefore a radiographic abnormality should be interpreted as consistent with tuberculous disease, but the diagnosis should be confirmed by clinical evaluation, particularly skin test in non-endemic area and bacteriological studies to establish the presence of tuberculosis, including a sensitive method for antigen detection (119). A normal chest X-ray study does not exclude foci of tuberculous infection in the lung, since these are present but indiscernible in most individuals with reactive skin test and those with acid fast bacilli in sputum (120). Although some radiologists attempt to describe the activity of a lesion on the basis of its radiographic appearance, the documentation of activity is best left to bacteriologic and clinical evaluation.

3. Tuberculin skin test

Koch's discovery of the tuberculin reaction in 1890 provide a diagnostic procedure and powerful epidemiologic tool, which revealed much more widespread infection than has been suspected previously.

Significance. The tuberculin test depends on the fact that persons infected with tubercle bacilli develop hypersensitivity to the proteins of organism contained in culture

filtrates (tuberculin). The hypersensitivity resulting from tuberculous infection is the cell-mediated delayed hypersensitivity type IV . Tuberculin reaction is highly specific for the tubercle bacillus and closely related Mycobacterium. It appears about 1 month after infection in man and persists for many years, often life-long ; hence the frequency of reactions in the population increases cumulatively with age. The persistence of tuberculin reaction probably depends on persistence of bacilli in dormant foci and reactivity may disappear following chemotherapy of recent infections.

Tuberculin. Two types of tuberculin are in use, old tuberculin (OT) and purified protein derivative (PPD).

OT was first preparted by Robert Koch in 1890. It is made from heated sterilized culture filtrate of tubercle bacilli, and concentrating to one tenth of its original volume. Since it is a relatively unrefined product with extraneous material present, a significant reaction is not always diagnostic for infection.

PPD was original developed by Florence Siebert in 1939 and has been designated as PPD-S (121). It is prepared from OT by precipitation several times with 50% saturated ammonium sulfate. PPD contains a mixture of tuberculoproteins having molecular weights of 2,000 and 9,000 (average mol. wt. 10,000).

Testing procedures (122) :

1. The Von Pirquet test : a scratching test
2. The Vollmer test : a patch test
3. The multiple-puncture test : tuberculin tine test
4. Mantoux test : the intradermal tuberculin skin

test. It is the standard initial procedure for the detection of tuberculous infection.

The test is read 48 to 72 hours later. The reading consists of the measurement of the greatest diameter of induration in millimeters, the erythema is not considered, the tuberculin specific material injected, and its strength.

Interpretation

There are three categories of Mantoux result for the PPD 5 TU test

Positive = 10 mm or more of induration.

Doubtful = 5 mm through 9 mm of induration.

Negative = 0 mm through 4 mm of induration.

Although there are approximately 9% of patients showing induration between 5 and 9 mm, 10% of adult population respond with less than 5 mm (123).

Advantage of tuberculin test

The tuberculin test can provide valuable information regarding (122) :

(1) The prevalence of tuberculosis in the community.

(2) The effectiveness of control measures against tuberculosis.

(3) The differential diagnosis of tuberculosis.

(4) The prevalence of opportunistic mycobacterial infections.

(5) Those who may require BCG vaccination.

(6) Those who may require chemoprophylaxis.

(7) The differential diagnosis and assessment of conditions which may influence Type IV reactivity.

Where tuberculosis is suspected clinically and the tuberculin skin test is negative, it is necessary to rule out general anergy as the cause of failure to react. For this, skin testing with trychophytin or candida antigen is most commonly used (124).

Disadvantage of tuberculin test

1. False negative results : In the presence of proven tuberculosis, inability to respond to the 5 TU of PPD tuberculin skin test can occur from the following reasons (125).

(1) In the presence of overwhelming disease.

(2) During the 6-8 week interval following first attack of M. tuberculosis, an acute Viral illness such as exanthemata or influenza, live virus vaccination.

(3) During prolonged corticosteroid therapy.

(4) In the presence of associated lymphoproliferative disease.

(5) With the sarcoidosis syndrome associated with tuberculous disease.

(6) In severely malnourished patients.

(7) In the distinct subset of tuberculosis patients with circulating adherent suppressor mononuclear cells ; these patients frequently show an increased number of peripheral blood monocytes.

(8) Using the out-dated tuberculin, inaccurate

technique, etc.

2. False positive results : false positives may be attributed to a number of technical or biologic causes including.

(1) Tuberculin is a biologic product, and M. tuberculosis shares antigens with other atypical mycobacteria, so that the tuberculin is not completely specific (90-93).

(2) BCG vaccination.

(3) Some sensitive individuals may develop local ulceration and necrosis or vesicle formation.

(4) Poor technique of injection.

4. Bacteriologic diagnosis

By far the most important diagnostic test is the examination of a spontaneously produced specimens of sputum by smear and culture for tubercle bacilli (126).

1. Specimen collection : When pulmonary tuberculosis is suspected, specimens should be a deep respiratory expectorate sputum. However, other specimens such as nebulized saline induced sputum, gastric aspirates, or bronchoscopy or transtracheal aspirations are also appropriate for mycobacterial culture or smear. Extrapulmonary tuberculosis, appropriate specimens include blood, pleural fluid, bronchial aspirates, bone marrow, purulent exudates, joint fluid, cerebrospinal fluid, and nasopharyngeal swab.

2. Direct microscopy : direct microscopic examination of acid-fast bacilli is the most rapid procedure for the detection of mycobacteria, but not specific for tubercle bacilli, since all species of mycobacteria are acid-fast. Thus,

the presence of bacilli in the sputum is still presumptive evidence of active tuberculosis.

It has been estimated that at least 10^5 AFB per ml of sputum are required for detection by staining. To find 1 AFB in every 10 fields one would require 10^5 bacilli per ml of sputum or to find 1 AFB per field on the average would require 10^6 bacilli per ml of sputum (127-129). This quantity usually is found only in sputum specimens obtained from patients with advanced disease, particularly in the presence of cavity. These estimations have been made under the assumption that the bacilli are evenly dispersed throughout the specimen. However it is known that bacilli are not evenly dispersed in a specimen, but are frequently found in clumps (129).

Acid-fast stained smears are also useful in following a patient's response to treatment, it can provide an early and quick evaluation of the response.

Two types of acid-fast stains are commonly used :

1. Carbolfuchsin methods, Ziehl-Neelsen and Kinyoun staining procedures differ in their staining principles. The former procedure requires heating to allow the carbolfuchsin to penetrate the mycobacterial cell wall. The latter is a "cold" staining method using an increased amount of phenol in solution to enhance penetration of the cell wall. Both methods stain the mycobacterial cells red against a methylene blue counterstain. The stained smears must be viewed using a 100 x oil immersion objective.

2. Fluorochrome method, using either auramine O or

auramine-rhodamine (rhodamine = second fluorochrome). Auramine O-stained mycobacteria are bright yellow against a dark background. 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 (130).

Fluorochrome staining has a definite advantage of being more sensitive than carbolfuchsin technique. Fluorochrome-stained smears can be scanned using a 25x 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 brightly colored mycobacteria and the dark background offers a distinct advantage in scanning the fluorochrome-stained slide.

Disadvantage of fluorochrome staining is the indiscriminate staining of non viable organism and mycobacteria killed by chemotherapy may be stained. The questionable fluorochrome results must be confirmed by carbolfuchsin method.

The recommendations of the American Lung Association for reporting mycobacteria seen on acid-fast stained smears are shown in Table 3 (128).

Table 3 Enumeration of AFB on fluorochrome stained slides (25X)

Number bacilli/slide	Slide report
0	negative
1-2	positive (require more sample)
3-9	positive (rare or +)

10 or more	positive (few or ++)
1 or more per field	positive (many or +++)

3. Culture : Recovery of Mycobacteria from clinical specimens requires an enriched medium. Ordinarily this is a modification of Lowenstein-Jensen's (L-J) medium containing egg, potato extract, glycerine and an inhibitory dye (malachite green) added to retard the growth of other bacterial contaminants. The medium is inoculated with decontaminated and concentrated sediment of clinical specimen. The culture is incubated at 37 C for as long as eight weeks and is examined weekly for the appearance of colonies. There are four types of media used for the recovery of mycobacteria. Egg based does not contain agar but is solidified by heating to 85 C-90 C for 30-40 mins, they are the L-J, most commonly used, Petragnani, and American thoracic society. Agar based, Middlebrook 7H10 and 7H11 media contain agar, enhance visualization of characteristic colonies as early as ten days after inoculation, provide prominent evidence of contamination, if present, since the medium is transparent. It has been reported that addition of sodium selenate to 7H11 medium enhances the growth of M. tuberculosis (131) by increased recovery rate and decreased recovery time and was rather found not to significantly improve either (132). Liquid media, Sula's medium, this medium is very useful for the culturing of small numbers of tubercle bacilli which present in body fluids. Selective media, commonly used selective media include L-J Gruft modification, Mycobactosel and selective Middlebrook 7H11 medium,

the antimicrobials contained in these media does not preclude the necessity for decontamination of specimens.

4. Decontamination : sputum is prepared befor culture by exploiting the unusual resistance of tubercle bacilli, which has high lipid content in cell wall, to strong alkalines and acids in order to liquefy and decontaminate. Then, the treated sputum was concentrated by centrifugation. The centrifuged sediment is used for smear and culture. Mycobacteria with its extremely long generation time (20-22 hrs) may be rapidly overgrown by contaminating bacterial flora whose generation time is only 40-60 mins. If the latter are not inhibited, bacterial accumulated waste products may potentially inhibit the growth of mycobacteria.

Commonly used agents for decontamination and concentration of specimen (35, 38, 133).

- n-Acetyl-L-cysteine plus 2% NaOH
- Dithiothreitol plus 2% NaOH
- Trisodium phosphate, 13%, plus benzalkonium chloride (Zephiran)
- NaOH 4%
- Trisodium phosphate, 13%
- Oxalic acid, 5%
- Cetylpyridium chloride, 1% plus 2% NaCl.

5. Detection of circulating antibody in mycobacterial infections

Attempts have been made to establish serological tests for the diagnosis of tuberculosis since the description by Arloing and Courmant (134) in 1904. The following main

procedures are discussed in chronological order of introduction into mycobacteriology.

Method used in serological studies of tuberculosis

1) Complement fixation test : Middlebrook (135) found that complement fixation test gave positive results with only 1.5% of sera from 226 non-tuberculous persons, only 67 of 134 (50%) test performed on 58 patients with bacteriologically proven active tuberculosis were positive.

2) Agglutination test : Arloing (134) was the first to show that liquid homogenous cultures of tubercle bacilli could be agglutinated by the serum of tuberculosis patients. Interest in the direct agglutination of M. tuberculosis as a diagnostic test for tuberculosis was reviewed in 1975 by Nicholls (136), who employed a phenolized suspension of M. tuberculosis H37Ra with normal sodium hydroxide treated for 30 min at 80° C, and agglutination titer was assessed. The initial results were encouraging ; the titers of sera were less than 125 in all of 40 healthy controls and in 98% of patients with other chest diseases, but were over 125 in 94% of tuberculosis. In a subsequent study (8) results inappropriate to the diagnosis were obtained in only 13 of 220 patients (5.9%). This test was evaluated rather by other workers as shown in the following Table, but they were unable to confirm the optimistic findings of the original studies of Nicholls (136).

Test	false negative negative results in tuberculosis	false positive positive results in patients with other diseases

Geddes et al (137)	31%	43%
Williams et al (138)	29%	52%
Mitchison et al (139)	30%	63%
Zimbabwe (140)	31%	80%

The experience accumulated from the agglutination test during the past 80 years shows that this test is unreliable and has not increased diagnostic yield in tuberculosis. However, Tanaka and colleagues described a technique employing suspensions of "wax D" instead of whole cells and reported that the titers correlated with the activity of the disease.

3) Haemagglutination Tests (HA) : The most extensively investigated of all the serological tests for tuberculosis used water-soluble mycobacterial antigen (141). Scott and Smith substituted commercially available old tuberculin. Such evaluations showed an enormous variation in their sensitivity and specificity. The percentage of sera giving positive reactions varied from 0 to 50 in the case of non tuberculous sera. It was thought that the production of IgG antibodies following infection was often delayed and it was stated that the main use of the test would be to distinguish moderate or far advanced tuberculosis from-non-tuberculosis.

Later, erythrocytes were used as carriers for 3 serologically active mycobacterial glycolipids 142. There were variations from person to person in these tests but the use of discriminant analysis, showed that 72% of 211 patients and 5% of 100 healthy contacts were positive, improved discrimination between patients and contacts. So it is important to employ a battery of test, each with a separate antigen.

4) Passive Agglutination, inert carrier particles : In addition to erythrocytes, inert particles of non-biological origin, such as kaolin, bentonite and latex, have been employed as carriers of mycobacterial antigens in sensitized carrier tests such as ; a) The phosphatide kaolin agglutination test (143).

b) Bentonic flocculation test (144).

c) The latex agglutination test (145).

The tests seemed to give promising results. However, the later study (146) did not appear to offer any substantial advantages.

5) Precipitation and Gel-Diffusion : Diffusion in gel techniques were first used for diagnosis by Parlett et al (150) using culture filtrates of M. tuberculosis H37Rv as antigen. Positive results were obtained with all 48 patients with tuberculosis and only 2 of 38 healthy individuals.

Several other workers, on the other hand, have found the gel diffusion test to be of much lower sensitivity. Burrell et al (147), sensitivity of gel diffusion test, that of HA test using protein antigen, and that of HA test using polysaccharide antigen was 12.5%, 50% and 80%, respectively ; Glenchur and Kettel (148) obtained positive results with only 24% of tuberculosis smear positive cases by plate diffusion method. These authors considered that sputum microscopy was a more valuable test than serology (147-150).

Reported differences in the efficacy may be due to the false interpetation of non-specific precipitation.

6) Fluorescent antibody test : The soluble antigen fluorescent antibody (SAFA) test developed by Toussaint et al

(151) for the diagnosis of tuberculosis gave higher levels of antibody in serum of patients with advanced disease than in those with minimal or moderately advanced disease (143). The antibody titer rose after the commencement of therapy. The mean fluorimeter readings of the test of normal healthy and those with minimal, moderately advanced and advanced disease were 31.6, 44.3, 44.6, and 58 arbitary units, respectively. The range of readings in each group was not given ; the significance of the differences and the discriminatory power of the test could not be ascertained.

It was assumed that the outer surface of the slowly growing Mycobacterium species (M. tuberculosis, BCG, M. kansasii and M. avium intracellular) stained poorly by fluorescent antibody stain, whereas rapidly growing species stained well with the appropriate antisera (152).

Nassau and Merrick (11), in 1970 used whole cells of M. tuberculosis H37Rv as an antigen and assessed the fluorescence visually by use of an ultraviolet microscopy. The test was positive in 96% of culture positive tuberculosis cases and in only 2.5% of healthy donors or patients with outer diseases (titer >1:20 = Positive). These results suggested that the test was a highly discriminatory one.

Mahfouz et al (153) used old tuberculin polymerized by treatment with ethyl chloroformate as an antigen in an immunofluorescence test. He suggested that this polymerization process may remove or destroy cross-reacting antigens.

Affronti et al (154) stated that "the SAFA test proved

to be more sensitive and specific than either type of skin test. The SAFA reactive antibodies appeared long before tuberculin reaction. The test was positive only 1 in 16 cases of tuberculin-positive control subjects and 7 in 9 patients with bacteriologically proven active tuberculosis. However, a relatively small number of sera was studies.

7) Radioimmunoassay (RIA) : Minden et al. (155) demonstrated that sonicated BCG bound to rabbits anti - BCG serum but also to normal rabbits serum and rabbit serum anti - a wide range of other bacteria. It means that there are shared antigen between M. bovis (BCG) and other bacterial species. The same technique has been used by Farr et al. (156) to quantitate antibodies to M. tuberculosis in sera. Only 40% active tuberculosis patients had binding level above the highest level in the control group. Similar discouraging results were obtained by Bardoma et al (157).

In 1975, Nassau and Parsons (12) used solid-phase radio-immunoassay technique to quantitate antibodies binding to the well of polystyrene microtitre trays coated with concentrated culture filtrate of M. tuberculosis H37Rv. The results were expressed as a percentage of the activity of a standard highly positive serum. Values greater than 30% were obtained from 63% of patients with tuberculosis, which were not only confirmed bacteriologically but were all positive in the fluorescent antibody test. Subsequently, the same study but used cytoplasmic extracts antigens was done by Mauch et al , in 1980. He found that 64% of patients with tuberculosis had binding levels of antibody above 95.3% confidence limits for healthy group.

In 1981, Winters and Cox (158) used plastic-coated metal beads as carriers for antigen in a solid-phase radio-immunoassay. The antigens used were BCG whole cells, M. tuberculosis whole cells, PPD, M. tuberculosis cell walls and BCG cell walls and these gave positive results with 59, 56, 52, 46 and 37% of sera from tuberculous patients, respectively.

Radio-immunoassay, although a powerful research tool, is unlikely to find much routine application in the study of tuberculosis outside major research centres because of the radioactive hazard, the cost of the equipment and reagents. Enzyme immunoassays are likely to replace RIA in many instances (159).

8) Enzyme immunoassay : Enzyme-linked immunosorbent assay (ELISA) is a primary binding test which has been sucessfully adapted to many serological systems. The ELISA (16) uses an antiglobulin conjugated to an enzyme to detect and quantitate antibodies bound to an 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 in 1976 by Naussau et al. (18) who used a concentrated culture filtrate of M. tuberculosis H37Rv as the antigen. A serum dilution of 1:100 gave good discrimination between patients and controls. They showed the ELISA to be a potentially useful method for the serodiagnosis with sensitivities of 84% and 67% in pulmonary tuberculosis and extrapulmonary tuberculosis respectively. 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 (27). In this study significant levels of antibody were detected from 80% of AFB positive cases of tuberculosis, 66% of AFB negative tuberculosis cases and 4% of control subjects. Similar results were reported by Grange et al. (22) who used an ultrasonicate of BCG as antigen. They quantitate the antibodies in the IgG, IgA and IgM classes and significant levels of antibody were found in 75.2, 31.4 and 11.8%, respectively, of sera from 153 cases of tuberculosis.

In 1980, Reggiardo et al (21) used ELISA to quantitate antibodies in tuberculosis to three serologically active glycolipids, A, B and C, extracted from BCG and purified by silicic acid chromatography. Positive results to one or more of the 3 antigens were found in 95.55 (44 of 46) of tuberculosis, in 90% (9 of 10 cases) of lepromatous leprosy, in 2 of 66 tuberculin-positive healthy control subjects and 1 of 24 tuberculin negative controls. Glycolipid A was most discriminative among the 3 antigens.

In 1982, Viljanen et al. (26) developed ELISA for determination of IgM, IgG, IgA antibodies aginst PPD, the mean antibody levels of all three immunoglobulin classes were found to correlate with the extent of the tuberculous infection. However, the concentration of IgM antibodies was lower than those of IgG and IgA antibodies which confirmed the study of Grange et al.

Stroebel et al. (20) used highly purified antigen "antigen 6" in ELISA and found out that the test with such antigen was not only highly sensitive (94%) but also specific

(100%) enough to be used for rapid serodiagnosis of extrapulmonary tuberculosis.

Benjamin et al. (23) has showed that when a highly purified antigen such as antigen 5 derived from unheated M. tuberculosis culture filtrate was employed in the ELISA, an antibody titer greater than or equal to 1:40 would have a 95.8% specificity in the PPD-positive control subjects but decrease to 79.9% specificity in the PPD-positive control subjects. However, the non-tuberculous mycobacteriosis group also showed 52.2% positive in this test which mean that antigen 5 does not exclusively belong to M. tuberculosis and is less powerful for interspecies discrimination by ELISA.

About 20% tuberculosis patients, the study reported by Grange et al. (160), do not have significantly elevated levels of antibodies to any ultrasonicated antigens unique to M. tuberculosis, antigens restricted to the slow growing mycobacteria and antigens common to all species in the genus. Consequently, the availability of highly purified soluble antigens specific for M. tuberculosis would not permit more cases of tuberculosis to be diagnosed serologically.

Monoclonal antibodies have been used lately to distinguish between M. tuberculosis and M. bovis and even between different strains of M. tuberculosis (161). These monoclonal antibodies have been used in a serodiagnostic test for tuberculosis. Hevitt et al devised a serological test on the basis of competitive inhibition by human sera of the binding of I-labelled murine monoclonal antibodies to a solid-phase bound

pressate of M. tuberculosis (162). The best results obtained with two monoclonal antibodies (71% positivity among 71 patients with smear-positive pulmonary tuberculosis) were no better than those with other methods.

6. Detection of early mycobacterial growth or mycobacterial antigen

Almost all of the mycobacteriologic services provided by Clinical Laboratory have a common problem, i.e. replication cycle takes approximately 18 hrs. (86, 145). Primary isolation of tubercle bacilli in culture takes at least 3 weeks, including isolation, identification and drug susceptibility test, and it takes at least 6 to 10 wks to get results. Although many culture media or culture techniques have been developed (131, 164-165), satisfactory improvement has yet to be achieved. It seems unlikely that any culture medium will significantly accelerate the replication rate of the bacilli. The best hope of accelerating the recognition of tubercle bacilli is thus through techniques that permit their detection and definitive identification after completion of only a few cycles of replication (87) or detect antigen directly from culture specimen.

1) Acid-fast smear

Among the techniques used in the bacteriological diagnosis of tuberculosis, especially in developing countries, microscopy is outstanding for its simplicity, speed and minimal cost. These 3 parameters made it a method of choice detecting approximately 70-80% of the cases of pulmonary tuberculosis.

Improved sensitivity could be obtained if one uses concentrated specimens.

Floatation method : sputum was floated on liquid hydrocarbons ; liquefied with hypochlorite, acid fast bacilli separated from an aqueous suspension by non-poly solvent such as xylol. It is reported that this technique increases by about 6 to 8 times the number of bacilli observed per microscopic field (166). The sensitivity of the flotation (43.3%) and culture method (50.0%) were significantly higher than those of direct smear (34.4%). (167), all specimens were stained by the Ziehl-Neelsen method.

Centrifugation Method : The sensitivity of acid-fast smears is directly related to the relative centrifugation force. Rickman and Mayer (133). When the relative centrifugal force (RFC) was 1260xg, the correlation of positive smears for AFB to positive culture was only 25%, where as increasing the RCF to 3800xg improved this correlation to 82%.

Membrane filter filtration : polycarbonate membrane filters have been used to concentrate sputum specimens for detecting AFB by microscopic examination (168-169). The sensitivity of polycarbonate membrane filter microscopic examination, centrifuged smears and direct smears were 80.2%, 62.2% and 55.8% respectively ; all sputa were culture positive and were stained by fluorescence acid-fast method (168).

2) Culturing

The transparent agar medium, Middlebrook 7H10 and 7H11, gives earlier results than opaque egg medium, because very

small colonies can be recognized microscopically (164). The simple plating the inoculum onto slide cultures or paper strips on solid medium (170) and detecting micro colonies by acid-fast staining after as short a time as 7 days. It has been reported that addition of sodium selenate to 7H11 medium enhanced the growth of M. tuberculosis (131) by increasing recovery rate and decreasing recovery time. Other report (132) was unable to confirm this.

