INTRODUCTION

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Tuberculosis is an infectious disease affecting primarily the lungs, but any other organ may be affected. It is primarily a disease of humans, but may occur in lower animals, particularly in cattles. In the latter case, the etiologic agent is usually transmitted to humans through the consumption of raw milk. However, airborne spread from cattle may occur *(Leff and Leff, 1986).*

It is estimated that, as we approach the year 2000, tuberculosis will produce the highest mortality of all bacterial diseases *(Cook, 1985).*

Definitive diagnosis of the disease is dependent on the isolation and identification of the tubercle bacillus, Presumptive diagnosis is based on detection of acid-fast bacilli in tissue sections or sputum smears. In certain cases, the demonstration of the tubercle bacilli may not be a practical way of diagnosis owing to the difficulties in obtaining the pathological specimens for examination, or may be due to the scarcity of the organisms which makes their detection difficult or due to other technical difficulties. Indirect methods of diagnosis may impart themselves in such cases. *(Pamra et al., 1977).*

The continued search for the value of serological tests in the diagnosis of tuberculosis has been in progress for many years. Attempts to employ various serodiagnostic techniques

for this purpose have been reported by many investigators but with little success and the results are still variable and unreliable and the occurrence of many false negative and some false positive reactions limited the value of these procedures for the serodiagnosis of tuberculosis *(Schubert and Brasher, 1967).*

Nassau, et al., (1976) used the enzyme-linked immunosorbent assay (ELISA) of *Engval and Perlmann* (1972) for the detection of antibodies to Mycobacterium Tuberculosis.

The basic reaction on which all immunoassays depends is the bond that forms between an antigen and an antibody. ELISA technique depends on the assumption that an antigen and antibody can be linked to an enzyme and the resulting conjugate is both immunologically and enzymatically active. Degradation of chromogenic or florogenic substrate by the enzymes yields a product which enables accurate detection of the presence of the enzyme *(Voller et al.,1976).*

Kalish et al (1983) reported that Enzyme linked immunosorbent assay (ELISA) test may be a useful parameter in the diagnosis of suspected cases of pulmonary tuberculosis; Also it has diagnostic potential for active pulmonary tuberculosis by elevation of immunoglobulin[G] antibody levels in sera of patients to mycobacterial purified protein derivative "P.P.D."

AIM OF THE WORK

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The aim of the present work is to evaluate ELISA Test in serodiagnosis of Pulmonary Tuberculosis.

This will be done by measuring the level of specific IgG in patients with Pulmonary tuberculosis to the purified protein derivative of tuberculin.

Review of Literature

REVIEW OF LITERATURE

THE MYCOBACTERIA

The tubercle bacilli belongs to the genus Mycobacterium, of the family Mycobacteriaceae order Actinomycetales, and class Schizomycetes. Members of this genus have the distinctive property of acid fastness, i.e. the ability, when once stained, to resist subsequent decolourization by mineral acid such as sulphuric acid or nitric acid. The degree of acidfastness differs from one species to another *(Buchanan and Gibbons, 1974).*

Classification of Mycobacteria:

The genus Mycobacterium is classified into four groups: true tubercle bacilli, atypical or tuberculoid bacilli, saprophrytic acid-fast bacilli, leprosy and John bacilli *(Topley and Wilson, 1975).*

1) True tubercle bacilli:

In 1882, Robert Koch discovered the mammalian tubercle bacilli in a preparation stained with alkaline methylene blue and counter-stained with vesuvin. Later investigations showed that the mammalian tubercle bacilli could be divided into two types - the human type, M. tuberculosis, and the bovine type; M bovis. The discovery of the avian type of tubercle bacilli, M. avian, was made during 1889-91. The existence of a fourth type - the cold-blooded type of tubercle bacillus was recognised as a result of a series of investigations carried out from 1889 to 1905. A fifth type, the murine type of tubercle bacillus, M. microti was isolated in 1937 by A.O. Wells from voles (Microtus agrestis)

suffering from natural tuberculosis. The term "tubercle bacillus" broadly includes these five types of mycobacteria which give rise to tuberculosis in man or animals. It is a non-motile, non-sporing organism and has no capsule. It possesses a thick cell-wall that contains lipids, proteins and polysaccharides with specific biological characteristics. (*Rao et al., 1981*).

2) Atypical mycobacteria:

Champan (1982), reported that several strains, (Pinner's organsims) some smooth and unpigmented, others rough and pigmented, and those pigmented organisms produce immune reactions in animals and that these reactions crossed both subcutaneously and serologically with antigens from human M. tuberculosis.

These organisms were partly or completely resistant to available antimicrobial agents. In a certain proportion of patients, they constituted the only organisms recovered both on initial and on all subsequent isolations. On the other hand, gastric washings of perfectly normal subjects also contained varieties of the same kind. However, with the increase of isolations of these saprophytic acid-fast bacilli, other terms were given to this group *(Atwell and prott, 1960).*

Julin (1960) criticized the name "Atypical mycobacterium" and preferred the name "Anonymous mycobacteria", while Paull (1973) used the term "Opportunist mycobacteria" in an environmental study of Mycobacteria. *Wilson and Milles* (1975) used the term "Tuberculoid bacilli" group for all Atypical mycobacteria that may cause disease in man and animals, but put the Saprophytic mycobacteria in a separate group.

Thomson (1932) had early divided Mycobacteria on the basis of gross cultural features (appearance and rapidity of growth on slopes by solidifying Long's synthetic medium with agar) into three fairly homogeneous groups:

Group I: Characterized by rapid growth on slopes, with greyish white dull wrinkled appearance, growth at 50°C and at 15°C, utilization of glycerol, dextrose and mannite as carbon source and they are gugonic.

Group II: Characterized by growing slowly with brilliantly pigmented, yellow or orange at 40°C, glycerol and mannite are utilized members which are eugonic while others dysgonic.

Group III: Rapidity of growth is midway between that of group I and II with yellow dry growth at 54°C and also at 6-8°C, glycerol, dextrose and mannite are utilized and all members are dysgonic.

On the other hand, *Runyon* (1959) classified the atypical mycobacteria on the basis of growth rate and pigment production into four groups:

Group I: Consists of the photochromogens, growing in colonies that produce little pigment, after exposure to light for one hour, and turn yellow orange in 48 hours. However, they require a complex media for their growth. They grow little faster than tubercle bacilli (3-4 weeks). This group includes M. kansasii, M. simiae and M. marinum.

Group II: Consists of scotochromogens (colonies are yellow orange in either light or dark). They grow slowly. This group includes M. scrofulaceum, M. szulgaie, M. gordonae and M. flavescence.

Group III: The non-chromogens colonies are colourless and grow slowly. This includes M