3) Selected ion monitoring

Recent technological advances have made it possible to recognize specific components in very small samples. Odham and colleagues (171) have detected tuberculostearic acid (10-methyloctadecanoic acid), which is unique for organisms belonging to Actinomycetales and that mycocerosis acid are specific for M. bovis and m. tuberculosis, either in sputa of tuberculous patients, or in washings of culture medium made 5 days after planting the medium with sputum concentrates, by methanolysis and analysis in a mass spectrometer coupled to a gas chromatogram. The specificity is not absolute, because tuberculostearic acid is produced by several mycobacterial and nocardial species. These authors suggest that the application of similar technique to detection of methyl mycocerostate would yield greater specificity but the signal-to-noise ratio is too low to be used practically. Thus, although not yet perfect, these methods accelerated recognition of tubercle bacilli in clinical specimen.

4) Radioimmunoassays.

Straus et al (172-173) have developed a radioimmunoassay for the defection of antigen in liquid medium that had been incubated with sputum concentrates for 4 to 25 days. The RIA was performed by using ^{125}I PPD and guinea pigs serum, anti-PPD as reagent in complication inhibition technique. Of the nine specimens that yield positive cultures all were also positive by RIA. The median time for detection of growth on culture was 33 days compared to only 8 days by RIA. Crossreacting immunoreactive material was detected in cultures of other mycobacterial species, but not in cultures of fungal and other bacterial species. The development of specific RIA for tuberculoproteins offers a new research and diagnostic approach. It may be that a great increase in both sensitivity and specificity can be accomplished by selection of an appropriate monoclonal antibody (161).

Kadival et al (174) standardised a RIA using a cell sonicate antigen of H37Rv strain of M. tuberculosis labelled ^{125}I anti BCG sera in a double antibody assay system. The assay can detect $1x10^{3}$ organisms/ml or 1 ng/ml of sonicate antigen. Antigen could be detected in biological samples like sputum, autoclaved and sonicated, in both smear positive as well as those which were only culture positive.

5) Radiometric detection of mycobacterial growth

Detection and recovery of mycobacterial growth by the radiometric method have been reported by Takahashi et al (175), Middlebrook and Reggiardo (176).

The BACTEC® system (Johnston Laboratories, Inc., Cockeysville, Maryland) uses liquid Middlebrook 7H12 medium containing ^{14}C labeled palmitic acid for the radiometric detection of mycobacterial growth. The ^{14}C label is evolved as $^{14}CO_2$ during growth and respiration. When the mycobacterial growth reaches a predetermined growth index, as determined by the amount of free $^{14}CO_2$ evolved above the liquid culture phase indicate active metabolism and growth, a positive result is recorded.

Bhardwaj, Shriniwas, and Balakrishnan 1980 (177); Bradley Nicholls, and Banfield 1979 (178) Gocclman N, Larsh H, Lendner T et al 1981 have shown that BACTEC® system to be as successful as conventional culturing method in detecting growth in specimens having a positive direct smear for acid-fast bacilli. Recovery times for M. tuberculosis by this system and conventional culturing system were 13.7 and 26.3 days, respectively. However, the BACTEC® system detected 71.8% of AFB smear negative cultures, whereas conventional cultural procedures detected 88.7%. The BACTEC® system medium was found to be more sensitive than anyone of the three conventional media evaluated ; L-J, 7H10, and 57H11 (179). The lower detection rate was felt to be a reflection of the specimen size : 0.1 ml was inoculated in the 7H12 broth and 1.5 ml was used with the conventional media.

There are two classes of indicator of drug action in the drug susceptibility test on the mycobacteria : inhibition of nucleic acid synthesis, as measured by uptake of radiolabeled uracil , and inhibition of catabolism of ^{14}C-labeled

carbohydrates and their intermediate products by measurement of evolution of labeled CO_2.

6) Enzyme-linked immunosorbent assay

ELISA was standardized to detect the presence of mycobacterial antigens in the CSF from patients of meningitis. (180). Antibody directed against certain fractions of M. tuberculosis-H37Rv sonicate was coupled to alkaline phosphatase and used for the assay. Of the 9 patients who were clinically diagnosed as tuberculous meningitis were reactive for mycobacterial antigen in the ELISA test, 32 proved to be of non tuberculous etiology were non-reactive for this test. The author suggested that, with some improvement, the ELISA appears to be a promising approach for a definitive diagnosis of tuberculosis.

Materials

1. Study population

Study population was categorized into 3 groups.

1. Patients with pulmonary tuberculosis.

Patients with pulmonary tuberculosis were from the central part of Thailand. They consisted of 86 patients, 65 males and 21 females, whose ages ranged from 18 to 78 years old. Patients were identified by chest physicians at the Tuberculosis Clinic with evidence based on respiratory and constitutional symptoms plus radiographic finding (postero-anterior radiographs 70 or 100 mm of the chest) compatible with pulmonary tuberculosis. They were recruited from Tuberculosis Center in Bangkok during May-October 1984 and none of them was previously treated. This group of patients were subsequently divided into 4 groups.

Group A (x+s+c+) consisted of 40 patients who had at least one sputum specimen positive for acid-fast bacilli on microscopic examination and positive culture for Mycobacterium tuberculosis.

Group B (x+s-c+) consisted of 10 patients whose sputum at the time of clinical diagnosis were negative at least twice for acid-fast bacilli in direct smears but sputum cultures subsequently grow out M. tuberculosis.

Group C (x+s-c+) consisted of 40 patients of group A and 10 patients of group B.

Group D (x+s-c-) consisted of 36 patients whose

sputum specimens were negative for M. tuberculosis at least twice times by both 2 direct smears and cultures at the time of clinical diagnosis.

2. Patients with non-mycobacterial pulmonary disease

Patients with non-mycobacterial pulmonary disease consisted of 40 subjects, 22 males and 18 females, whose ages ranged from 14 to 84 years old. They were diagnosed on the basis of clinical, radiographical and bacteriological findings. Sputum cultures were negative for M. tuberculosis and atypical mycobacterium in Lowenstein-Jensen Medium. In some of these patients, the etiologies of the current respiratory tract illness were known before or shortly after collection of sample and the causes of pulmonary disease were substantiated by the exhibition of the typical response when treated accordingly. Most of them (70%) suffered from bacterial pneumonia.

3. Healthy subjects

Fifty healthy subjects enrolled in this study. They were verified by history taking, physical examination to assure no recent deterioration in health status and by normal chest roentgenogram. Sputum culture must be negative for acid-fast bacilli by both microscopical examination and culture. Most of them were staff at Siriraj Hospital.

2. Clinical specimens

Serum and sputum specimens were collected from each

patient and healthy subject. Five ml of blood was obtained and allowed to clot at room temperature for 1 hour. Then the serum was removed and stored at -70°C in small aliquots which were thawed once or only twice as needed. Sputum was collected during the symptomatic period by spot expectorating into two containers one for smear and culture and the other for antigen detection by ELISA method.

3. History and symptomatology

Information about the patient's illness was extracted from their chest clinical notes and direct interview. For each suspect a questionnaire was recorded : (1) the name and address. (2) sex, (3) age, (4) duration of symptom, cough and/or sputum, the occurrence of haemoptysis, weight loss.

4. Equipments

1) polystyrene microtiter plate (NUNC).

2) Titertrek Multiskan photometer.

3) Automatic pipette.

5. Reagents

5.1 reference or standard antisera for IgG anti-PPD determination

5.1.1. Reference serum for positive anti -PPD antibody from Mycobacterial Research Laboratory, Division of Microbiology, Faculty of Veterinary Science, Chulalongkorn University, which were kindly supplied by Dr. Kriengsag Saitanu. These serum gave high titer (1:256, 1:512, 1:1024) for anti-PPD

by hemagglutination test and also were serum of the PPD skin test positive subject. They served as the reference serum for calibration of the normal pooled standard serum and the pulmonary tuberculosis positive pooled standard serum.

5.1.2 Normal pooled standard serum

Serum from 20 healthy subjects were pooled and divided into small aliquots to serve as standard for preparing checker board titration in the determination of optimal condition for IgG anti-PPD detection and serve as negative standard ELISA values in each plate

5.1.3 Pulmonary tuberculosis pooled standard serum

Serum from 12 known pulmonary tuberculosis patients which were screened for positive anti-PPD (ELISA values more than 500) by ELISA method using reference serum in 5.1.1 as standard were pooled and divided into small aliquots serving as positive standard ELISA values in each plate.

5.2 Purified protein derivative (PPD) of human tuberculin.

PPD of human tuberculin in neutralized and freeze-dried coded PPD 298 was obtained from Tuberculin Section (243). Biological Products and Standards Department, Ministry of Agriculture, Fisheries and Food. Central Veterinary Laboratory. Weybridge, Surrey, England. PPD was used as the coating antigen for ELISA method in the determination of IgG anti-PPD.

5.3 Reference bacterial antigens

5.3.1 Reference M. tuberculosis sonicated antigen.

M. tuberculosis H37Rv (NCTC 7416) was cultivated on Sula's liquid medium at 37°C for 6 weeks by daily shaking and maintained by serial subcultures. The organisms were killed by autoclaving at 120°C for 15 min. The culture filtrate and the pellet of bacilli were separated by centrifugation at 3,000 rpm for 10 min, the pellet was washed three times with 0.01M PBS buffer, pH 7.5 and finally resuspended in the same buffer. The antigen was extracted by sonication for 20 min with Soniprep (Soniprep 150 Ultrasonic Disintegrator). Using 5 min sonicating intervals with 2 min cooling to prevent over-heating. During sonication the preparation was always kept at 4°C in the ice bath. The sonicated M. tuberculosis antigen was centrifuged at 10,000 rpm for 60 min and the clear supernatant was then separated. Protein content of this solution was estimated by Lowry's method (188) using BSA as standard protein. This solution was stored at -70°C in divided small aliquots to serve as stock reference M. tuberculosis sonicated antigen using in the experiment of mycobacterial standard curve for the quantity of antigen and the precision of the assay.

5.3.2. Reference M. tuberculosis antigen in sonicated normal human sputum.

M. tuberculosis H37Rv culture was quantitated by counting the acid-fast stained organisms which was smeared on glass slide with know volume of the suitable dilution of the culture, strongly shaken with small glass beads in PBS pH 7.2. The M. tuberculosis H37Rv 10^6, 10^5, 10^4, 10^3, 10^2 and 10 organisms/ml were prepared.

Sputum from 10 normal healthy subjects were pooled. Each 1 ml of the normal healthy pooled sputum was mixed with 1 ml of 10^6, 10^5, 10^4, 10^3, 10^2 and 10 organisms/ml of M. tuberculosis H37Rv. The mixture was then autoclaved and sonicated as in 5.3.1. The supernatant was separated by centrifugation at 2,000 rpm, in order to prepare the reference M. tuberculosis antigen in sonicated normal human sputum the same as that of testing sputum.

Reference M. tuberculosis antigen in sonicated normal human sputum were prepared for using in the experiment of the sensitivity of ELISA method for mycobacterial antigen determination.

5.3.3 Non- mycobacterium bacterial and mycobacterium non-tuberculosis sonicated antigen

Clinical isolates of Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, P. pseudomallei, Staphylococcus aureus, S. epidermidis, α-Streptococcus spp, β-Streptococcus group A, γ-Streptococcus, Neisseria spp. and Nocardia asteroides were obtained from the Division of Infectious Disease, Siriraj Hospital. Three strains of each organisms were cultured in Mueller-Hinton broth at 37°C for 24 hours. The quantity of each culture was adjusted by diluted with PBS PH 7.2, five ml of 10^9, 10^8 and 10^7 organisms/ml of each culture were prepared. After that each culture was autoclaved and sonicated with the same method as in 5.3.1 but the supernatant was separated by centrifugation at 2,000 rpm (as in the preparation of the supernatant of sonicated sputum).

M. fortuitum, M. qodonae, M. scrofulaceum and M.

vaccae, which were obtained from Dr. Kriengsag Saitanue. (Mycobacterial Research Laboratory. Division of Microbiology. Faculty of Veterinary Science. Chulalongkorn University) were suspended in PBS pH 7.2 and strongly mixed with glass beads. The organisms were quantitated by the same method in 5.3.2 then autoclaved and sonicated as the same method in 5.3.1.

The non-mycobacterium bacterial and mycobacterium non tuberculosis sonicated antigen were prepared to serve as negative control in the ELISA for the experiment of the specificity of ELISA method for mycobacterial antigen determination.

5.4. Reference antisera

5.4.1. Goat IgG anti-H37Rv culture filtrate

a) Preparation of Goat IgG anti-H37Rv culture filtrate

Goat anti- H37Rv culture filtrate serum (kindly provided by Dr. J. Kenneth McClatchy, Mycobacterium Culture Collection, National Juvish Hospital and Research, Colorado, U.S.A.) was diluted with an equal volume of 0.85% (W/V) NaCl solution. In order to precipitate globulin, an equal volume of cold saturated $(NH_4)_2SO_4$ was slowly added to the diluted serum at a ratio of 1:1 with gentle stirring at 4°C for overnight. The precipitate was separated by centrifugation at 300 x g for 30 min and dissolved in 0.85% NaCl and adjust to the original volume of serum used, then reprecipitated 2-3 times with the same procedure as above. The final precipitate was dissolved in a small volumn

of 0.85% NaCl solution and by dialyzed against several changes of NaCl solution at 4° C. This globulin was used for further purification of IgG.

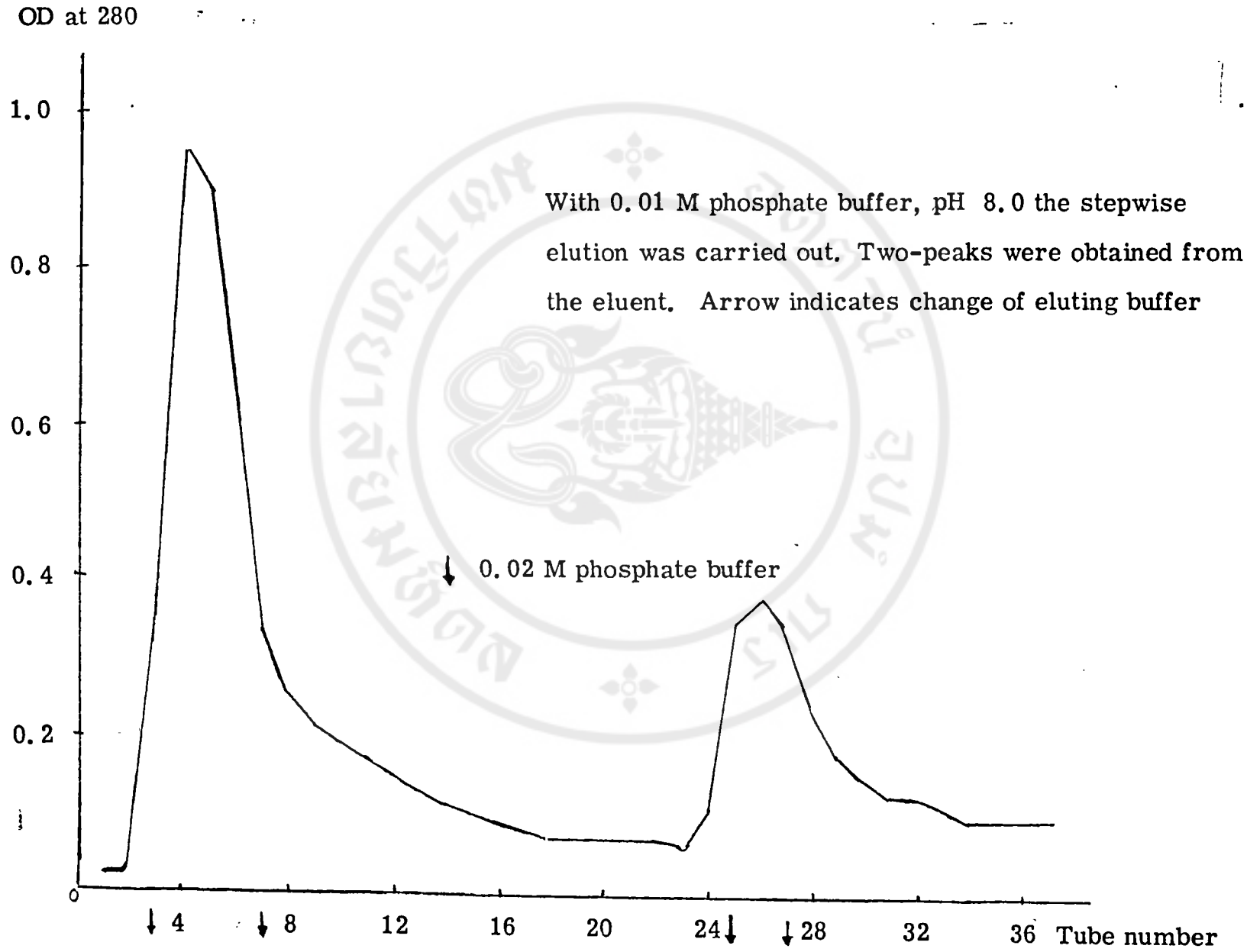
b) Purification of Goat IgG anti- H37Rv culture filtrate.

Goat IgG was purified from the globulin fraction by DEAE-cellulose column chromatography (181-183). The DEAE-cellulose (Whatman DE-52) was suspended and decanted several times in water to remove fine particles. The remainder was stirred in 15 volumes of 0.5N HCl, left in the acid for at least 30 min., then washed with distilled water until the pH of the water was 4. After that the cellulose was resuspended in 15 volumes of 0.5 N NaOH for 30 min then washed again Washing was repeated until the effluent was near neutral. the cellulose was finally equilibrated in 0.01 M phosphate buffer, pH 8.0. The equilibrated cellulose was packed into a column essentially as suggested by the manufacturer.

The globulin fraction to be chromatographed was previously equilibrated in 0.01 M phosphate buffer, pH 8.0 (starting buffer) before being applied to the column. IgG which did not adsorb to cellulose at this condition pass through the column with the starting buffer. Another portion of proteins containing the mixture of IgG and other immunoglobulins were eluted by 0.02 M phosphate buffer, pH 8.0. The elution pattern is shown in Figure 1.

The IgG fraction eluted with 0.01 M buffer was pooled from tubes No 3-45 and concentrated to 3-5 ml by Diaflo (Amicon).

Fig 1 chromatography of goat IgG on DEAE



With 0.01 M phosphate buffer, pH 8.0 the stepwise elution was carried out. Two-peaks were obtained from the eluent. Arrow indicates change of eluting buffer

The purified Goat IgG was lyophilized and stored until used. Purified goat IgG stock solution was made from freeze and dried purified goat IgG to contain 60 mg/ml.

5.4.2. Rabbit IgG anti-BCG

Rabbit IgG anti-BCG purchased from Dako patts, Denmark, was used as secondary antibody to react with mycobacterial antigen in the ELISA method for mycobacterial antigen detection. The antisera was in the form of immunoglobulin fraction prepared by salting out and ion exchange chromatography.

METHODS

I. The development of sensitive and reliable methods for rapid large scale diagnosis of pulmonary tuberculosis compared to conventional culture and microscopic methods based on :

1. Enzyme-linked immunosorbent assay for IgG antibody to PPD

Principle

The principle of this technique is the same as described by Kalish (24) and Leelarasamee (184). It is a solid-phase indirect ELISA technique.

Purified protein derivative of tuberculin coated to a solid phase and the tested serum added is allowed to bind to the coated PPD. IgG antibody to PPD is quantitated by anti human IgG alkaline phosphatase conjugate. The amount of IgG anti-PPD in the sample is proportional to the amount of alkaline phosphatase-

conjugated goat anti serum to human IgG.

Determination of optimal conditions in ELISA for IgG anti PPD :-

a. Determination of optimal concentration of PPD and of dilution of serum by indirected method.

The optimal concentrations of PPD and of dilution of tested serum were determined by checker board titration :

1) Polystyrene microtiter plates were used. A 100 ul of PPD at concentration of 40, 20, 10, 5, 2.5, 1.25, 0.62 and 0.31 µg/ml in carbonate/bicarbonate buffer (pH 9.6) was coated onto each well of duplicate horizontal rows, incubated for 2 hr at 37°C in humid chamber, and then kept overnight at 4°C.

2) The plate was washed 5 times (5 min per wash) with phosphate buffer saline pH 7.5 containing 0.15% Tween 20 (PBS-Tween 20), and then skaken dry.

3) A 200 µl of 2% BSA in coating buffer was added to each well and let stand for 2 hr at 37°C.

4) The plate was washed as in step No 2

5) A 100 µl of diluted pulmonary tuberculosis pooled standard serum at 1:50, 1:125, 1:250, 1:500, 1:1,000 and 1:2,000 in PBS/ 0.05% Tween 20/0.5% BSA, was added onto each well of duplicate vertical column of PPD coated plate. The normal pooled standard serum was assayed by the same procedure as shown in diagram and incubated for 2 hr at 37°C.

6) The plate was washed again as in step No 2

7) A 100 µl of goat anti-human IgG alkaline

phosphatase conjugate at 1;1,000 in PBS/Tween 20/BSA was added and incubated for 2 hr at 37°C.

B) The plate was washed as in step No 2 for 6 times.

9) The final step consisted of a 45 min at 37° C incubated with 100 µl of substrate solution (p-nitrophenyl phosphate 1mg/ml in 10% diethanolamine buffer (pH 9.8). The reaction was stopped by 50 µl of 3N NaOH.

10) The result was determined by reading the absorbance at 405 nm against conjugate substrate blank.

The lowest concentration of PPD and the highest dilution of tested serum that gave the best result which best differentiation between normal pooled standard serum and pulmonary tuberculosis pooled standard serum was selected as the optimal conditions.

b. Determination of the optimal incubation temperature and time

After the optimal dilution of each reagents were obtained. Four different conditions for incubation were tests as follow :

condition	PPD coated plate	serum	anti IgG alkaline phosphatase conjugate
1	37°C 2 hr	37°C 1 hr	37°C 1 hr
2	37°C 2 hr and 4°C O/N	37°C 1 hr	37°C 1 hr
3	37°C 2 hr and 4°C O/N	37°C 2 hr	37°C 1 hr
4	37°C 2 hr and 4°C O/N	37°C 2 hr	37°C 2 hr

O/N = overnight

Each condition was tested with pulmonary tuberculosis pooled standard serum and normal pooled serum. The condition that gave the highest absorbance and could best differentiate between positive IgG anti-PPD and negative IgG anti-PPD was used for further testing.

2. Enzyme-linked immunosorbent assay for mycobacterial antigen detection.

Principle

The principle of this technique is a solid-phase modified double antibody sandwich method employing H37Rv culture filtrate goat antiserum to coat the plate, and sonicated BCG cell rabbit antiserum to couple with mycobacterial antigen trapped by the former antiserum. Mycobacterial antigen is determined by Goat anti-rabbit IgG alkline phosphatase conjugate. The amount of mycobacterial antigen in the sputum or in reference antigens is proportional to the amount of anti -IgG alkaline phosphatase conjugate.

Determination of optimal condition in ELISA for mycobacterial antigen detection

a. Determination of optimal concentration for coating antibody, goat IgG anti-H37Rv culture filtrate, and the secondary anti-body, rabbit IgG anti-BCG sonicated cell.

The optimal concentrations of coated antibody and the second antibody were determined by checker board titration as followed.

1) A 100 µl of goat IgG anti-H37Rv culture filtrate

diluted to 1:500, 1:1,000, 1:2,000, 1:4,000, 1:8,000, 1:16,000 and 1:32,000 in carbonate/ bicarbonate buffer pH 9.6 (120, 60, 30, 15, 7.5, 3.7, and 1.8 µg/ml) was coated onto each well of duplicate horizontal rows of microtiter plate. The plate was incubated for 2 hr at 37°C in humid chamber, and then kept overnight at 4°C.

2) The plate was washed 5 times with PBS/Tween 20 and then shaken dry.

3) A 200 µl of 2% BSA in coating buffer was added to each well, and allowed to stand for 2 hr at 37°C before washing as in step No 2.

4) A 100 µl of 1,000 ng of standard reference sonicated mycobacterium antigen in 1 ml of PBS/T/BSA was added onto the antigen well and also PBS/T/BSA was added onto the PBS well. The plate was incubated for 2 hr at 37°C and then kept overnight at 4°C before washing as in step No 2.

5) A 100 µl of rabbit IgG anti-BCG sonicated cell diluted to 1:5,000 ; 1:10,000 ;1:20,000 ; and 1:30,000 in PBS/T/BSA was added onto each well of duplicate vertical column of antibody coated plate. The plate was incubated for 4 hr at 37°C, and then washed as in step No 2.

6) A 100 ul of goat anti rabbit immunoglobulin conjugated with alkaline phosphatase diluted to 1:1,000 dilution in PBS/T/BSA was added to each well and allowed to then incubate for 1 1/2 hr at 37°C before washing as in step No 2.

7) In the final step, 100 µl of p-nitrophenyl phosphate substrate was added to all wells and incubated for 45

min at 37°C. The reaction was stopped by 50 µl of 3 N NaOH.

The result was determined by reading the absorbance at 405 nm with Titertek Multiskan photometer.

The lowest concentration of goat IgG anti-H37Rv culture filtrate and the highest dilution of rabbit IgG anti-BCG sonicated cell that gave the best differentiation between the presence or absence of standard reference mycobacterial sonicated antigen and also best differentiation between pooled AFB positive treated sputum and pooled normal healthy treated sputum was selected as the optimal dilutions

b. Determination of optimal concentration and incubation condition for goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase.

The optimal concentration and incubation condition of antibody conjugated with enzyme were determined by the same steps as in the determination of optimal concentration of coated antibody and secondary antibody :- Microtitre plate was coated with 1:1,000 dilution of goat IgG anti-H37Rv culture filtrate, the standard reference mycobacterial antigen at 500 ng/ml was added. In control well diluent was added instead of the antigen and the plate was incubated at 37°C for 2 hr, and then kept overnight at 4°C. The 1:20,000 dilution of rabbit IgG anti BCG sonicated cell was added and incubated at 37°C for 4 hours. The dilution at 1:1,000, 1:1,500, 1:2,000, 1:3,000 of antibody enzyme conjugate was added, 100 µl per well, then incubated at 37°C for 30, 60, 90, and120 mins. The following steps were the same as described above. The highest dilution of conjugated antibody

which yielded the highest sensitivity and the lowest optical density of conjugated control was selected.

c. Determination of the optimal condition for incubation steps in ELISA.

After the optimal dilutions of each reagent were obtained, different conditions for incubation were tested as followed ;

condition	first layer antibody (Goat IgG/g) 1:6,000	antigen or sample (mycobacterial Ag)	secondary antibody (Rabbit IgG) 1:20,000
1	37°C 2 hr and 4°C O/N	37°C 2 hr 4°C O/N	4°C vary incubation time from 1, 2, 3, 4 and 5 hr
2	37°C 2 hr and 4°C O/N	37°C 2 hr 4°C O/N	27°C vary incubation time from 1, 2, 3, 4 and 5 hr
3	37°C 2 hr and 4°C O/N	37°C 2 hr 4°C O/N	37°C vary incubation time from 1, 2, 3, 4 and 5 hr.
4	37°C 2 hr and 4°C O/N	37°C 2 hr 4°C O/N	37°C 4 hr
5.	37°C 2 hr and 4°C O/N	4°C vary incubation times from 1,2,3,4, 5 hrs and O/N	37°C 4 hr
6	37°C 2 hr and 4°C O/N	25 C vary incubation times from 1,2,3,4,5 hr and O/N	37°C 4 hr

7	37°C 2 hr and 4°C O/N	37°C vary incubation times from 1,2,3,4,5 hr and O/N	37°C 4 hr
8	37°C 2 hr and 4°C O/N	37°C vary incubation times from 1,2,3,4 and 5 hr before 4°C O/N	37°C 4 hr

Each condition was tested with mycobacterial reference antigen at 250 ng/ml. In control well the diluent (PBS-T-BSA) was used. The first antibody coating plate was constant at 37°C for 2 hr and then 4°C O/N for each condition . The incubation times and temperatures for antigen incubating and the second antibody step were allowed to vary. The optimal conditions of each step were selected as the same criteria of other step.

Determination of precision, sensitivity and specificity of the ELISA method for mycobacterial antigen detection.

a. Precision

The precision of the ELISA method for mycobacterial antigen detection was evaluated by analysis the reference M. tuberculosis sonicated antigen at 2 concentrations, 1,000 ng/ml and 15.6 ng/ml and subjected to within-assay and between-assay.

Within-assay : each of the two samples was tested twelve times in a single run.

Between-assay : the two samples were tested daily for 10 days.

b. Sensitivity of the ELISA method for mycobacterial antigen determination.

Reference M. tuberculosis sonicated antigen and

reference M. tuberculosis in sonicated normal human sputum at various concentrations was analyzed 10 times daily for 10 days for mycobacterial antigen determination. The lowest concentration of antigen which were still in the acceptable range defined the sensitivity of the method.

c. Specificity of the ELISA method for mycobacterial antigen determination

Eleven clinical isolates of non mycobacterium bacteria ; E. coli, K. pneumoniae, Ps. aeruginosa, Ps. pseudomallei, S. aureus, S. epidermidis, α-Streptococcus, β-Streptococcus group A, γ-Streptococcus, Neisseria spp and N. asteroides ; and 4 species of mycobacterium-non tuberculous ; M. fortuitum, M. godonae, M. scrofulaceum and M. vaccae ; at 10^7 , 10^8 , and 10^9 organisms/ml in the form of sonicated antigens were tested for the specificity of this developed ELISA method.

II. The application of ELISA methods for rapid large scale diagnosis of pulmonary tuberculosis compared to the conventional culture and microscopic methods :-

1. The application of ELISA method for the determination of IgG anti-PPD

Serum obtained from patients with pulmonary tuberculosis and with non mycobacterial pulmonary disease together with those from healthy control group were assayed for IgG antibody activity to PPD by an ELISA in a double-blinded experiment.

The levels of antibody to PPD in the IgG class were quantified by enzyme-linked immunosorbent assay (ELISA) as described previously (22, 24), the optimal condition and reagent concentration was titrated from the first part of this study. Basically after the serum was assayed by the conventional ELISA, the presupmtively positive specimens were repeated again and confirmed by ELISA which used 2% Bovine serum albumin in carbonate/bicarbonate buffer instead of the coating antigen, (PPD).

Assay of IgG antibody to PPD in serum is shown in Figure 2. The procedure of ELISA for the IgG anti-PPD detection were the same as that previous describe in the past of the development of method.

The following control reaction were performed in tripicate, i.e.

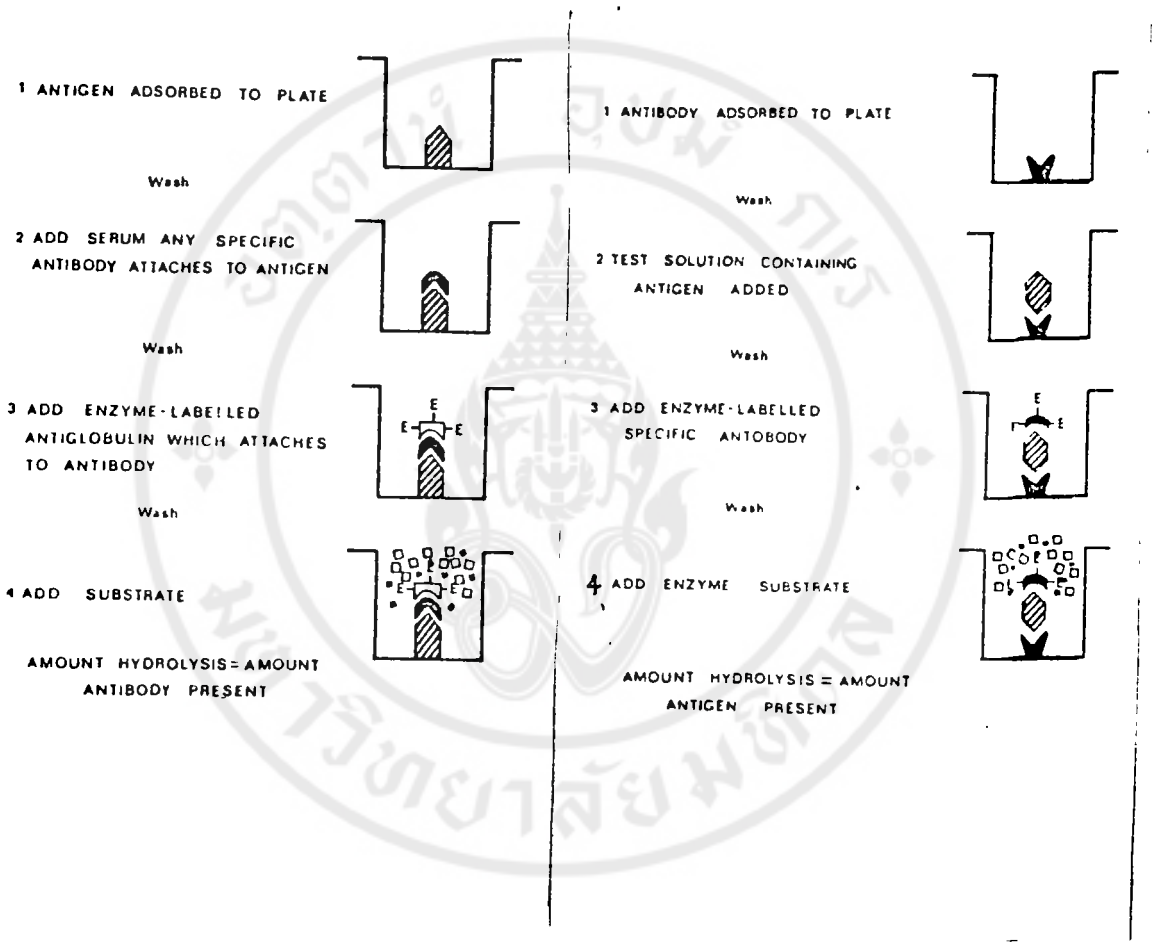
1. Conjugate-substrat : Using conjugated enzyme, followed by substrate was added to the antigen coated wells.

2. Substrate blank : Only substrate was applied into the antigen coated wells.

3. Negative control serum, positive control serum and the cut off control serum.

The result was determined by reading the absorbance of the contents in the wells with Titertek Multiskan photometer against conjugated-substrate blank at 405 nm and calculated as ELISA values which was the absorbance at 405 nm x 1,000.

The result was interpreted as positive if the ELISA values was greater than 300 (mean + 2SD of 20 healthy subjected), values lower than 300 was deripvated as negative for IgG anti-



- Fig. 2 Indirect ELISA method for assay of antibody

- Fig. 3 Double antibody sandwich ELISA method for assay of antigen

PPD.

2. The application of ELISA to the mycobacterial antigen detection.

Sputa from the same 3 groups of study population mentioned in anti-PPD detection, after apparently inspection were thoroughly investigated by microscopic examination for acid-fast bacilli, mycobacteria culture identification, and mycobacteria antigen detection by ELISA. In addition, sputum culture was performed in some patients with non mycobacterial pulmonary disease for isolation of other causative agents.

2.1 Direct inspection of sputum.

Sputa were inspected with naked eye and for volume, colour consistency and the appearence of mucus and saliva, or purulent material.

2.2 Microscopic examination

Smear preparation : a sterile stick was used to spread a small portion of purulent sputum on two glass slides as thin as possible. The prepared slides were air dried and fixed by passing three times through the flame.

Staining procedures : one of the slides was stained by Ziehl-Neelsen staining procedures, the other was stained by Auramine phenol.

Examination by microscopy : the Auramine phenol slide was first examined for at least 100 microscopic fields and then followed by Ziehl-Neelsen stain. The results of examination recorded as 0 (negative), 1+, 2+ and 3+ which were equal to 0-2 AFB/slide, 3-9 AFB/slide, >10 AFB/slide and >10 AFB/oil field respectively.

2.3 Cultural method

Sputum samples were digested and decontaminated by the swab technique. Two sterile swabs were used to mix each sputum. Each swab was placed in a tube containing excess 5% oxalic acid for exactly 25 minutes to decontaminate normal flora and other pathogenic organisms. They were then neutralized using 5% sodium citrate for 10 minutes. The treated swabs were inoculated on two slants of Lowenstein-Jensen egg medium. They were cultured at 37°C in an atmosphere of 10% CO_2. They were examined weekly for 8 weeks before being discarded as negative, and the time of appearance of the growth in each duplicated cultures were noted. All positive cultures were identified for M. tuberculosis by subculturing and observing the rate of growth at 37 C and niacin test with a commerically available paper strip.

2.4 Assay of mycobacterial antigen in sputum

2.4.1 Preparation of sputum for mycobacterial antigen detection

Three ml. of each sputum (in 13 x 100 mm tube) were autoclaved at 121°C, 15 lbs for 15 minutes in order to kill the tubercle bacilli that may be contained in sputum and to liquify the sputum. The autoclaved sputum was then sonicated for 20 min with Soniprep (Soniprep 150 Ultrasonic Disintegrator, MSE Sonicator Instruments Manor Royal, England). Using 5 min sonicating intervals with 2 min cooling to prevent over-heating of the extract preparation. During sonication, the preparation was always kept at 4°C by the use of ice bath. The treated

sputum was then centrifuged at 1,500 rpm for 10 min to separate the unbroken cells and debris (i.e. epithelial cells, white blood cells, mucous, throat flora organism and tubercle bacilli). The supernatant which represented crude mycobacterial antigens was stored in small aliquots at -20° C until used.

2.4.2 ELISA for mycobacterial antigen detection

ELISA for mycobacterial antigens detection was prepared and set up as describe in the first part of this thesis. The principle of the technique is the modified double sandwich ELISA for the detection of antigen as described by Yolken et al (187). After the treated sputum was assayed by the conventional ELISA, the presumptively positive specimens were repeated again and were confirmed by confirmatory ELISA which used normal rabbit antibody instead of BCG hyperimmune rabbit antibody.

Assay of mycobacterial antigen in sputum is shown in Figure 3. The procedures of this assay were the same as the previously described in the past of the development of method.

The following control reactions were performed simultaneously, i.e.,

1. Conjugate-substrate : Using conjugated enzyme, followed by substrate was added to the antibody coated well.

2. Substrate blank only substrate was applied into the antibody coated wells.

3. Negative control, cut off control sample and positive controls. The reference M. tuberculosis H37Rv antigens in serial dilution were included in the test.

The result was determined by reading the absorbance of

the contents in the wells with Titertrek Multiskan photometer against conjugated-substrate blank at 405 nm and calculated as ELISA values which was the absorbance at 405 nm x 1,000.

Interpretation of results : Any sample with the repeatedly mean ELISA values (absorbance at 405 x 1,000) greater than the mean ELISA of the normal control sputum plus 2 standard derivation would be judged to contain mycobacterial antigen, this cut off point was subsequently determined as 300.

Result

I. Development of methods

1. Enzyme-linked immunosorbent assay for IgG antibody to purified protein derivative of tuberculin (PPD)

The optimal concentration of reagent and condition of ELISA for IgG antibody to PPD are shown in Tables 4-5 and in Figure 4. It was concluded that for subsequent study, the following conditions will be followed : PPD concentration, 10 µg/ml (2 hr at 37 ^{o} C, and then kept overnight at 4 ^{o} C) ; serum dilution, 1:500 (2 hr at 37 ^{o} C) ; anti-IgG alkaline phosphatase conjugate dilution, 1:1,000 (2 hr 37 ^{o} C).

Table 4. Evaluation the optimal concentration of PPD and the dilution of sera in indirect micro-ELISA method for IgG anti-PPD detection.

Case	PPD ug/ml	Optical density of IgG anti-PPD				
		Serum dilution				
		1:125	1:250	1:500	1:1,000	1:2,000
Positive pooled serum	40	1.282	1.022	.795	.631	.493
	20	1.161	.973	.770	.592	.428
	10	1.120	.970	.719*	.568	.440
	5	1.109	.853	.655	.539	.408
	2.5	1.185	.861	.680	.541	.433
	1.25	.850	.701	.542	.450	.375
	6.2	.733	.611	.471	.413	.335
	3.1	.713	.600	.485	.400	.371
Normal pooled serum	40	.418	.332	.300	.279	.230
	20	.488	.311	.260	.252	.215
	10	.471	.302	.230*	.223	.210
	5	.508	.300	.248	.219	.211
	2.5	.568	.372	.250	.220	.198
	1.25	.559	.368	.240	.218	.209
	6.2	.441	.313	.220	.221	.197
	3.1	.508	.352	.230	.198	.189

*The lowest concentration of plate and the highest serum dilution that gave the best result for IgG anti-PPD are 10 ug/ml for PPD and 1:500 dilution respectively.

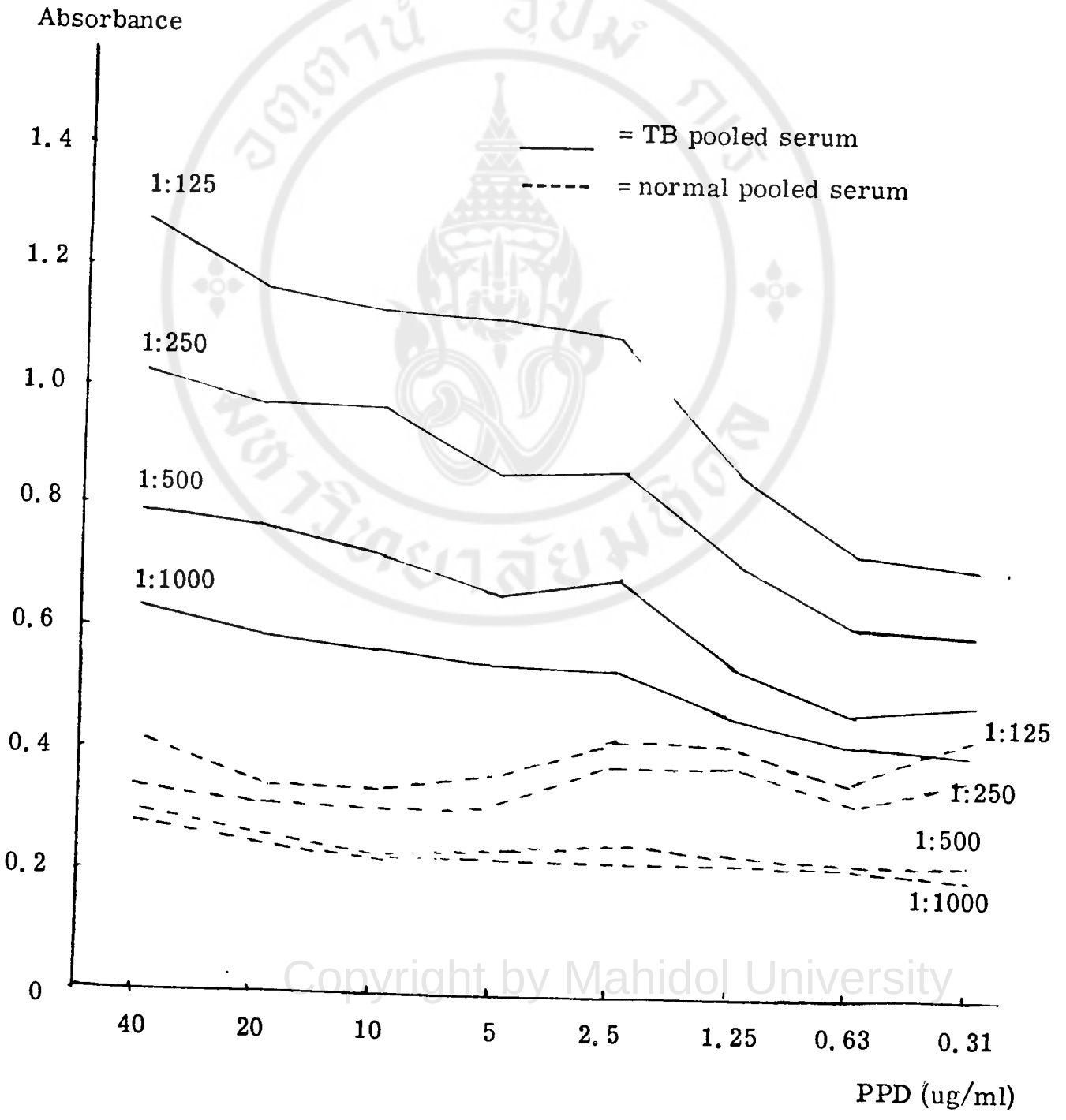
Table 5. Distribution of optical density values by various conditions in indirect ELISA method for IgG anti-PPD.

Conditions Reference	Optical Density of IgG Anti-PPD	
	Pooled + ve serum	Normal pooled serum
1) PPD 37° C 2 hrs serum 37° C 1 hr Ab-E 37° C 1 hr	.533	.190
2) PPD 37° C 2 hrs and 4° C O/N serum 37° C 1 hr Ab-E 37° C 1 hr	.556	.198
3) PPD 37° C 2 hrs and 4° C O/N serum 37° C 2 hrs Ab-E 37° C 1 hr	.653	.220
4) PPD 37° C 2 hrs and 4° C O/N serum 37° C 2 hrs Ab-E 37° C 2 hrs	.720 *	.230 *

* The optimal condition for the incubation periods for IgG anti-PPD was condition 4.

Ab-E = Goat anti human IgG conjugated enzyme

Fig 4 Evaluation the optimal concentration of PPD and dilutions of tested serum in ELISA method



2. Enzyme-linked immunosobent assay for mycobacterial antigen detection

2.1 The optimal concentration of reagent and condition of ELISA for mycobacterial antigen detection are shown in Tables 6-7 and in Figures 5-10. It was concluded that the following conditions will be followed : For goat IgG anti H37Rv culture filtrate dilution, 1:1,000 or 60 ug/ml (2 hr at 37° C, and then kept overnight 4° C in Figure 5) ; rabbit IgG anti-BCG sonicated cell dilution, 1:20,000 (4 hr 37° C in Figure 10 and in Table 7) ; rabbit globulin conjugated alkaline phosphatase dilution, 1:1,000 (1 1/2 hr 37° C in Figure 6) ; standard reference microbacterial antigen or unknown sample, the condition (Figure 7)is the same as in the first stept.

Table 6. Distribution of optical density values of mycobacterial antigen condition in modified double antibody sandwich method.

Case	Optical density				
	goat IgG dilution	rabbit IgG dilution 1:5,000	1:10,000	1:20,000	1:40,000
standard mycobacterial antigen (500 ng/ml)	1:500	1.433	1.316	1.186	.900
	1:1,000	1.303	1.157	1.039	.803
	1:2,000	1.100	1.007	.853	.599
	1:4,000	.920	.858	.702	.501
	1:8,000	.817	.716	.594	.446
	1:16,000	.711	.629	.499	.413
	1:32,000	.661	.553	.451	.400
PBS	1:500	.510	.303	.240	.220
	1:1,000	.400	.240	.229	.211
	1:2,000	.395	.263	.233	.205
	1:4,000	.364	.258	.235	.198
	1:8,000	.421	.293	.256	.205
	1:16,000	.391	.286	.245	.180
	1:32,000	.384	.257	.217	.198

Table 7. Evaluation the optimal concentration and condition of goat anti rabbit immunoglobulin conjugated with alkaline phosphatase in mycobacterial antigen detection by ELISA method.

Case	Optical density				
	incubation per mins	dilution of conjugated			
		1:1,000	1:1,500	1:2,000	1:3,000
standard mycobacterial antigen (250 ng/ml)	30	.546	.461	.390	.330
	60	.819	.646	.556	.453
	90	.899	.756	.660	.553
	120	1.106	.879	.753	.633
PBS	30	.186	.169	.159	.153
	60	.259	.199	.193	.187
	90	.260	.226	.206	.179
	120	.366	.266	.198	.223

Fig 5 Evaluation the optimal dilutions of goat anti-H37Rv and rabbit anti-BCG in ELISA method

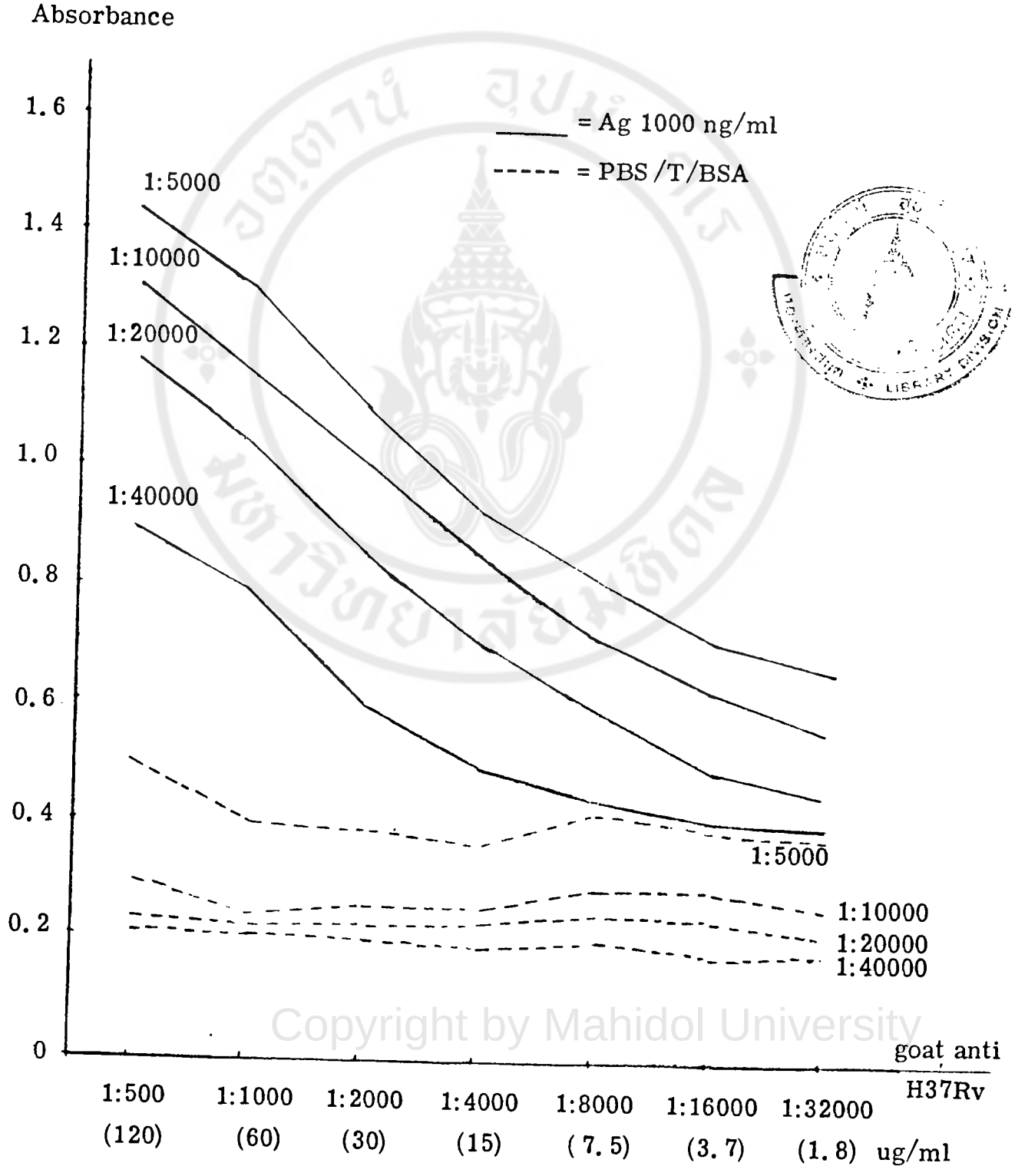
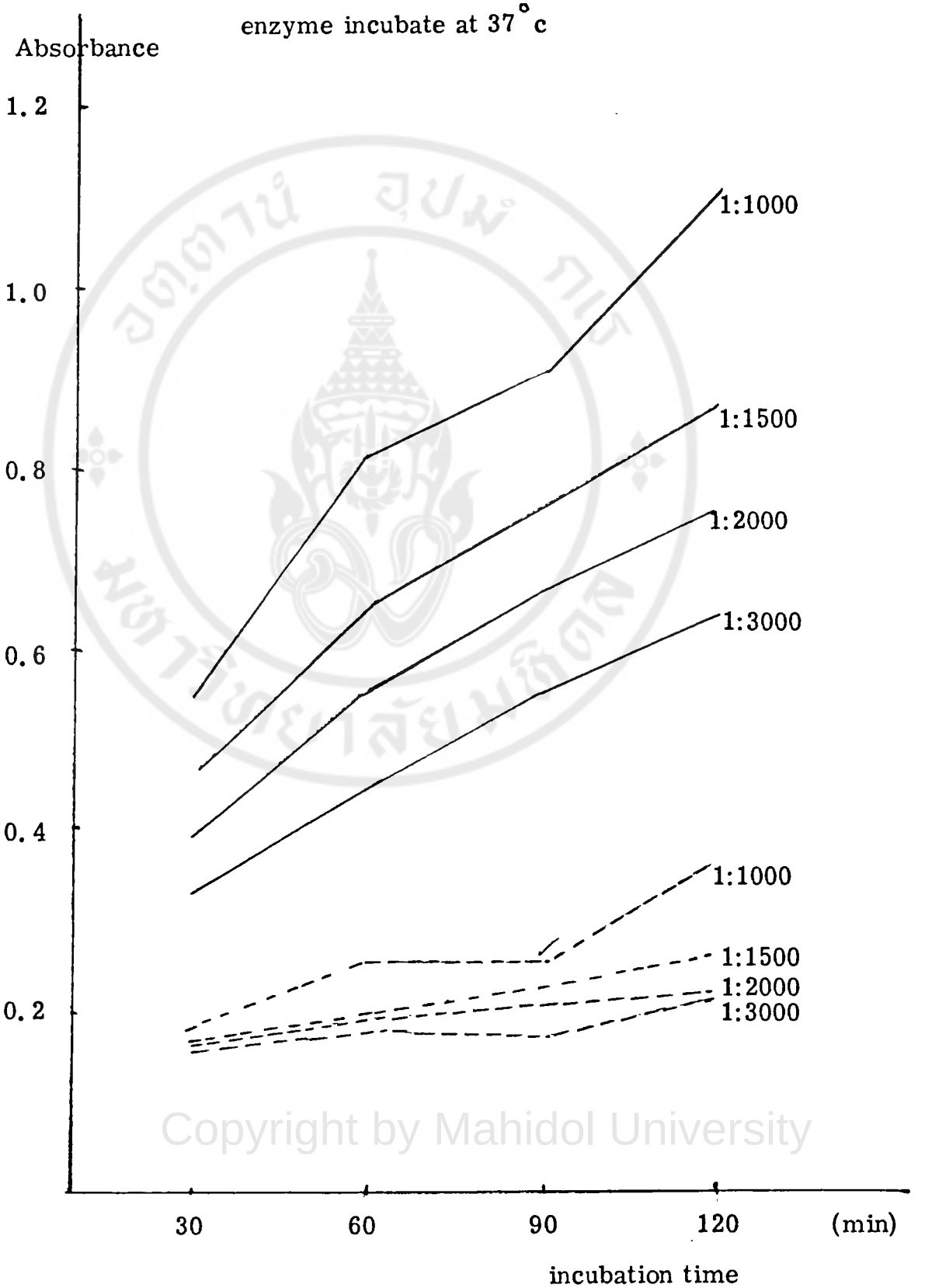


Fig 6 Evaluation the optimal dilutions and time of goat anti-rabbit globulin conjugated enzyme incubate at 37°c



ation the optimal incubation time and temperature of Ag in ELISA method

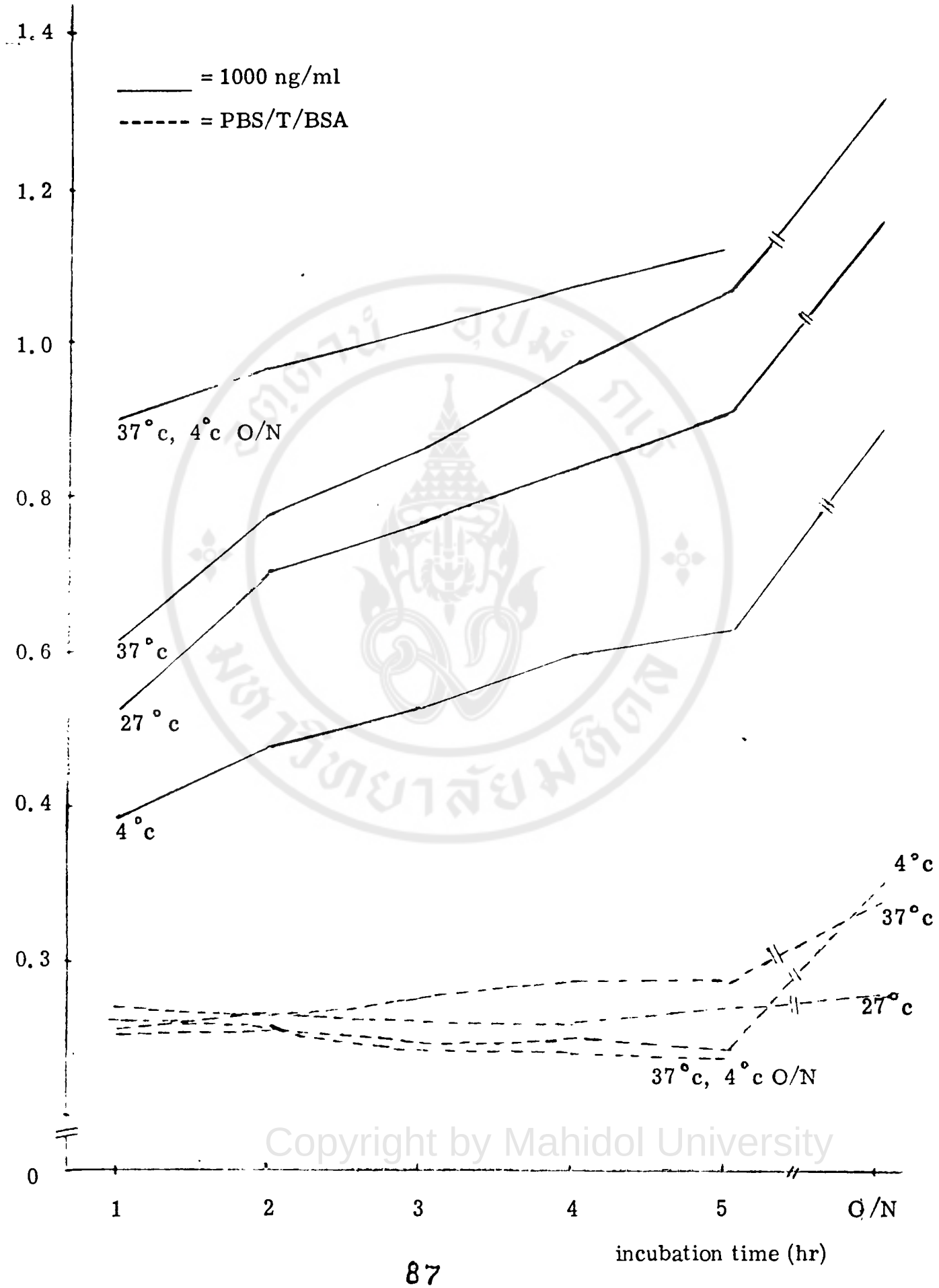


Fig 8 Evaluation the optimal concentration and time of rabbit Ig G anti-BCG incubate at 4°c

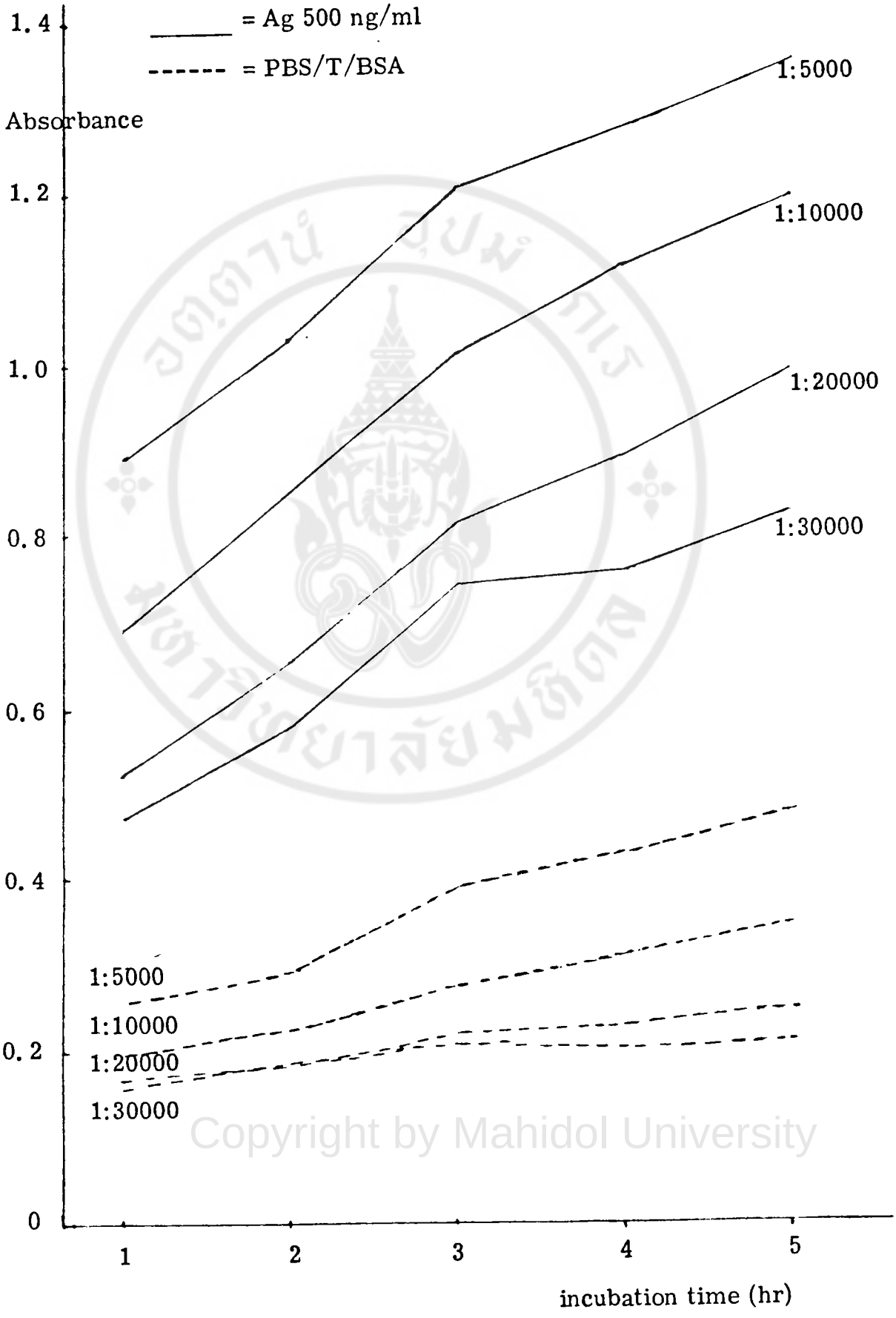


Fig 9 Evaluation the optimal concentration and time of rabbit Ig G anti-BCG incubate at 27 °c

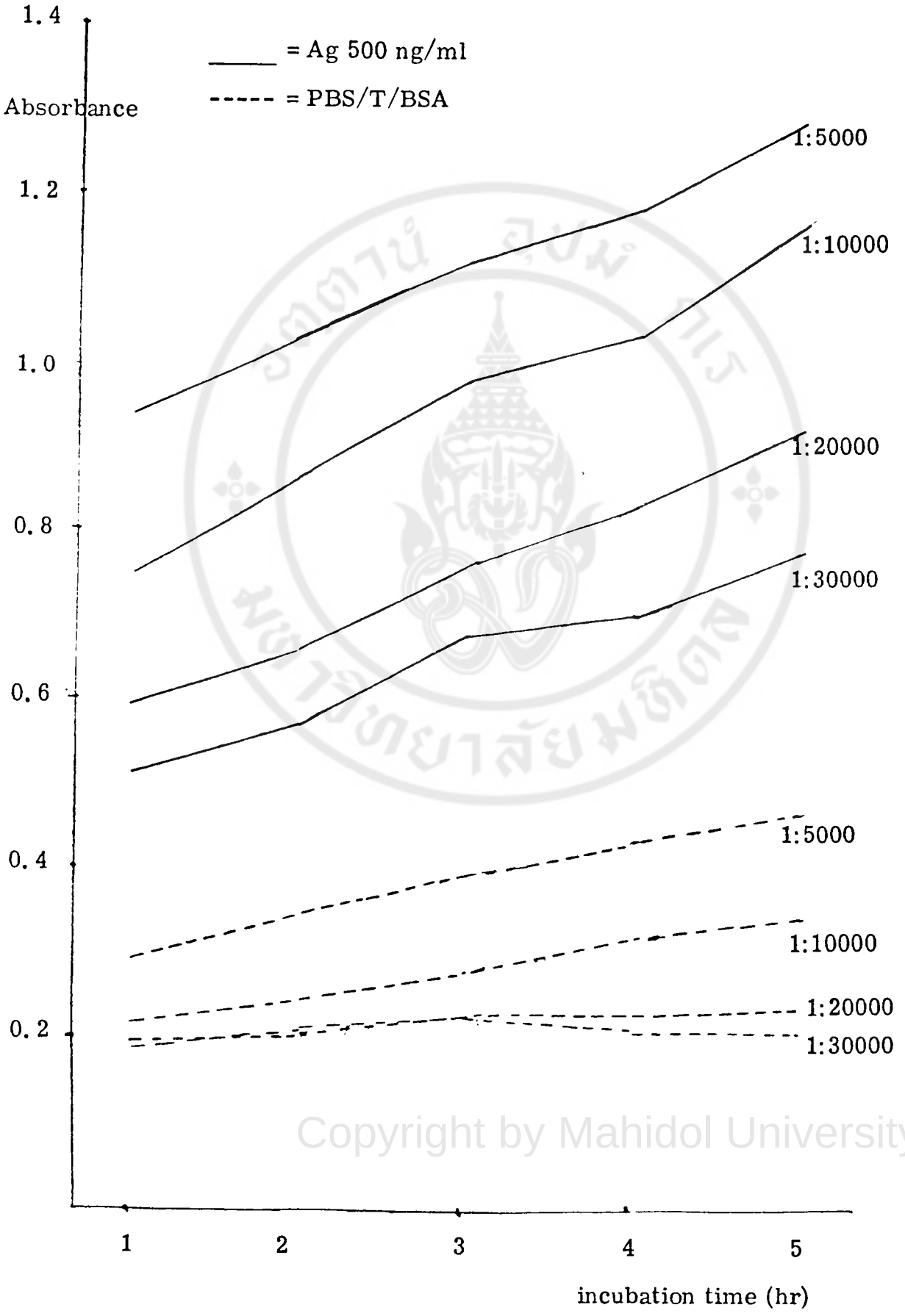
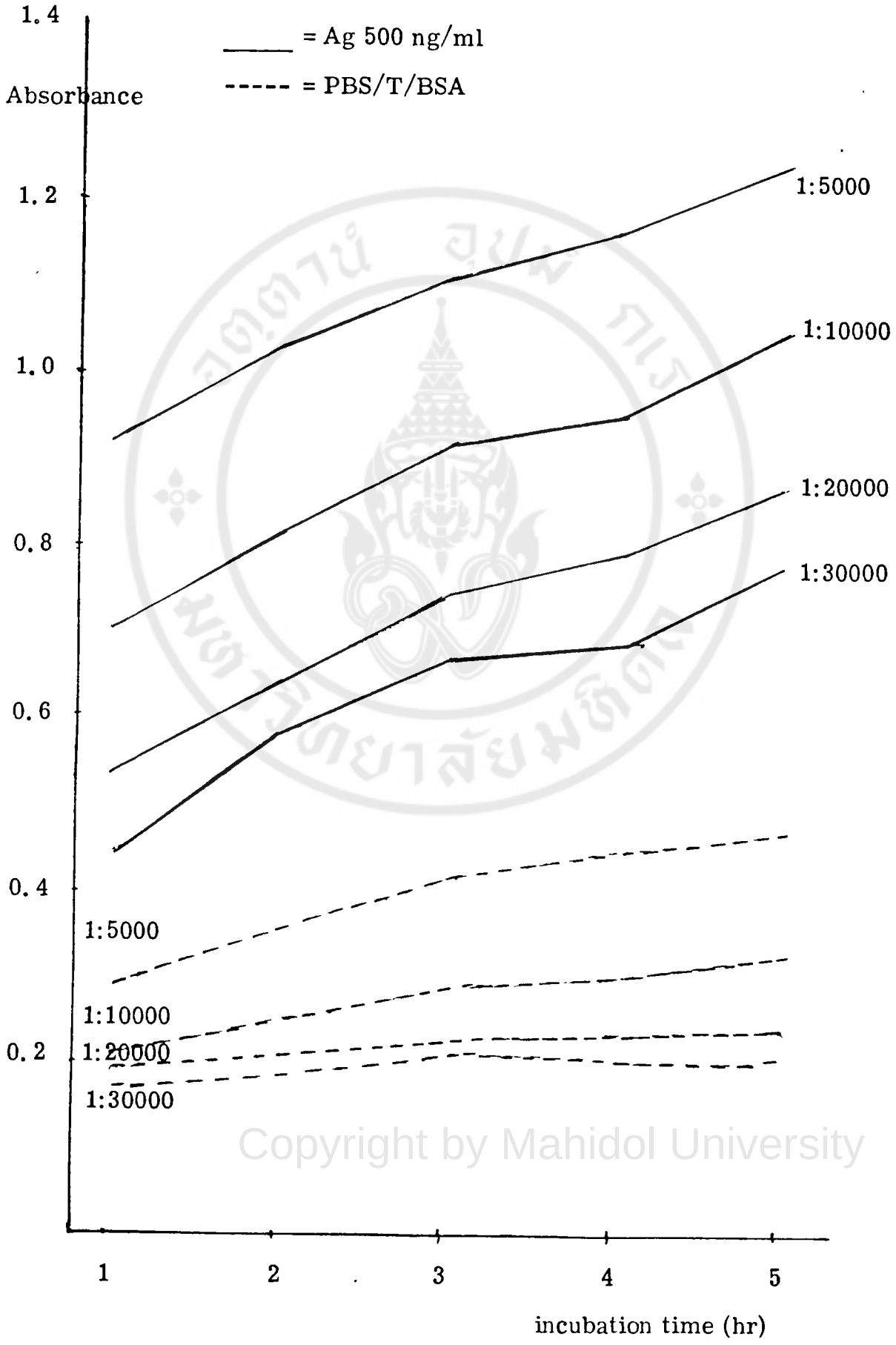


Fig 10 Evaluation the optimal concentration and time of rabbit IgG anti-BCG incubate at 37° c



2.2 Precision, sensitivity and specificity of the developed ELISA method for mycobacterial antigen detection.

a. Precision

The studies of the within assay precision of the ELISA method using reference sonicated antigen at different concentration showed satisfactory coefficients of variation (4.2-10.3%) (Table 8). The between assay precision studies also gave the accepted coefficients of variation (5.9 - 10.4%) (Table 8).

b. Sensitivity

The lowest amount of mycobacterial antigen which could be detected was 62.5 ng/ml of reference M. tuberculosis sonicated antigen which was equivalent to 1.9×10^3 cells/ml of M. tuberculosis. This concentration was significantly different from zero at 95% confidence level (Table 9, Figure 11).

In the study of reference M. tuberculosis antigen in sonicated normal human sputum, the lowest limit of detection was 10^3 cells/ml of M. tuberculosis. The concentration was significantly different from zero at 95% confidence level (Table 10 Figure 12).

c. Specificity

The specificity of the ELISA method for mycobacterial antigen determination widely varied for non-mycobacterium bacteria with its ELISA values ranged from 148-222. The ELISA

values did not increased even when the high concentration of organisms 10^9 cells/ml were used. However, it was found that their is a high degree of cross reaction in mycrobacterial antigen detection between M. tuberculosis and atypical mycobacterium (Table 11).

Table 8. Precision of the ELISA method for mycobacterial antigen detection

Sonicated mycobacterial antigen	No	ELISA values		C.V. %
		Mean	SD	
Within assay				
standard 1,000 ng/ml	12	1,062.7	45.0	4.2
standard 15.6 ng/ml	12	296.8	30.6	10.3
Between assay				
standard 1,000 ng/ml	10	1,085.5	64.3	5.9
standard 15.6 ng/ml	10	269.0	28.1	10.4

Table 9. Sensitivity of the ELISA method for the detection of mycobacterial antigen using reference M. tuberculosis sonicated antigen.

ELISA values	Concentration of reference antigen in ng/ml (cells/ml)								
	1,000 (3×10^4)	500 (1.5×10^4)	250 (7.5×10^3)	125 (3.8×10^3)	62.5 (1.9×10^3)	31.2 (9.5×10^2)	15.6 (4.8×10^2)	7.9 (2.4×10^2)	3.9 (1.2×10^2)
1	1,194	943	723	546	444	357	276	-	-
2	1,208	943	710	547	429	351	261	-	-
3	1,066	888	655	454	370	295	215	177	-
4	1,081	842	597	436	355	293	210	172	-
5	1,052	784	590	472	393	315	269	238	217
6	1,052	776	596	424	334	276	344	213	188
7	1,095	870	649	480	379	324	273	239	192
8	1,024	816	617	455	335	301	255	220	143
9	1,044	762	588	459	443	393	357	329	255
10	1,039	744	484	426	471	364	330	297	255
Mean	1,085	836	620	469	395	327	269	235	213
SD	64.3	75.6	68.6	44.2	48.8	37.7	45.7	54.4	35.2
No of test	10	10	10	10	10	10	10	8	6
Different between ELISA values	-	249	216	151	74	68	58	34	22
P values P>0.05	s	s	s	s	s	NS	NS	NS	NS

ANOVA one way classification

Table 10 Sensitivity of the ELISA method for mycobacterial antigen determination using reference M. tuberculosis antigen in sonicated normal human sputum

ELISA values	Number of M. tuberculosis in treated sputum (cells/ml)					
	10^6	10^5	10^4	10^3	10^2	10^1
Mean	> 1855	1855	974	583	235	223
Standard deviation	60.5	45.0	51.0	37.7	32.0	30.1
No. of test	10	10	10	10	10	10
Different between ELISA values	-	> 881	881	391	348	12
Statistical test P - values (F test)* P > 0.05	-		S.	S.	S.	N.S.

* ANOVA one way classification

Table 11 Specificity of the ELISA method for mycobacterial antigen determination

ELISA values / organism	Number of organism (cells/ml)		
	10^7	10^8	10^9
Non-mycobacterium bacteria			
E. coli	222	204	212
K. pneumoniae	198	189	203
Ps. aeruginosa	211	186	189
Ps. pseudomallei	194	200	237
S. aureus	182	166	203
S. epidermidis	178	150	195
α - Streptococcus	181	148	179
β - Streptococcus group A	174	166	262
γ - Streptococcus	179	160	181
Neisseria s.p.p.	182	186	296
N. asteroides	726	1221	1534
Mycobacterium - non-tuberculous			
M. fortuitum	806	1100	1305
M. gadonae	1248	1300	1769
M. scrofulaceum	994	1395	1965
M. vaccae	716	826	1296

Fig 11 standard curve of mycobactirial Ag
by ELISA technique
(reference M. tnberculosis sonicated antigen)

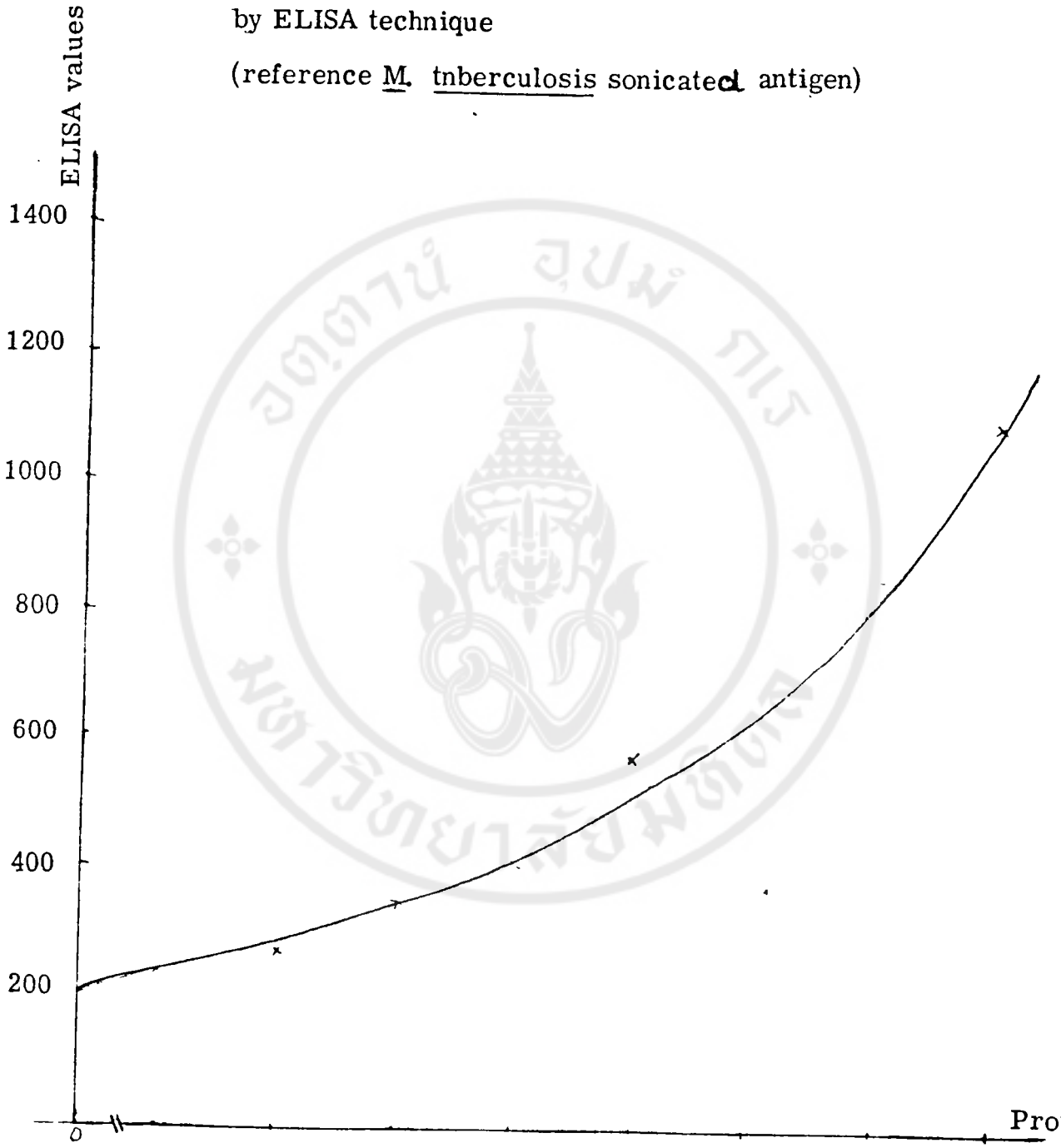
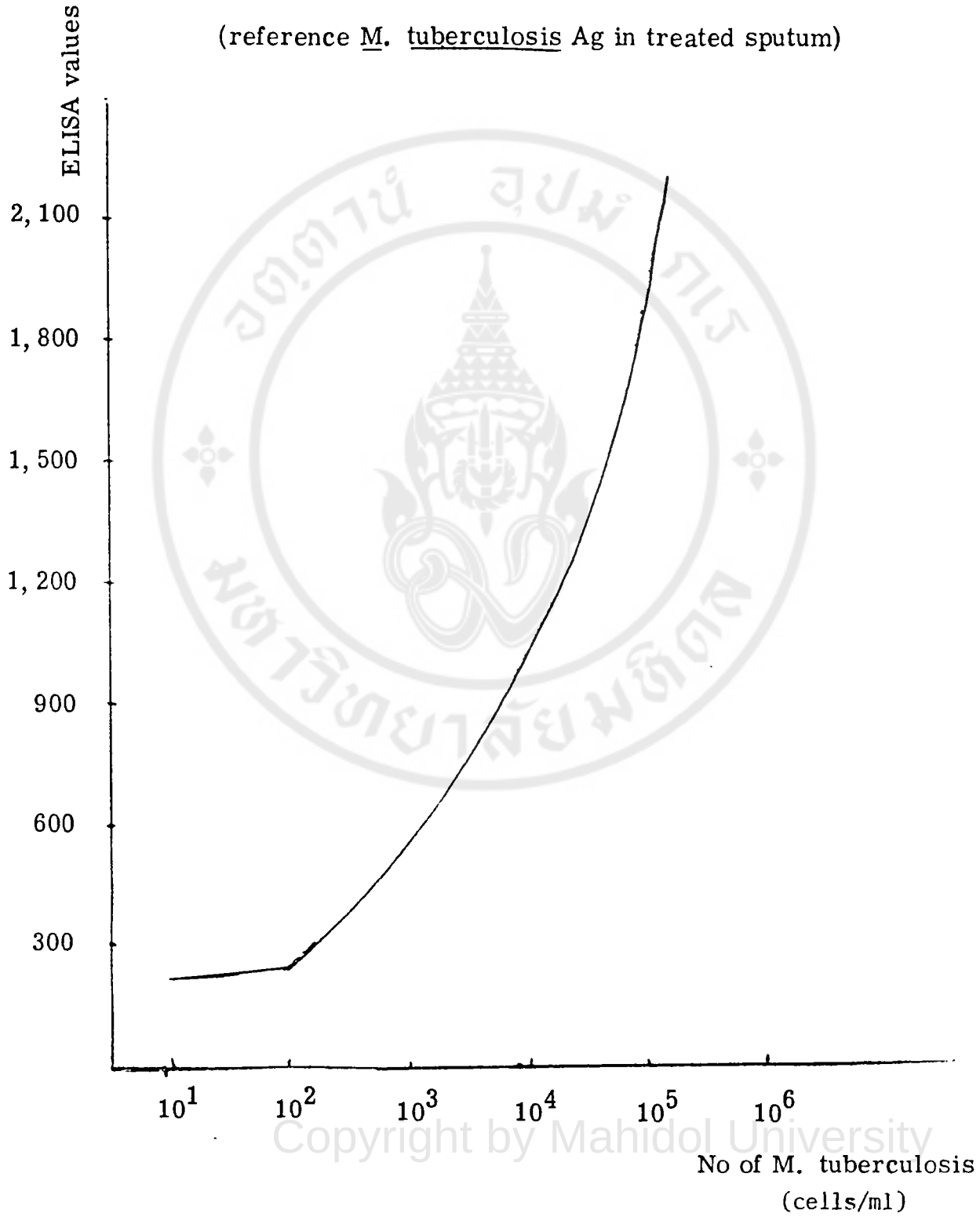


Fig 12 Standard curve of mycobacterial Ag
by ELISA technique
(reference M. tuberculosis Ag in treated sputum)



II. Application of the developed ELISA methods to the determination of IgG anti-PPD and mycobacterial antigen

1. General

A total of 126 patients were enrolled in this study and of these, 86 and 40 cases could be categorized into pulmonary tuberculosis and non-mycobacterial pulmonary diseases respectively. Serum and sputum specimens were also obtained from 50 healthy subjects. Most of the cases were Thai males and lived in Bangkok. The demographic data are shown in Table 12. Of 86 pulmonary tuberculosis suspects, all had chest radiography compatible with pulmonary tuberculosis, 40 cases (46.5%) and 50 cases (58.1%) had positive smears and culture respectively, only 10 cases (11.6%) had negative smear but positive culture, and sputa in 36 cases (41.9%) were negative by both smear and culture.

Table 13 showed the various diagnoses in the non-mycobacterial pulmonary diseases. Of 40 cases, 25 cases (62.5%) suffered from acute bacterial pneumonitis, and only 12 cases (30%) had non-infectious pulmonary diseases.

2. Clinical details between patients with definite pulmonary tuberculosis and patients with possible pulmonary tuberculosis.

A summary of clinical details of subjects in each group is shown in Table 14. Radiographic finding showed cavitation in 67.5% and 36.1% of the definite pulmonary tuberculosis patients (x+s+c+)and the possible pulmonary tuberculosis (x+s-c-) respectively. The extent of pulmonary lesions in most of the forms group (47.5%) involved three lobes

or more, but most of the later group (47.2%) has only one lobe involved. Haemoptysis could be elicited in 60% of the definite pulmonary tuberculosis and 36.1% of the possible pulmonary tuberculosis. There are significant difference in radiographic finding, cavitation and extent of pulmonary lesions ; in bloodly sputum ; and in fever symptom, between these two groups. But there are no significant difference in symptomatology such as, cough, chest pain, weight loss ; and the duration of disease. History of having haemoptysis and or cavitation on chest film was found in 92.5% of positive smear and culture and 55.5% of negative smear and culture, a cough of one to twelve was elicited from all the positive smear and from 97.2% of TB suspects whose sputa were negative for smear and culture. Duration of disease was between 2-3 months in one half of each groups.

Table 12 Percentage distribution of cases and control subjects by age, sex, residence,

	patients with definite pulmonary tuberculosis (X+S+C+) n = 40	Patients with possible pulmonary tuberculosis (X+S-C-) n = 36	patients with non-mycobacterial pulmonary disease (X-S-C-) n = 40	P-value*
Age				
Age in years				
10-29	9	8	10	
30-49	17	16	7	
⩾ 50	14	12	23	
mean	41.8	41.9	42	> 0.05
standard deviation	14.6	14.2	14.6	
range	20 - 78	18 - 72	14 - 84	
Sex				
male/female	31/9	28/8	22/18	< 0.05
ratio	3.4/1	3.5/1	1.2/1	
Residence				
Bangkok/others	28/12	16/20	-	< 0.05
Contact				
Yes	8 (25%)	10(38.5%)	-	> 0.25
No	32	26		

* Chi-square method

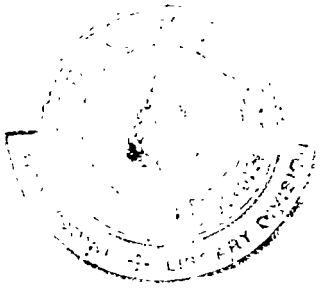
Table 13 Distribution of non-mycobacterial pulmonary disease cases by diagnosis (n = 40)

Diagnosis	Number of cases
Pulmonary infections	
acute bacterial pneumonitis	25 (62.5%)
infected bronchiectasis	3 (7.5%)
Chronic obstructive pulmonary disease	3 (7.5%)
Asthma	4 (10.0%)
Malignancy	
bronchogenic carcinoma	3 (7.5%)
metastatic carcinoma	2 (5.0%)

Table 14 Percentage distribution of clinical details of subjects in each study group

	patients with definite pulmomary tuberculosis (X+S+C+) n = 40	patients with possible pulmonary tuberculosis (X+S-C-) n = 36	P.value
Radiographic finding			
cavitation - present	27 (67.5%)	13 (36.1%)	< 0.01*
- absent	13 (32.5%)	23 (63.9%)	
Extent of pulmonary lesions			
one lobe	6 (15%)	17 (47.2%)	< 0.01%*
two lobes	15 (37.5%)	13 (36.1%)	
three lobes or more	19 (47.5%)	6 (16.6%)	
History and symptomatology			
Haemoptysis			
Yes	24 (60%)	13 (36.1%)	
No	16 (40%)	23 (63.9%)	
Haemoptysis $\bar{c}$ cavitation	12 (30%)	6 (16.6%)	
Haemoptysis and/or cavitation	37 (92.5%)	20 (55.5%)	
Fever			
Yes	39 (97.5%)	25 (69.4%)	< 0.001*
No	1 (2.5%)	11 (30.6%)	

Cough			
Yes	40 (100%)	35 (97.2%)	> 0.1*
No	0	1 (2.8%)	
Chest pain			
Yes	29 (72.5%)	28 (77.8%)	> 0.5
No	11 (27.5%)	8 (22.2%)	
Weight loss			
Yes	26 (65%)	23 (63.9%)	> 0.5
No	14 (35%)	13 (36.1%)	
patients ≥ 4 symptoms	21 (52.5%)	21 (58.33%)	
Duration of disease			
< 2 months	22 (55%)	20 (55.5%)	> 0.05
3-6 months	13 (32.5%)	10 (27.8%)	
> 6 months	5 (12.5%)	6 (16.7%)	



3. IgG anti-PPD and mycobacterial antigen levels in various study groups.

All ELISA values for anti-PPD IgG and mycobacterial antigen in sputa and their distribution in various study groups are presented in Tables 12A-12B and 13A-13C, and in Figures 13-16. Summary of statistical data are shown in Tables 17-18 and 19-20 for mycobacterial antigen.

Sera at the 1:500 dilutions of definite pulmonary tuberculosis patients proven by chest roentgenogram and sputum-smear-positive (x+s+) yielded highest mean ELISA value of 497.9, highest standard deviation of 462.5 and longest range of 57-1,791. The pulmonary tuberculosis suspects characterized by chest radiograph compatible with pulmonary tuberculosis but sputum-negative by both smear and culture (x+s- or x+c-) and non-mycobacterial pulmonary disease (x-s- or x-c-) yielded mean ELISA values of 256.9 with standard deviation of 150.8 and 263.3 with 139.9 respectively, the maximum ELISA values were 562 and 603 respectively. The analysis of variance (Kruskal-Wallis test) of the means obtained from three groups of samples. Samples in both experiments ; one using X-ray and AFB smear technique as reference (Table 17), the other using X-ray and culture method as reference (Table 18) ; P values were greater than 0.05. This demonstrated that there were no statistically significant difference between anti-PPD IgG in the various groups of pulmonary tuberculosis and the control group (non mycobacterial pulmonary disease).

Undilute-treated sputum of definite pulmonary

tuberculosis (x+s+) had highest mean ELISA values of mycobacterial antigen, greatest standard deviation, and also widest range, these values are 787, 363.6 and 292-1,530 respectively. In the pulmonary tuberculosis suscepts (x+s- or x+c-), the mean ELISA value, standard deviation, and range of ELISA value were 413.5, 312.4 and 94-1,438 respectively. In two control groups ; non mycobacterial pulmonary and normal healthy subjects ; three values were 169.3, 77.8 and 78-448 respectively in the first control group and 179.8, 80.4 and 64-408 respectively in the second control group. The analysis of variance (kruskal-Wallis test) of the means of mycobacterial antigen detected by ELISA method obtained from two groups of tuberculosis and two control groups revealed P-values of 0.0001 adn less than 0.05. This showed that there were statistically significant difference among the mycobacterial antigen levels obtained from four study groups as shown in table , that using X-ray and smear as reference methods or in table , that using X-ray and culture as reference. In additional there were statistically significant difference of antigen levels obtained from pulmonary tuberculosis group (x+s+) and the control group ; between those obtained from pulmonary tuberculosis group (x+c+) and the control group ; this significant difference also presented between those obtained from the tuberculosis suspects (x+s-c-) and the control group. However, there was significant difference between mycobacterial antigen level of tuberculosis group (x+s+) and that of the tuberculosis suspects (x+s-c-), but there was no statistically significant difference between two control groups, non-mycobacterial pulmonary disease and normal

healthy subjects.

From Table 18 it can be seen that an ELISA values at 300 was condidered as a cut-off point for both IgG anti-PPD and mycobacterial antigen detection. By ELISA method IgG anti-PPD could be demonstrated in one half (20 of 40 cases) of definite pulmonary tuberculosis (x+s+c+), 40% (4 of 10 cases) 36.1% (13 of 36 cases) in pulmonary tuberculosis (x+s-c+), and pulmonary tuberculosis suspects with only the chest X-ray compatible with tuberculosis (x+s-c-). However, IgG anti-PPD could be detected in 30% of control group. By ELISA method mycobacterial antigen was measurable in about 80-95% of pulmonary tuberculosis (x+s+c+) or (x+s-c+). Beside these about 50% of pulmonary tuberculosis suspects who had the chest X-ray compatible with tuberculosis (x+s-c-) had detectable mycobacterial antigen in their sputa. The false positive mycobacterial antigen was only 5% in non-mycobacterial pulmonary group.

Table 15A : ELISA Values of IgG anti-PPD and mycobacterial antigen in definite pulmonary tuberculosis patients proven by chest roentgenogram and positive sputum smear and culture

(X+S+C+) n = 40

No	ELISA Values		No	ELISA Values	
	IgG anti-PPD	mycobacterial antigen		IgG anti-PPD	mycobacterial antigen
1	487	1009	21	143	972
2	254	336	22	565	422
3	1782	435	23	637	596
4	982	636	24	285	569
5	547	685	25	993	292
6	520	884	26	235	792
7	685	938	27	159	1530
8	1791	756	28	166	1264
9	92	1504	29	148	996
10	311	1032	30	448	868
11	226	1524	31	497	306
12	132	629	32	230	550
13	121	297	33	91	735
14	350	385	34	468	1010
15	187	783	35	853	302
16	209	342	36	57	1097
17	292	1268	37	164	865
18	881	421	38	991	460
19	1020	715	39	1686	826
20	122	937	40	112	1482

Table 15B ELISA Values of IgG anti-PPD and mycobacterial antigen in pulmonary tuberculosis patients proven by chest roentgenogram and negative sputum smear but positive culture.

(X+S-C+) n = 10

No	ELISA Values	
	IgG anti-PPD	mycobacterial antigen
1	348	394
2	724	176
3	75	403
4	76	400
5	1637	526
6	68	439
7	245	358
8	131	458
9	231	195
10	888	305

Table 15C ELISA Values of IgG anti-PPD and mycobacterial antigen in pulmonary tuberculosis suspected by chest radiograph compatible with pulmonary tuberculosis with negative smear and culture

(X+S-C-) n = 36

No	ELISA Values		No	ELISA Values	
	IgG anti-PPD	mycobacterial antigen		IgG anti-PPD	mycobacterial antigen
1	416	501	19	83	1438
2	279	407	20	499	794
3	212	211	21	99	533
4	41	150	22	117	163
5	147	558	23	495	458
6	69	203	24	232	200
7	134	269	25	281	355
8	121	143	26	139	461
9	429	94	27	136	129
10	264	112	28	348	196
11	298	347	29	188	701
12	347	260	30	523	656
13	438	155	31	62	471
14	68	484	32	441	296
15	164	165	33	350	863
16	317	282	34	108	318
17	386	165	35	190	1084
18	562	222	36	268	1042

Table 15D ELISA Values of IgG anti-PPD and mycobacterial antigen in non-mycobacterial pulmonary patients

(X-S-C-) n = 40

No	ELISA Values		No	ELISA Values	
	IgG anti-PPD	mycobacterial antigen		IgG anti-PPD	mycobacterial antigen
1	251	96	21	33	187
2	603	448	22	83	152
3	247	92	23	48	231
4	347	192	24	220	282
5	309	154	25	222	139
6	360	205	26	280	125
7	280	125	27	228	78
8	281	126	28	82	216
9	400	245	29	293	138
10	446	291	30	300	145
11	261	106	31	253	98
12	537	382	32	330	175
13	358	208	33	283	128
14	273	123	34	54	188
15	344	194	35	57	121
16	248	98	36	69	160
17	381	231	37	110	172
18	264	114	38	68	148
19	591	107	39	270	115
20	214	139	40	251	96

Acute bacterial pneumonitis	=	No 1 - 25
Infected bronchiectasis	=	26 - 28
Chronic obstruction pulmonary disease	=	29 - 31
Asthma	=	32 - 35
Bronchogenic carcinoma	=	36 - 38
Metastatic carcinoma	=	39 - 40

Table 15E ELISA Values of mycobacterial antigen in sputa from healthy control group

(X-S-C-) n = 50

No	ELISA Values of mycobacterial antigen	No	ELISA Values of mycobacterial antigen
1	169	26	205
2	127	27	156
3	285	28	195
4	120	29	100
5	88	30	175
6	151	31	212
7	103	32	309
8	99	33	153
9	99	34	362
10	159	35	76
11	199	36	176
12	134	37	94
13	214	38	293
14	64	39	148
15	164	40	190
16	272	41	146
17	133	42	245
18	237	43	365
19	106	44	116
20	142	45	300
21	141	46	148
22	117	47	211
23	254	48	112
24	264	49	126
25	128	50	408

Table 16A Distribution of ELISA Values of IgG anti-PPD (1:500 dilution) from tuberculous and non-mycobacterial cases

ELISA Values	Patients with pulmonary tuberculosis				patients with non-mycobacterial pulmonary disease
	(X+S+) n = 40	(X+C+) n = 50	(X+S-C+) n = 10	(X+S-C-) n = 36	(X-S-C-) n = 40
0 - 99	3	6	3	6	8
100 - 199	10	11	1	10	1
200 - 299	7	9	2	7	18
300 - 399	2	3	1	6	8
400 - 499	4	4	-	5	2
500 - 599	3	3	-	2	2
600 - 699	2	2	-		1
700 - 799	-	1	1		
800 - 899	2	3	1		
900 - 999	3	3	-		
1000 - 1099	1	1	-		
1100 - 1199	-	-	-		
1200 - 1299	-	-	-		
1300 - 1399	-	-	-		
1400 - 1499	-	-	-		
> 1500	3	4	1		

Table 16B Distribution of ELISA Values of mycobacterial antigen in sputa from pulmonary tuberculosis patients

ELISA Values	Patients with pulmonary tuberculosis			
	(X+S+) n = 40	(X+C+) n = 50	(X+S-C+) n = 10	(X+S-C-) n = 36
0 - 99	-	-	-	-
100 - 199	-	2	2	8
200 - 299	2	2	-	10
300 - 399	5	8	3	3
400 - 499	4	8	4	5
500 - 599	3	4	1	3
600 - 699	3	3		1
700 - 799	5	5		2
800 - 899	4	4		1
900 - 999	4	4		-
1000 - 1099	4	4		2
1100 - 1199	-	-		-
1200 - 1299	2	2		-
1300 - 1399	-	-		-
1400 - 1499	1	1		1
> 1500	3	3		-

Table 16C Distribution of ELISA Values of mycobacterium antigen in sputa from non-mycobacterial pulmonary patients and healthy subjects

ELISA Values	Patients with non-mycobacterial pulmonary disease n = 40	healthy subjects n = 53
0 - 99	5	6
100 - 199	25	28
200 - 299	8	14
300 - 399	1	1
400 - 499	1	2
500 - 599	-	2
> 600	-	-

Fig 13 ELISA values of Ig G anti-PPD in serum of various study groups

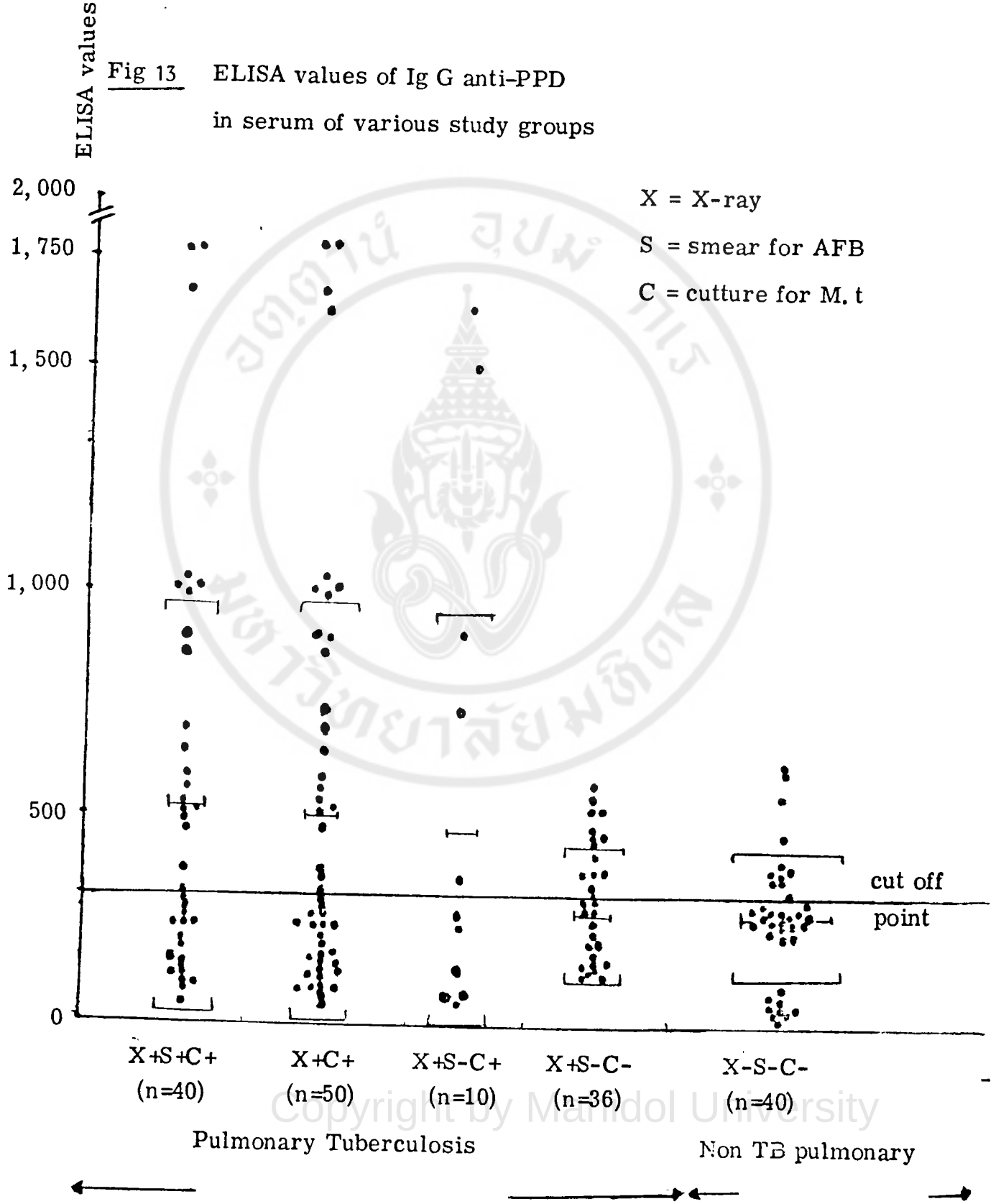


Fig 14 Distribution of Ig G anti-PPD (1:500 dilution) in serum by various study groups

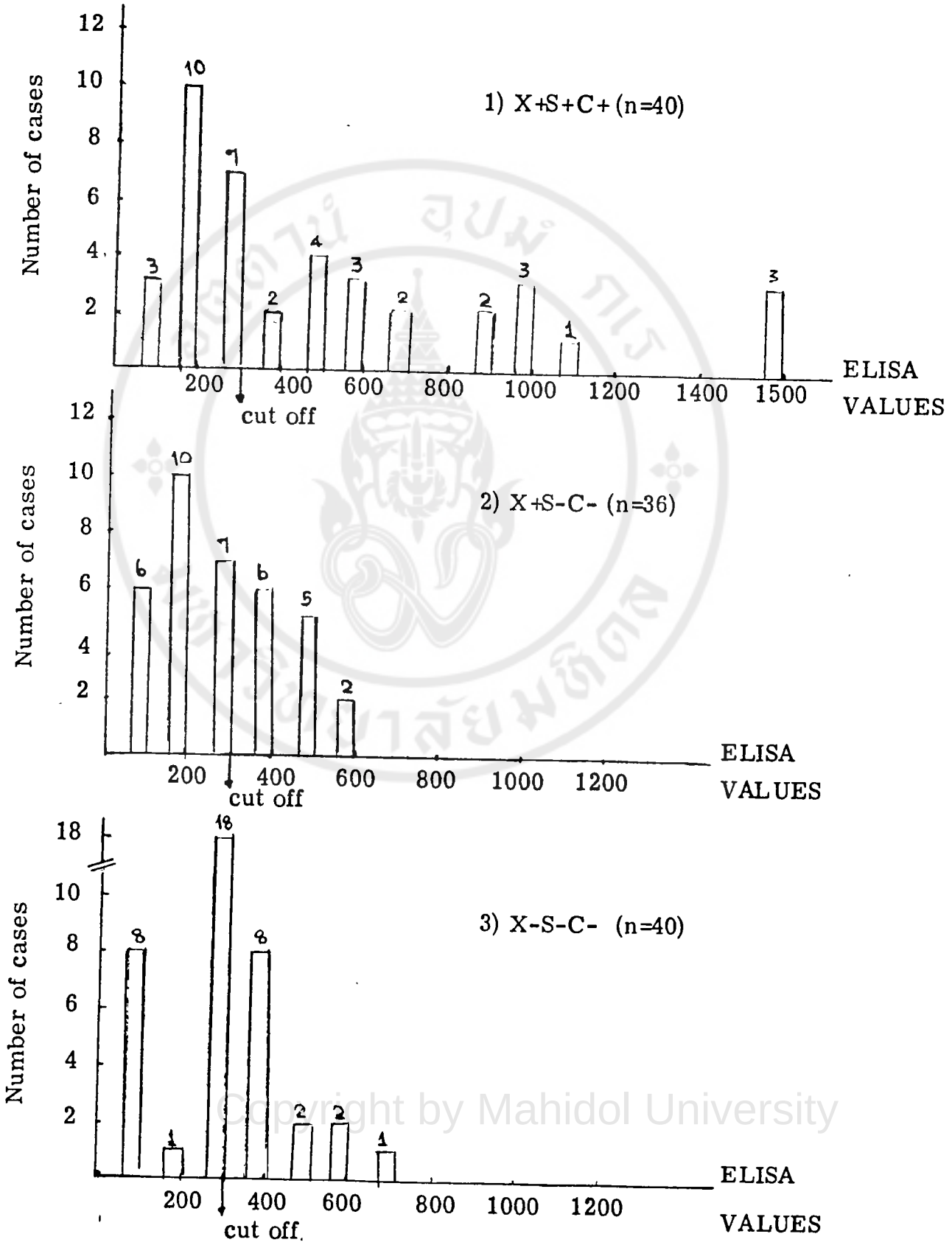


Fig 15 ELISA values of mycobacterial antigen in sputum of various study groups

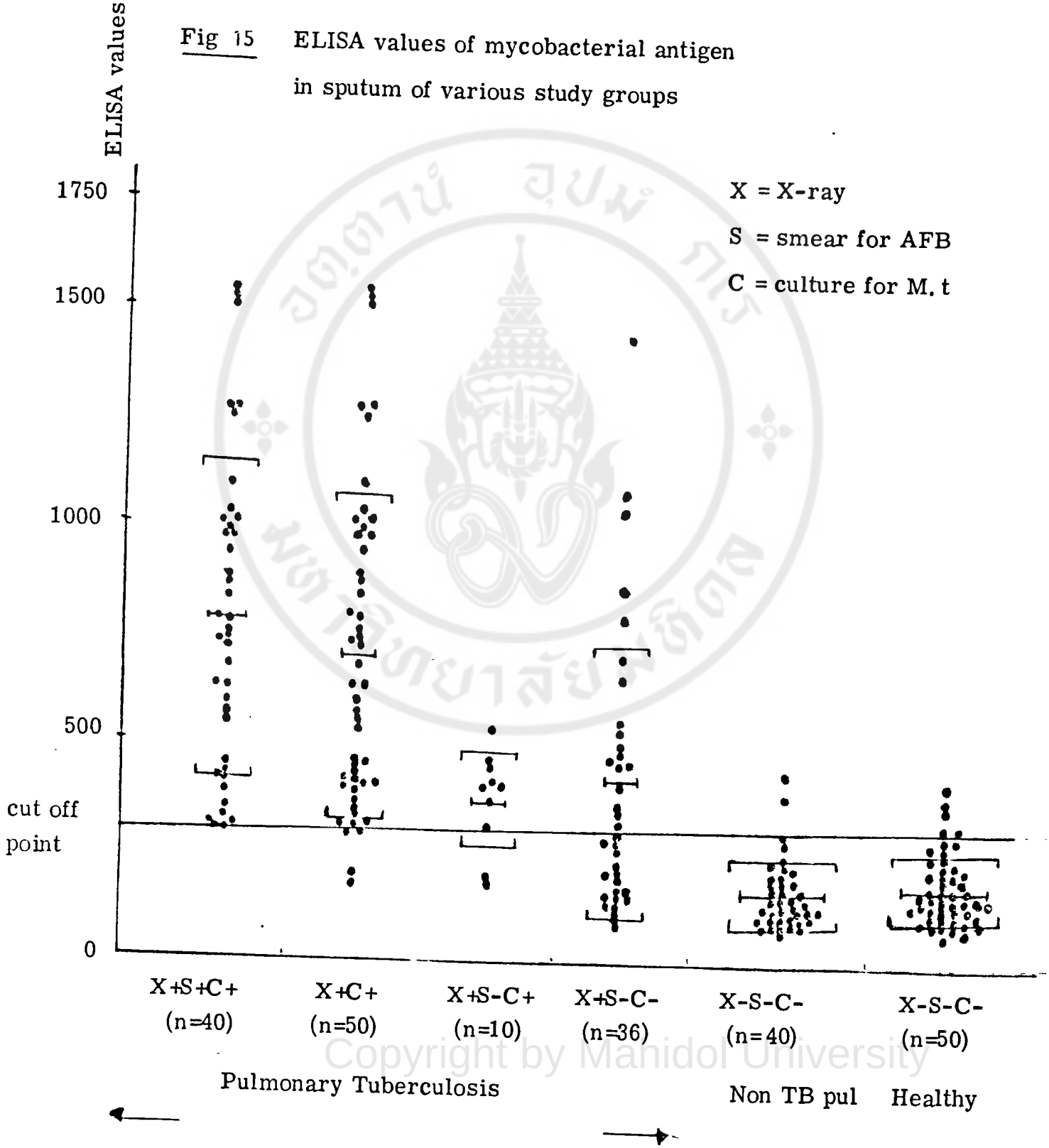


Fig 16 Distribution of mycobacterial antigen in sputum by various study groups

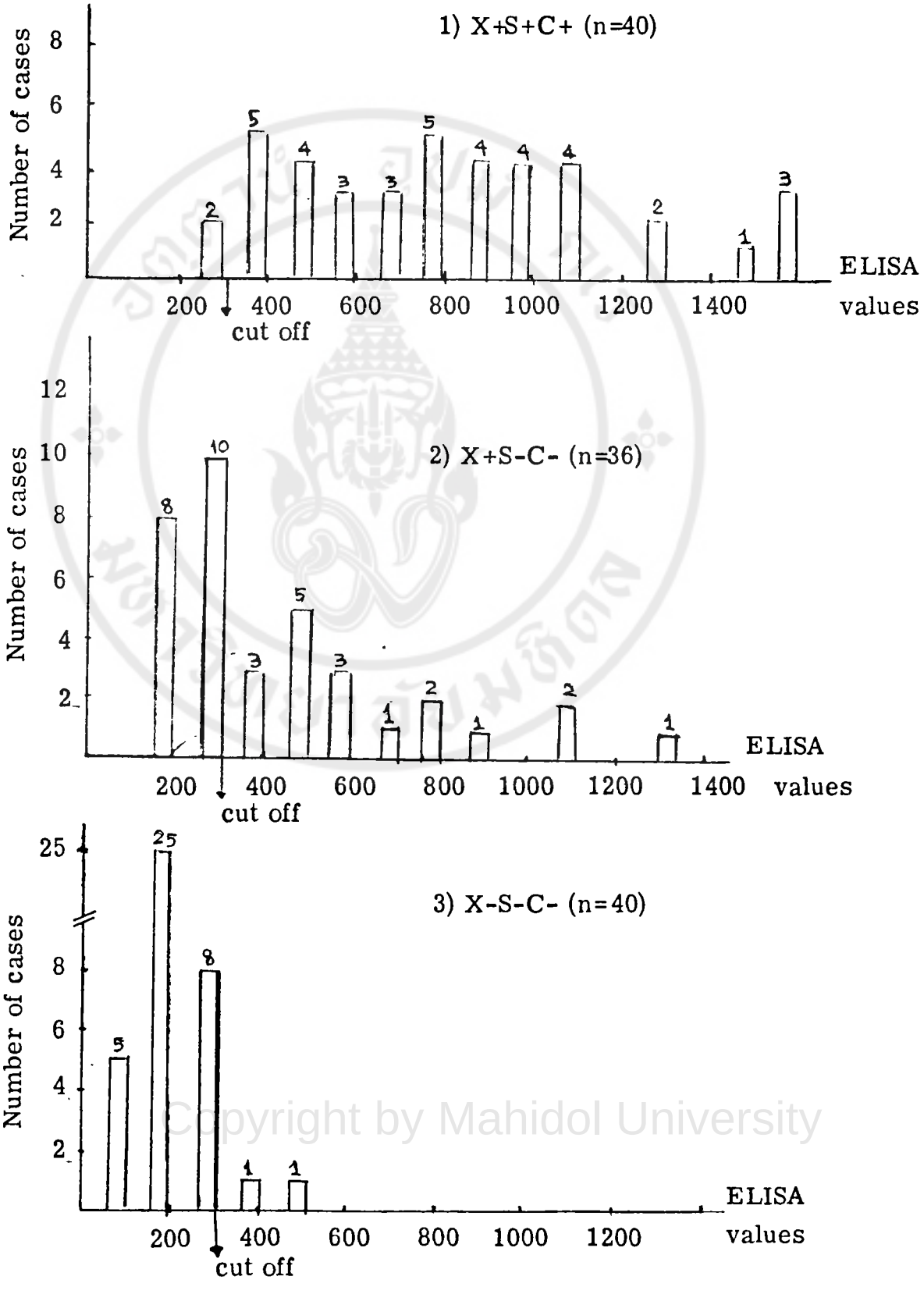


Table 17 Statistical data of IgG anti-PPD among the smear-positive, the smear-negative pulmonary tuberculosis and control groups.

Groups	No of serum	Median	Mean ± SD	Min - Max
Pulmonary tuberculosis				
X+S+ (1)	40	301.5	497.9 ± 462.5	57 - 1791
X+S- (2)	36	273.5	256.9 ± 150.8	41 - 562
Non-mycobacterial pulmonary				
X-S- (3)	40	278.5	263.2 ± 139.9	33 - 603

Kruskal - Wallis test

P value (1 VS 2 VS 3) = 0.064 , > 0.05

Table 18 Statistical data of IgG anti PPD among the culture-positive, the culture-negative pulmonary tuberculosis and contral groups.

Group	No of serum	Median	Mean ± SD	Min - Max
Pulmonary tuberculosis				
X+C+ (1)	50	288.5	486.8 ± 466.6	57 - 1791
X+C- (2)	36	273.5	256.9 ± 150.8	41 - 562
Non-mycobacterial pulmonary				
X-C- (3)	40	278.5	263.2 ± 139.9	33 - 603

Kruskal - Wallis test

P value (1 VS 2 VS 3) = 0.116 , > 0.05

Table 19 Statistical data of mycobacterial antigen among the smear-positive, the smear-negative pulmonary tuberculosis and control groups.

Group	No of sputum	Median	Mean $\pm$ SD	Min - Max
Pulmonary tuberculosis				
X+S+ (1)	40	759.5	787 $\pm$ 364.6	292 - 1530
X+S- (2)	36	307.0	413.5 $\pm$ 312.4	94 - 1438
Non-mycobacterial pulmonary				
X-S- (3)	40	212.0	169.3 $\pm$ 77.8	78 - 448
Healthy subjects				
X-S- (4)	50	156.0	179.8 $\pm$ 80.4	64 - 408

Kruskal - Wallis test ; α = 0.05

P value (1VS 2 VS 3 VS 4) = 0.0001 , < 0.05

Mann - Whitney U - test ; α = 0.05/6/2 = 0.0042

P value (1 VS 2) = 0.0000 , < 0.004

(1 VS 3) = 0.0000 , < 0.004

(1 VS 4) = 0.0000 , < 0.004

(2 VS 3) = 0.0000 , < 0.004

(2 VS 4) = 0.0000 , < 0.004

(3 VS 4) = 0.4404 , > 0.004

Table 20 Statistical data of mycobacterial antigen among the culture-positive, the culture-negative pulmonary tuberculosis and contral groups.

Group	No of sputum	Median	Mean ± SD	Min - Max
Pulmonary tuberculosis				
X+C+ (1)	50	632.5	702.7 ± 370.2	176 - 1530
X+C- (2)	36	307	413.5 ± 312.4	94 - 1438
Non-mycobacterial pulmonary				
X-C- (3)	40	212	169.3 ± 77.8	78 - 448
Healthy subjects				
X-C- (4)	50	156	179.8 ± 80.4	64 - 408

Kruskal - Wallis test ; $\alpha = 0.05$

P value (1 VS 2 VS 3 VS 4) = 0.0001 , < 0.05

Mann - Whitney U - test ; $\alpha = 0.05/6/2 = 0.0042$

P value (1 VS 2) = 0.000 , < 0.004

(1 VS 3) = 0.000 , < 0.004

(1 VS 4) = 0.000 , < 0.004

(2 VS 3) = 0.000 , < 0.004

(2 VS 4) = 0.000 , < 0.004

(3 VS 4) = 0.4404 , > 0.004

Table 21 Percentage distribution of positive IgG anti-PPD and or mycobacterial antigen in various study groups.

Group	No of study	Ab+	Ag+	Ab+and Ag+	Ab+or Ag+
Pulmonary tuberculosis					
X+S+C+	40	20(50%)	38(95%)	19(47.5%)	39(97.5%)
X+S-C+	10	4(40%)	8(80%)	3 (30%)	9(90%)
X+S-C-	36	13(36.11%)	18(50%)	5(13.9%)	26(72.2%)
Non-mycobacterial pulmonary					
X-S-C-	40	12(30%)	2(5%)	2 (5%)	12(30%)

Ab = IgG anti PPD

Ag = mycobacterial antigen

\+ = positive , ELISA value greater than 300

4. Diagnostic values of ELISA method for pulmonary tuberculosis

Since the vast majority of serum samples taken from healthy subjects (Blood Bank donors) had ELISA values of IgG anti-PPD less than 300, and almost all sputa from healthy subjects had ELISA values of mycobacterial antigen less than 300. It was decided that ELISA value of 300 should be adopted as the cut-off point; the significant level in differentiating positive and negative tests for both antibody and antigen detection.

The percentage of positive and negative obtained from sera and sputum of patients with active pulmonary tuberculosis (x+s+c+) and non-mycobacterial pulmonary disease for IgG anti-PPD were 50% (sensitivity) and 70% (specificity) respectively, for mycobacterial antigen were 95% (sensitivity) and 95% (specificity) respectively.

The values of IgG anti-PPD by ELISA method for diagnosis of pulmonary tuberculosis were demonstrated in term of sensitivity, specificity, positive predictive values, negative predictive values, the accuracy and the prevalence as shown in Table 22. These values are also shown in Tables 24A-24D for other study groups. The diagnostic values of mycobacterial antigen detection in sputum by ELISA method are demonstrated in Tables 23 and 25A-25D.

The likelihood ratio for various ranges of ELISA values, the sensitivity and the specificity of IgG anti-PPD at various values are demonstrated in Tables 26 and 28A respectively. These values were also shown in Table 27 and 28.2 for mycobacterial antigen detection.

Table 22 Diagnostic values of the IgG anti-PPD detected by ELISA method for pulmonary tuberculosis among various study groups at ELISA values of 300

Groups	Sensitivity	Specificity	Predictive Values		Accuracy	Prevalence
			positive	negative		
x + s +	50.0 %	70.0 %	62.5 %	58.3 %	60.0 %	50.0 %
x + c +	48.0 %	70.0 %	66.7 %	51.8 %	57.8 %	55.6 %
x + s ± c ±	43.0 %	70.0 %	75.5 %	36.4 %	51.6 %	68.3 %
x + s - c -	36.1 %	70.0 %	52.0 %	54.9 %	53.9 %	47.4 %

Table 23 Diagnostic values of the mycobacterial antigen detected by ELISA method for pulmonary tuberculosis among various study groups at ELISA values of 300

Groups	Sensitivity	Specificity	Predictive Values		Accuracy	Prevalence
			positive	negative		
x + s +	95.0 %	95.0 %	95 %	95.0 %	95.0 %	50 %
x + c +	92.0 %	95.0 %	95.8 %	90.5 %	93.3 %	55.6 %
x + s ± c ±	74.4 %	95.0 %	97.0 %	63.3 %	79.7 %	67.2 %
x + s - c -	50.0 %	95.0 %	90.0 %	67.9 %	73.7 %	47.4 %

Table 24A Diagnostic values of the IgG anti-PPD detected by the ELISA method for pulmonary tuberculosis demonstrated by fourfold table at the ELISA values of 300 among the patients with compatible chest roentgenogram for pulmonary tuberculosis and positive smear in sputum

cases / ELISA Value	Pulmonary tuberculosis Patients with positive both smear and culture in sputum (x + s +)	Patients with non-mycobacterial pulmonary disease (x - s -)	Total
300 or over	20	12	32
less than 300	20	28	48
Total	40	40	80

Sensitivity	=	50.0 %
Specificity	=	70.0 %
Positive predictive Value	=	62.5 %
Negative predictive Value	=	58.3 %
Accuracy	=	60.0 %
Prevalence	=	50.0 %

Table 24B Diagnostic values of the IgG-anti PPD detected by ELISA method for pulmonary tuberculosis among the patients with compatible chest roentgenogram for pulmonary tuberculosis and positive-culture in sputum

cases / ELISA Value	Pulmonary tuberculosis patients with sputum - culture-positive (x + c +)	Patients with non-mycobacterial pulmonary disease (x - c -)	Total
300 or over	24	12	36
less than 300	26	28	54
Total	50	40	90

Sensitivity	= 48.0 %
Specificity	= 70.0 %
Positive predictive value	= 66.7 %
Negative predictive value	= 51.8 %
Accuracy	= 57.8 %
Prevalence	= 55.6 %

Table 24C Diagnostic values of the IgG anti-PPD detected by ELISA method for pulmonary tuberculosis among the pulmonary tuberculosis suspects proven by chest roentgenogram

cases / ELISA Value	Patients with chest radiograph compatible with pulmonary tuberculosis (x + s ± c ±)	Patients with non-mycobacterial pulmonary disease (x - s - c -)	Total
300 or over	37	12	49
less than 300	49	28	77
Total	86	40	120

Sensitivity	=	43.0 %
Specificity	=	70.0 %
Positive predictive value	=	75.5 %
Negative predictive value	=	36.4 %
Accuracy	=	51.6 %
Prevalence	=	68.3 %

Table 24D Diagnostic values of the IgG anti-PPD detected by ELISA method for pulmonary tuberculosis among the patients with compatible chest roentgenogram for pulmonary tuberculosis but negative both smear and culture in sputum

cases / ELISA Value	Pulmonary tuberculosis Patients with negative both smear and culture (x + s - c -)	patients with non-mycobacterial pulmonary disease (x - s - c -)	Total
300 or over	13	12	25
less than 300	23	28	51
Total	36	40	76

Sensitivity	=	36.1 %
Specificity	=	70.0 %
Positive predictive value	=	52.0 %
Negative predictive value	=	54.9 %
Accuracy	=	53.9 %
Prevalence	=	47.4 %

Table 25A Diagnosis values of the mycobacterial antigen detected by ELISA method for pulmonary tuberculosis among the patients with compatible chest roentgenogram for pulmonary tuberculosis but negative both smear and culture in sputum.

cases / ELISA Value	Pulmonary tuberculosis patient with sputum negative both smear and culture (x + s - c -)	Patient with non-mycobacterial pulmonary disease (x - s - c -)	Total
300 or over	18	2	20
less than 300	18	38	56
Total	36	40	76

Sensitivity	=	50 %
Specificity	=	95 %
Positive predictive value	=	90 %
Negative predictive value	=	67.9 %
Accuracy	=	73.68 %
Prevalence	=	47.37 %

Table 25B. Diagnosis Values of the mycobacterial antigen detected by ELISA method for pulmonary tuberculosis among the pulmonary tuberculosis suspects proven by chest roentgenogram

ELISA Value / cases	Patients with chest radiograph compatible with pulmonary tuberculosis (x + s ± c ±)	Patients with non-mycobacterial Pulmonary disease (x - s - c -)	Total
300 or over	64	2	66
less than 300	22	38	60
Total	86	40	128

Sensitivity	=	74.42 %
Specificity	=	95.00 %
Positive predictive value	=	96.97 %
Negative predictive value	=	63.33 %
Accuracy	=	79.69 %
Prevalence	=	67.19 %

Table 25C Diagnostic Values of the mycobacterial antigen detected by ELISA method for pulmonary tuberculosis among the patients with compatible chest roentgenogram for pulmonary tuberculosis and positive-culture in sputum.

cases / ELISA Value	Pulmonary tuberculosis patients with sputum culture - positive (x + c +)	Patients with non-mycobacterial pulmonary diseases (x - c -)	Total
$\geqslant$ 300	46	2	48
$<$ 300	4	38	42
Total	50	40	90

Sensitivity	= 92 %
Specificity	= 95.0 %
Positive predictive value	= 95.8 %
Negative predictive value	= 90.5 %
Accuracy	= 93.3 %
Prevalence	= 55.6 %

Table 25D Diagnostic values of the mycobacterial antigen detected by ELISA method for pulmonary tuberculosis demonstrated by fourfold table at the ELISA values of 300 among the patients with positive chest roentagenogram for pulmonary tuberculosis and positive smear in sputum.

cases / ELISA Value	Pulmonary tuberculosis patients with positive both smear and culture (x + s +)	Patients with non-mycobacterial pulmonary disease (x - s -)	Total
300 or over	38	2	40
less than 300	2	38	40
Total	40	40	

Sensitivity	= 95 %
Specificity	= 95 %
Positive predictive value	= 95 %
Negative predictive value	= 95 %
Accuracy	= 95 %
Prevalence	= 50 %

Table 26 Likelihood ratios for various ranges of ELISA values of IgG anti-PPD in patients with definite pulmonary tuberculosis (X+S+C+)

	Patients with definite pulmonary tuberculosis		Patients with non-mycobacterial pulmonary disease		Likelihood ratio
range of ELISA Values	number	proportion (A)	number	proportion (B)	(A/B)
0 - 299	20	20/40	27	27/40	0.74
300 - 599	9	9/40	12	12/40	0.75
600 - 899	4	4/40	1	1/40	4.0
900 - 1199	4	4/40	0	0/40	> 100
> 1200	3	3/40	0	0/40	> 100
	40		40		

Table 27 Likelihood ratios for various ranges of ELISA values of mycobacterial antigen in sputum from patients with definite pulmonary tuberculosis (X+S+C+)

	Patients with definite pulmonary tuberculosis		Patients with non-mycobacterial pulmonary disease		Likelihood ratio
range of ELISA Values	number	proportion (A)	number	proportion (B)	(A/B)
0 - 299	2	2/40	87	87/93	0.05
300 - 599	12	12/40	7	7/93	3.99
600 - 899	12	12/40	0	0/93	> 100
900 - 1199	8	8/40	0	0/93	> 100
1200 - 1499	3	3/40	0	0/93	> 100
> 1500	3	3/40	0	0/93	> 100
	40		93		

Table 28A The Values of sensitivity and specificity of the ELISA for IgG anti-PPD at various ELISA values (in pulmonary tuberculosis patient with X+S+C+)

ELISA Values less than	100	200	300	400	500	600	700	800	900	1000
sensitivity (%)	92.5	67.5	50.0	45.0	35.0	27.5	22.5	22.5	17.5	10.0
specificity (%)	20.0	22.5	70.0	87.5	92.5	97.5	100.	100.	100.	100.

Table 28B The Values of sensitivity and specificity of the ELISA for mycobacterial antigen at various ELISA values (in pulmonary tuberculosis with X+S+C+)

ELISA Values less than	100	200	300	400	500	600	700	800	900	1000
sensitivity (%)	100.0	100.	95.0	82.5	72.5	65.0	57.5	45.0	35.0	30.0
specificity (%)	12.5	75.0	95.0	97.5	100.	100.	100.	100.	100.	100.

5. The time required to diagnose tuberculosis patient by ELISA method for both antibody and antigen detection compared with conventional culture and microscopic methods.

The time required to detect visible growth of M. tuberculosis in culture are summarized in Table 29, and the distribution of ELISA values of IgG andti-PPD and mycobacterial antigen by incubation time of culture are shown in Table 30. In (x+s+c+) group, sixty five per cent of all isolates were apparent within the first 3 weeks of incubation, 90% within 7 weeks and 7.5% were contaminate from culture. In (x+s-c+) group, only 10% of all isolates were apparent within the first 3 weeks and up to 6 weeks only 90% were culture positive.

IgG anti-PPD in serum can be run completely within one day by indirect ELISA method and it took only 2 days for performing mycobacterial antigen detected in sputum by the double antibody sandwich ELISA method.

Table 29 The time required to detect visible growth of M. tuberculosis in culture

(on Löwenstein-Jensen's medium at 37 °C)

study group	No Positive	Cumulative per cent of detectable colonial appearance by day						
		8-14	15-21	22-28	29-35	36-42	43-49	50-56
Pulmonary tuberculosis								
with x+s+c+	40	12.5	65.0	77.5	82.5	87.5	90	92.5
with x+s-c+	10	0	10	10	70	90	100	100

Table 30 Distribution of ELISA values of IgG anti PPD and mycobacterial antigen by incubation time of culture (on Löwenstein-Jensen's medium at 37° C in (x+s+c+) group)

IgG anti PPD	Time required for visible growth of M. tuberculosis by week		
	<3 wks	>4 wks	contaminated
number of case	26(65%)	11(27.5%)	3(7.5%)
min-max	112-1,686	91-1,791	209-448
mean	430.4	652.8	316.3
SD	375.5	649.8	121.3
medium	269.5	486.0	292
number positive (ELISA values>300)	12(46.2%)	6(54.5%)	1(33.3%)

P>0.05

Mycobacterial Ag	Time required for visible growth of M. tuberculosis by week		
	<3 wks	>4 wks	contaminated
number of case	26(65%)	11(27.5%)	3(7.5%)
min-max	279-1,530	292-1,504	342-1,268
mean	791.8	765.0	826
SD	373.8	353.3	464.4
medium	749	756	868
number positive (ELISA values>300)	25(96.2%)	10(90.9%)	3(100%)

P>0.05

6. Factors associated with levels of anti PPD-IgG and mycobacterial antigen in sputum

a) Enumeration of AFB in sputum

There was a strong association between ELISA values of IgG anti-PPD and the number of tubercle bacilli presented on acid fast stained smears of sputa of definite pulmonary tuberculosis patient i.e., the more the number of AFB, the lower the IgG antibody level against PPD and the lower the standard deviation ($p<0.05$) as presented in Table 31A. Analysis of variance (Mann-Whitney U-test) of the means obtained from three groups showed P-value to be 0.0349. The IgG anti-PPD from pulmonary tuberculosis with number of AFB equal to or more than one per oil field (3+) all fell below the cut off point. Sixty five per cent of pulmonary tuberculosis with number of AFB 3-9 per slide (1+) have IgG anti PPD above the cut off point.

The ELISA value from mycobacterial antigen also showed strong association with the number of AFB in sputum of defenite pulmonary tuberculosis i.e., the more the number of AFB, the greater the mycobacterial antigen detected by ELISA method. Percentage of mycobacterium that was greater than 700 were found by the same method of analysis of variance of the means obtained from three groups of different bacterial density in the smears (1+, 2+, 3+) P-values was <0.05 (Table 31B). These demonstrated that there were statistically significant difference of IgG anti PPD or mycobacterial antigen among sub groups of pulmonary tuberculosis that were classified according to the number of AFB in sputum. The level of IgG anti-PPD were 625.6 504.5 (n=20) in

AFB 3-9/slide (1+), 370.4 387.5 (n=17) in AFB > 10/slide (2+) and 236.7 99.3 (n=3) in AFB > 1/field (3+). The level of mycobacterial antigen were 644.3 336.5, 927.7 341.6 and 1,253 278 in pulmonary tuberculosis patient with AFB 1+, 2+ and 3+ respectively. Only 35% of tuberculpsis patients with AFB 1+ had mycobacterial antigen more than 700 but 82% and 100% of patients with AFB 1+ and AFB 2+ respectively had the values for these antigen greater than 700.

b) Radiographic finding

Radiographic finding had no association with IgG anti-PPD in serum or mycobacterial antigen in sputum both in term of the presence or absence of cavitary and the extent of pulmonary lesions as demonstrated in Tables 32-33 and in Figures 17-18 (P>0.05 by Mann-Whitney U test in case of sample <2 groups, by Kruskal-Wallis test in case of sample >3 groups). No association between radiographic finding and IgG anti-PPD or mycobacterial antigen was also presented in pulmonary tuberculosis suspects who had only the chest X-ray compatible with tuberculosis.

c) haemoptysis

Whether the sputum contained blood or not had no influence on level of IgG anti-PPD or mycobacterial antigen, as shown in Table 34 and in Figures 17-18. (P-values = 0.7614 for igG anti PPDm P-values = 0.9340 for mycobacterial antigen.

Table 31A. Distribution of ELISA values of IgG anti-PPD by number of AFB on acid-fast stained smears in pulmonary tuberculosis patients (x+s+c+)

	Enumeration of AFB on fluorochrome stained slides		
	3-9/slide (+)	>10/slide (++ or +++)	>1/field (+++)
IgG anti-PPD			
Number of cases (%)	20(50%)	20(50%)	3(7.5%)
ELISA values			
min-max	106-1,719	57-1,686	122-292
mean	625.60	370.35	236.67
SD	504.52	387.49	99.32
medium	533.50	235	226
ELISA values>300	13(65%)	7(41%)	0(%)
	t = 1.79 , d.f. = 38		
	P^{*} = 0.0349		

* Mann-Whitney U-Test

Table 31B. Distribution of ELISA values of mycobacterial antigen by number of AFB on acid-fast stained smears in pulmonary tuberculosis patients (x+s+c+)

Tests	Enumeration of AFB on fluorochrome stained slides		
	3-9/slide (+)	>10/slide (++ or +++	>1/field (+++)
Mycobacterial antigen			
Number of cases (%)	20(50%)	20(50%)	3(7.5%)
ELISA values			
min-max	292-1,264	306-1,530	969-1,524
mean	644.25	929.75	1,253.00
SD	336.46	341.60	278.80
medium	559.50	865	1,268
ELISA values>300	19(95%)	20(100%)	3(100%)
	t = -2.66 d.f. = 38 P = 0.0080		

Table 32A Distribution of ELISA values of IgG-anti PPD by cavitation in pulmonary tuberculosis patients

Tests	(x+s+c+) cavitation in lung		(x+s-c-) cavitation in lung	
	Yes	No	Yes	No
IgG anti-PPD				
Number of cases (%)	27(67.5%)	13(32.5%)	13(36.11%)	23(63.89%)
ELISA values				
min-max	57-1,791	92-1,782	41-523	69-562
mean	506.78	479.69	262.38	253.91
SD	467.42	470.26	147.20	156.04
medium	292	311	279	190
ELISA values>300	12(44.88%)	7(53.85%)	5(38.46%)	8(34.78%)
	t = -0.17 , d.f.= 38		t = -0.16 , d.f. = 34	
	P = 0.9195		P = 0.8822	

Table 32B Distribution of ELISA values of mycobacterial antigen by cavitation in pulmonary tuberculosis patients

Tests	(x+s+c+) cavitation in lung		(x+s-c-) cavitation in lung	
	Yes	No	Yes	No
Mycobacterial antigen				
Number of cases (%)	27(67.50%)	13(32.50%)	13(36.11%)	23(63.88%)
ELISA values				
min-max	297-1,524	292-1,530	150-1,042	94-1,438
mean	758.89	845.38	405.77	417.87
SD	352.52	396.48	266.31	341.29
medium	735	884	355	296
ELISA values>300	26(96.29%)	12(92.31%)	(7(53.85%)	11(47.83%)
	t = 0.70 , d.f. = 38		t = 0.11 , d.f. = 34	
	P = 0.4441		P = 0.7922	

Table 33A Distribution of ELISA values of IgG anti-PPD by extent of pulmonary lesions in pulmonary tuberculosis patients

	x+s+c+ Extent of pulmonary lesions			
	one lobe	two lobes	>three lobes	
x+s+c+				
IgG anti-PPD				
Number of cases (%)	6(15%)	15(37.5%)	19(47.5%)	
ELISA values				
min-max	92-685	57-1,782	122-1,791	
mean	342.33	468.73	570.21	
SD	255.50	453.83	520.34	
medium	322.50	311	292	
ELISA values>300	3(50%)	8(53.3%)	9(47.4%)	P = 0.597
x+s-c-				
IgG anti-PPD				
Number of cases (%)	17(47.2%)	13(36.1%)	6(16.7%)	
ELISA values				
min-max	69-499	41-438	68-562	
mean	230.65	272.62	297.67	
SD	150.77	130.54	201.59	
medium	147	298	234.5	
ELISA values>300	5(29.4%)	6(46.2%)	2(33.3%)	P = 0.614

Table 33B Distribution of ELISA values of mycobacterial antigen by extent of pulmonary lesions in pulmonary tuberculosis patients

Group	Extent of pulmonary lesions			
	one lobes	two lobes	>three lobes	
x+s+c+				
Mycobacterial antigen				
Nubmer of cases (%)	6(15%)	15(37.5%)	19(47.5%)	
ELISA values				
min-max	306-1,504	292-1,097	336-1,530	
mean	1,018.33	617.33	847.89	
SD	443.46	290.06	349.48	
medium	917	569	783	
ELISA values>300	6(100%)	13(86.7%)	19(100%)	P = 0.076
x+s-c-				
Mycobacterial antigen				
Number of cases (%)	17(47.2%)	13(36.1%)	6(16.7%)	
ELISA values				
min-max	112-1,438	94-1,042	165-1,084	
mean	469.24	299.31	503	
SD	340.22	246.42	335.78	
medium	458	211	446	
ELISA values>300	10(58.8%)	4(30.8%)	4(66.7%)	P = 0.148

Table 34A Distribution of ELISA values of IgG anti-PPD by haemoptysis in pulmonary tuberculosis patients

Tests	(x+s+c+) haemoptysis		(x+s-c-) haemoptysis	
	Yes	No	Yes	No
IgG anti-PPD				
Number of cases (%)	24(60%)	16(40%)	13(36.1%)	23(63.9%)
ELISA values				
min-max	57-1,782	91-1,791	62-523	41-562
mean	525.75	456.31	290.69	237.91
SD	486.03	436.74	157.47	147.03
medium	301.5	302	298	190
ELISA values>300	12(50%)	8(50%)	6(46.2%)	7(30.4%)
	t = -0.46,d,f,=38		t = -1.01, d.f. = 34	
	P = 0.7614		P = 0.3479	

Table 34B Distribution of ELISA values of mycobacterial antigen by haemoptysis in pulmonary tuberculosis patients

Tests	x+s+c+ haemoptysis		x+s-c- haemoptysis	
	Yes	No	Yes	No
Mycobacterial antigen				
Number of cases (%)	24(60%)	16(40%)	13(36.11%)	23(63.88%)
ELISA values				
min-max	292-1,530	336-1,504	94-1,438	112-1,084
mean	780.67	796.50	415.69	415.09
SD	374.52	360.97	350.98	296.68
medium	809	745.50	347	296
ELISA values>300	22(91.67%)	16(100.00%)	7(53.84%)	11(47.82%)
	t = 0.13 , d.f. = 38		t = 0.04 , d.f. = 34	
	P = 0.9340		P = 0.8952	

Fig 17 ELISA values of Ig G anti-PPD by the extent of pulmonary lesions, the presence of cavitation and haemoptysis sputum in tuberculosis (X+S+C+)

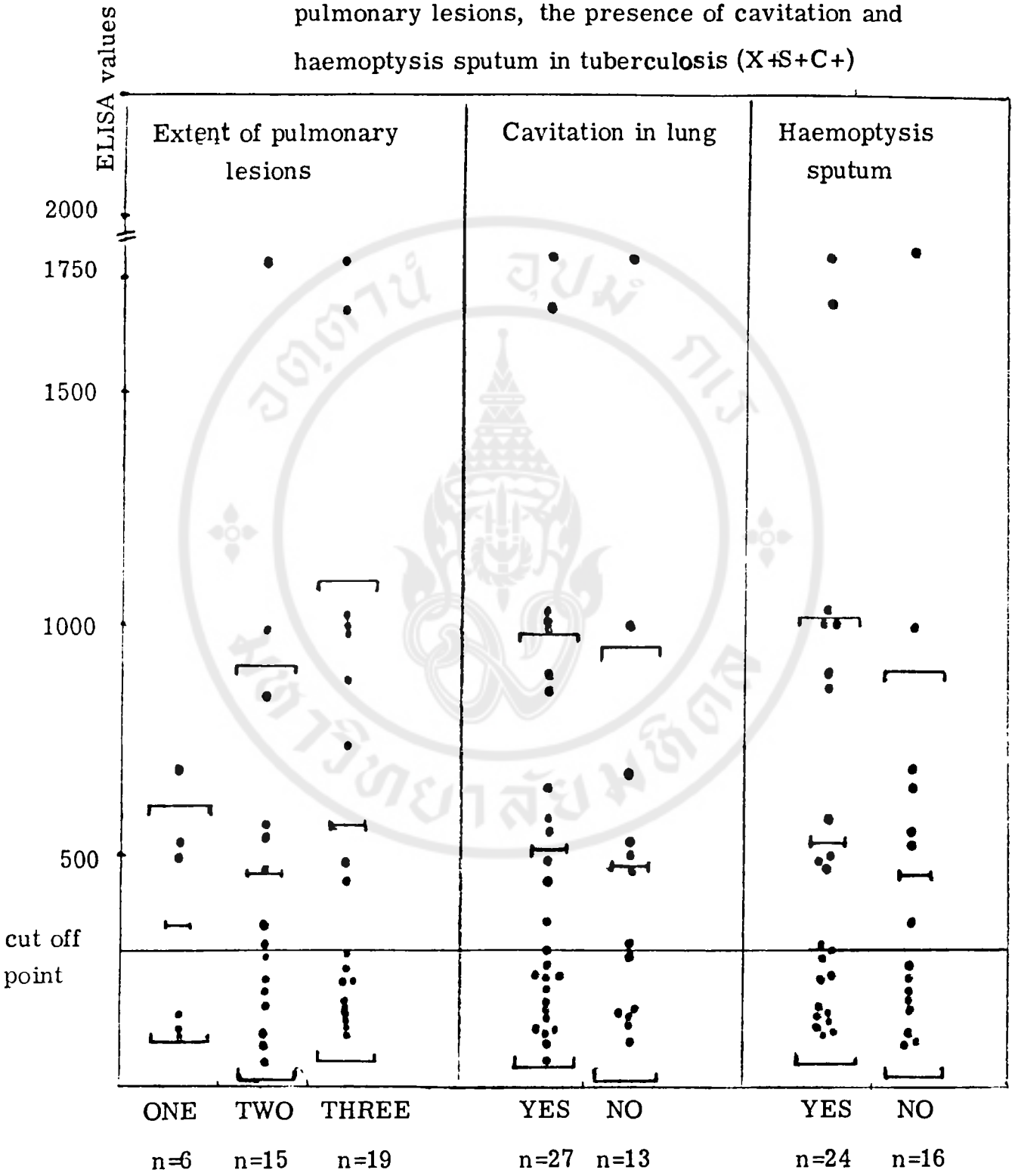
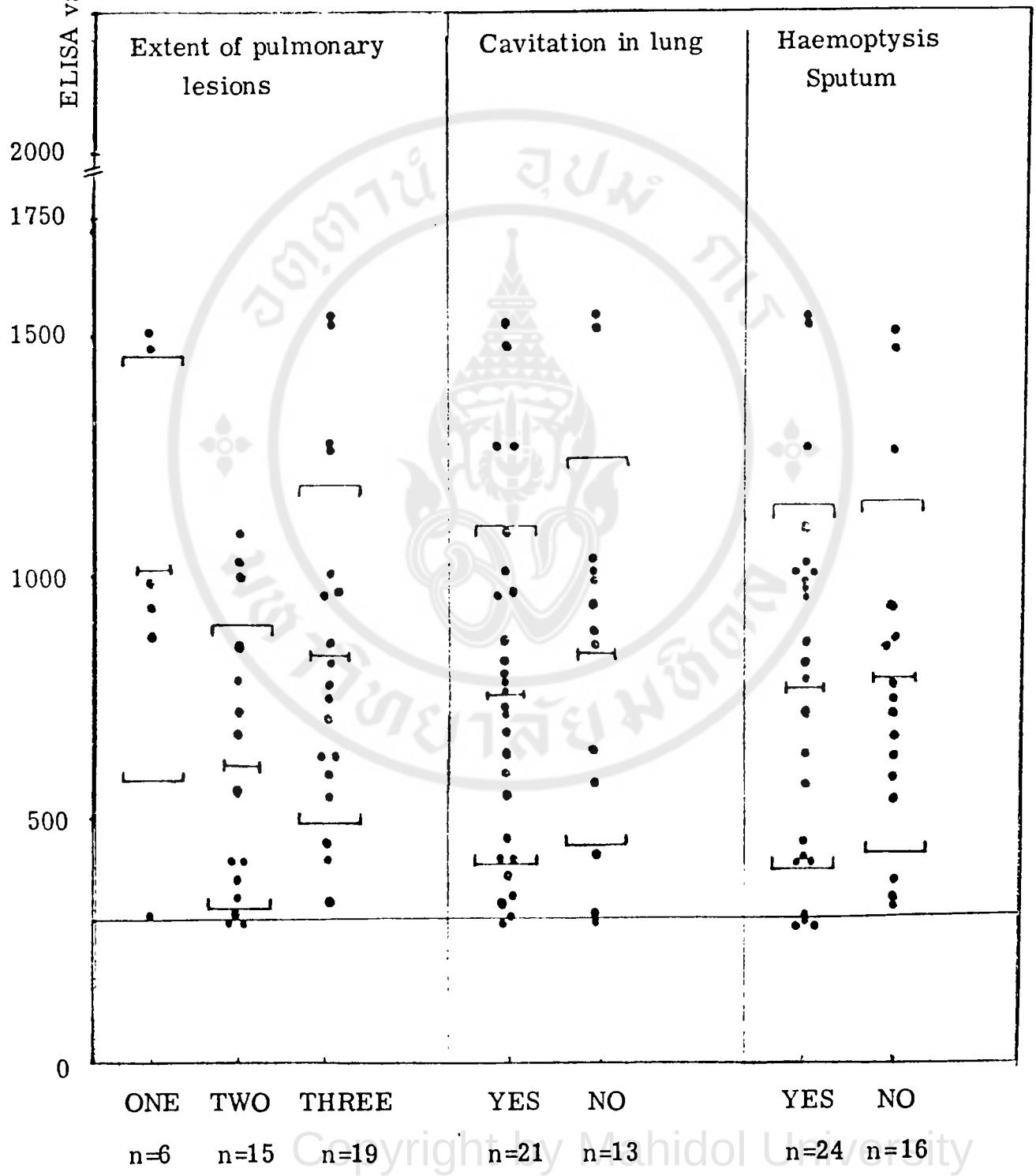


Fig 18 ELISA values of mycobacterial antigen by the extent of pulmonary lesions, the presence of cavitation and haemoptysis sputum in tuberculousis (X+S+C+)



Discussion

The signal to enroll a patient in the study occurred when a chest physician at tuberculosis clinic had already made the decision to evaluate a patient for pulmonary tuberculosis from the evidence that based on respiratory and constitutional symptom i.e. cough, chest pain, fever, weight loss or haemoptysis for 2 weeks or more plus radiographic finding compatible with pulmonary tuberculosis. Definite pulmonary tuberculosis was established by a positive culture and/or positive smear which were achievable in minority of the patients. In many cases, pulmonary tuberculosis was suspected when patients had chest X-ray compatible with pulmonary tuberculosis without positive smear and culture. It is in this later group that accurate diagnosis is a big problem which challenges us to develop a technique using ELISA

The cut off value of 300 for the ELISA test for the detection of both antibody and antigen was chosen because it represented the arithmatic mean plus two standard deviations exhibited by a group of normal healthy subjects (from "check up" Clinic in Siriraj Hospital for control in anti-PPD IgG detection, and by blood donors at Siriraj for control in antigen detection), and as well the optimal values of sensitivity or specificity were got from this cut-off point as demonstrated in Table 28A-28B.

The result was reported as ELISA values which was determined by averaging the absorbance of readings of the duplicates of a single serum dilution (1:500) and multiplied by 1,000. This method had the advantage over the serial dilution

procedure for end-point titration technique (23) as less antigen and conjugated antibody were required for testing. Moreover, the technical errors, e.g., from serial dilution was eliminated. In addition, multiplying by 1,000 makes the results easy to analyse or report.

Two different diagnostic ELISA methods for pulmonary tuberculosis were assessed. One was the indirect ELISA for detection of anti-PPD IgG, which based on the procedure described by Kalish (24) and leelarasamee (184) the other was the modified double antibody sandwich ELISA for detection of mycobacterial antigen in sputum which was set up for the first time. In the past, radioimmunoassay was set up by Kadival (174) to detect tuberculous antigen and ELISA for mycobacterial antigen detection in the cerebrospinal fluid was established by Vinceta Bal (180).

The ELISA method for anti-PPD IgG assay described in this study is slighly different from that of Kalish (24). In the present study the optimum concentration of PPD coating plate was 10 ug/ml, 1:500 serum dilution, was used instead of serial dilution. Incubation time and temperature at each stage were different but were appropriate according to our previous study (184). Only IgG antibody was measured in this study because previous works with this assay (26, 99) had shown that IgM antibody could be seldomly demonstrated in tuberculous patients and that IgG gave highest discrimination between active from non active and non-tuberculous pulmonary disease (22).

When the ELISA method was used for the detection of IgG anti-PPD in the sera from various tuberculous groups (Tables

14-15), the mean level of 497.9 was found in (x+s+) group, while 256.9 and 263.2 were found in (x+s-) and control groups respectively. Although the mean ELISA value of (x+s+) group seemed to be higher than that of the other groups, the different was not statistically significant. This was not caused by marked variation in the ELISA values of tuberculous group, because for statistical analysis Kruskal-Wallis test was used instead of ANOVA for one way classification. The results most probably reflect the immunological status of individual patient. Using ELISA value 300 as the cut-off point, it was found that only 48-50% of bacteriologically positive patients (x+s+ or x+c+) were serological positive as compared with 30% (12 of 40 patients) in those with non-mycobacterial pulmonary disease, so the specificity was only 70%. Only 36% of bacteriologically negative pulmonary tuberculosis suspects were serological positive (Table 21) at the selected cut off point.

Several explanations may account for the poor sensitivity of the ELISA in this study. The reasons for low levels of antibody in some cases of tuberculosis are

1) Generalized immunosuppression.

2) Specific inhibition of lymphocyte responding to relevant antigens.

3) Immune complex formation with deposite in tissue or removal of complexes by phagocytic cells.

4) Highly effective degradation of antigen by macrophages, thus, hiding tubercle bacilli from immune system.

5) Genetically determined poor humoral immune response.

6) Immunologically reactive cases with very little antigen present, such as the effect of a tuberculin test performed prior to the correction of serum.

Immunosuppression, whether occurred naturally or caused by therapeutic procedures, infection by a wide range of opportunistic pathogens including Mycobacteria. In such cases the infection is often severe with numerous organisms in the lesions, poor cellular response and poor prognosis (198). This explanation is consistent with the result in Table 31A which revealed inversed association between the level of serum anti-PPD IgG and the number of AFB presented in sputum. Although sereral workers (Choucroun (190) and Kirby (191)) have shown that antibody levels are higher in patients with advanced disease than in those with minimal disease. A few studies reported an absence of antimycobacterial antibodies in very advanced disease, such as the studies reported by Fried, Sasano and Toussaint (151).

The formation of immune complexes as a cause of apparently low levels of circulating antibodies was postulated by Gerstl (192) In this study it was found that antibody levels fluctuated markedly in the tuberculous groups (all of them were newly diagnosed, non-treated cases of tuberculosis) but not in the tuberculous suspects nor in the control group. Cole (9), using a simple latex agglutination test, also found that antibody levels fluctuated markedly in newly diagnosed cases of tuberculosis but not in patients with longer established disease. Consequently, it was suggested that the fluctuations in antibody levels might result from 1) an increasing bacillary load which

lead to higher antibody titers as suggested by Wallace (144), and 2) such an increasing bacilli would reduce the levels of circulating antibody through the formation of immune complex as suggested by Young and Leonard (193).

The reasons for the "false positive" reactions, e.g. occurring in persons not suffering from active tuberculosis include :-

1) Antibodies produced in response to contact with environmental Mycobacteria.

2) Antibodies produced to antigens common between Mycobacteria and other bacteria.

3) Cryptic infection by environmental mycobacteria in other diseases.

4) Unproven mycobacterial etiology of certain other diseases.

5) An increase in the background levels of antibodies due to a polyclonal stimulation of antibody production.

6) A non-immune reaction.

One of the major problems associated with this serodiagnostic test is the number of persons who have circulating specific antibody to M. tuberculosis antigen without apparent evidence of disease (non-mycobacterial diseases and healthy individuals). Indeed, sensitive tests such as radio-immunoassay (155) and ELISA (23, 184) can demonstrate the presence of such antibodies in these two groups. It is always difficult to judge the significance of such results since they may reflect previous

infection, or may be related to cross reaction or non-specific reaction. Most of the non mycobacterial pulmonary patients in this study had various underlying diseases in which some of them may really have tuberculous infection but simply could not be ascertained by insensitive routine diagnostic method. About 70% of these patients had pulmonary infection with bacteria as major causative agents, the mean value of anti-PPD IgG in this group was 292.5 with a range of 33-603. The values in the non-mycobacterial pulmonary disease groups who were non infectious was only 194.8 with a range of 54-330. Consequently, it can be suggested that the second reason for the false positive reaction of antituberculosis as described previously is true, however our sample size was too small to substantiate this reason

The reason for the false positive antigen detection

There is a high degree of chemical similarity or identity among polysaccharides isolated from various Mycobacteria (82, 84, 92-93). Corynebacteria and Nocardia have been shown to contain polysaccharides similar to those found in Mycobacteria (82, 84-85). In this study it was found that there is a high degree of cross-reaction in mycobacterial antigen detection between M. tuberculosis and atypical mycobacterium and also has cross reaction in Nocardia spp as shown in Table 11. However, the incidence of atypical mycobacterium pulmonary disease is rare (194). From 1969 to 1978, only 24 patients were suspected of having pulmonary disease caused by atypical mycobacteria. Seven were infected with M. avium-intracellulare, six with M. avium, six with M. scrofulaceum, two with M. fortuitum and one with M.

gordonae, one with M. simiae, M. kansasii was found in only one patient. It is apparent that atypical mycobacterial disease is rare in Thailand, during which time 43,889 patients were examined for tuberculosis and of these 10,892 patients were culture positive for M. tuberculosis. The rarity of atypical mycobacterial disease in Thailand may be a result of the high prevalence of tuberculosis and the wide spread use of BCG vaccination (118).

The abnormal radiographic, physiological and pathological changes caused by pathogenic atypical mycobacteria cannot be distinguished from those produced by human tubercle bacillus (119), as well as, there is not different between the M. tuberculosis antigen detection and that of atypical mycobacteria by ELISA method as in this study. In these cases culture and identification will distinguish these causative agents.

It had been also reported that there is cross reaction in tuberculous antibodies from M. tuberculosis infected patients and that of atypical mycobacterial pulmonary patients.

However, there had no significant cross reaction or false positive results when normal throat bacterial flora including Streptococci, Neisseria spp etc. were present in sputum and also no false positive in pathogenic bacteria that may contain in the sputum.

The determination of IgG anti-PPD in serum can be completed within one day by indirect ELISA method. In (x+s+c+) group ; this method could detect 12 of 26 cases (46.2%) who had

positive M. tuberculosis culture from sputum within 3 weeks, 6 of 11 cases (54.5%) who required more than 4 weeks for M. tuberculosis positive culture, and also 1 of 3 cases (33.3%) whose culture results could not be interpreted because of other bacterial contamination. Four of 10 cases (40%) of (x+s-c+) group could be diagnosed by the same method.

It took only 2 days to quantitate the amount of mycobacterial antigen in the sputum by the ELISA. In (x+s+c+) group ; this procedure could diagnose pulmonary tuberculosis in 25 of 26 cases (96.2%), and 10 of 11 cases (90.9%) from whom M. tuberculosis were isolated within 3 weeks and after 4 weeks respectively. Moreover, all 3 cases (100%) in the (x+s+c+) group whose culture was contaminated or 8 of 10 cases (80%) in (x+s-c+) group, mycobacterial antigen could be detected by this method.

More time is needed to perform antigen detection than antibody detection but the sensitivity and specificity of the former (95% sensitivity and 95% specificity) is much higher than the latter (50% sensitivity and 70% specificity). In this study, fifty cases of 86 pulmonary tuberculosis suspects, 40 (46.51%) and 50 (58.1%) cases had positive smear and culture respectively, only 10 cases (11.6%) had negative smear but positive culture and sputa in 36 cases (41.9%) were negative by both smear and culture.

By ELISA method IgG anti-PPD could diagnose pulmonary tuberculosis in 50% of (x+s+c+), 40% of (x+s-c+), 36.1% of (x+s-c-). The false positive antibody detection was 30% in non-mycobacterial pulmonary group.

By ELISA method mycobacterial antigen detection could

identify pulmonary tuberculosis in 95% of (x+s+c+), 80% of (x+s-c+) and 50% of (x+s-c-). The false positive was only 5% in (x-s-c-).

The preliminary study of ELISA for detection of mycobacterial antigens appeared to be reasonably sensitive, specific and rapid for pulmonary tuberculosis diagnosis when compared with anti-PPD detection, smear, or culture.

SUMMARY

The developments of an indirect ELISA for immunoglobulin G antibody detection and a modified double antibody sandwich method for Mycobacterium tuberculosis antigen detection were assessed in 3 groups of pulmonary tuberculosis patients. It was found that 48% (24 of 50 cases) were positive for antibody and 92% (46 of 50 cases) for antigen in pulmonary tuberculosis patients proven by chest roentgenogram and positive sputum culture, while 50% (20 of 40 cases) for antibody and 95% (38 of 40 cases) for antigen in pulmonary tuberculosis patients proven by chest roentgenogram and acid fast staining, and 36.1% (13 of 36 cases) for antibody, 50% (18 of 36 cases) for antigen in those with only positive chest roentgenogram. In comparison with non mycobacterial pulmonary patients or healthy control group 30% (12 of 40 cases) were antibody positive but only 5% (2 of 40 cases) of the patients or 8% (4 of 50) of healthy subjects were antigen positive for ELISA. From this study, the developed method seem to be more sensitive, simpler and rapid than chest X-ray, the insensitive AFB smear and the time consuming culture, especially for the large scales diagnosis of pulmonary tuberculosis.

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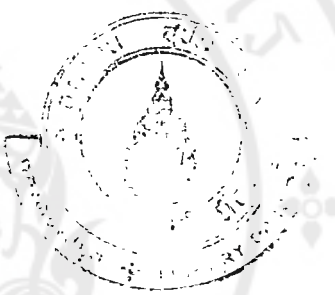

Appendix

Reagent for ELISA microtiter plate were as follow

1) Carbonate-bicarbonate 0.05M pH 9.6

$NaCO_2$	1.59	g
$NaHCO_3$	2.93	g
NaN_3	0.2	g
distilled water (DW)	1,000	ml

Stored at 4 °C, stability 14 days.

2) Phosphate buffered saline-tween (PBS/T) pH 7.4

NaCL	8	g
$KHPO_4$	0.2	g
$NaHPO_4 . 12HO_2$	2.9	g
(or $NaHPO_4$ anhydrous	1.15	g)
KCL	0.2	g
NaN_3	0.2	g
DW	1,000	ml
Tween 20 (polyoxyethelene 20 sorbitan mono-oleate)	1.5	ml

Stored at 4 °C, stability 2 months

3) Carbonate-bicarbonate buffer/BSA

BSA (bovine serum albumine)	20.0	g
carbonate-bicarbonate buffer	1,000	ml

4) PBS/T/BSA

BSA	10.0	g
PBS/T	1,000	ml

5) 10% diethanolamine buffer pH 9.8

diethanolamine	97	ml

DW	800	ml
$MgCL_2 . 6H_2O$	100	mg
NaN_3	0.2	g
1NHCl adjust until pH 9.8		
DW qs	1,000	ml

Stored at 4^{o} C in the dark

6) Substrate : p-nitrophenyl phosphate

	1	mg/ml
Sigma 104 pNpp (5 mg)	1	tablet
(store at -20^{o} C in the dark)		
10% diethanolamine buffer	5	ml

Prepare the reagent shortly before use

7) 3M NaOH

NaOH	10	g
DW	62.5	ml

Stored at room temperature

Reagent for Acid-Fast staining

Ziehl-Neelsen stain ;

1) Carbol-fuchsin :

basic fuchsin	1.0	g
ethanol, 95%	10.0	ml
phenol solution 5% in carbolic acid	100	ml

Dissolve the basic fuchsin in the ethanol ; mix the basic fuchsin solution with phenol solution. Let stand for several days and filter before use

2) 3% Acid alcohol

conc HCl	3	ml
ethanol 95%	97	ml

3) Methylene blue counterstain :

methylene blue chloride	3.0	g
DW	100	ml

Auramine Fluorochrome stain :

1) Phenolic auramine

solution A : dissolve 6 g auramine O in 100 ml of 95% ethanol

solution B : dissolve 60 g of phenol in 1,900 ml of DW

phenolic auramine was prepared by adding 100 ml of solution A slowly to 1,900 ml of solution B, let stand for 1 to 2 days.

Stored at room temperature in the dark.

2) 1% acid alcohol

NaCl	2	g
DW	24	ml
conc HCl	1	ml
ethanol 95%	75	ml

3) Potassium permanganate

$KmnO_4$	0.1	g
DW	100	ml

Ziehl-Neelsen staining procedures.

1. Cover the heat-fixed film with strong carbol fuchsin.

2. Heat the stain-covered slide with a flame to steaming but do not allow to dry, and kept it steaming for 5 minutes, replenished the stain if necessary.

3. Wash with water, and allow to drain.

4. Decolorize with 3% acid-alcohol until no more stain appears.

5. Wash with water, and allow to drain

6. Counterstain with methylene blue for 1 to 2 mins.

7. Wash with water, and air dry.

8. Examine with 100 x oil immersion objective over the entire smear.

Mycobacteria are stained red and the background ligh blue.

Auramine phenol staining procedures

1. Cover-heat-fixed, dried smear with carbol auramine and allow to stain for 15 mins.

2. Wash with water and drain.

3. Decolarize with 1% acid-alcohol for 5 mins.

4. Wash with water, drain.

5. Flood smear with potassium permanganate for 10 second and not more than 1 min.

6. Wash with water, drain.

7. Examine with 25 x objective using a mercury vapor burner and BG-12 filter or a strong blue light.

Mycobacteria are stained yellowish-orange against a dark background.

Culture media

1) Lowenstein-Jensen medium

$KHPO_4$ anhydrous	2.40	g
$MgSO_4 \cdot 7H_2O$	0.24	g

$MgCO_3$	0.60	g
Asparagine	3.60	g
Potato flour	30.00	g
Glycerol	12.00	ml
DW	600.00	ml
Homogenized whole eggs	1,000	ml
Malachite green 2% aqueous	200.00	ml

1. Dissolve the salts and asparagine in the water.

2. Admix the glycerol and potato flour, autoclave at 121° C for 15 mins., and cool to room temperature.

3. Scrub eggs in 5% soap solution. Allow to stand for 30 mins. in soap solution : then rinse thoroughlt in cold running water.

4. Immurse in 70% ethyl alcohol for 15 mins.

5. Break eggs into a sterile flask. Homogenize by hand shaking and filter through four layers of gauz.

6. Add homogenized eggs to the potato salt mixture.

7. Prepare the malachite green and admix thoroughly.

8. Dispense 6 to 8 ml screw-capped tubes.

9. Slant and inspissate at 85 C for 50 mins.

10. Incubate for 48 h at 37° C to check sterility and store at 4°to 6° C.

2) Sula's liquid medium

Na_2HPO_4	2.5	g
$KHPO_2{}_4$	1.5	g
Sodium citrate	1.5	g
$MgSO_4$	0.5	g
Asparagin	2.0	g

Alanin	0.15	g
Glycerin	25.00	ml
Ferriammonium citrate	0.05	g
malachite green (0.2%)	1.00	ml
DW aq	1,000.00	ml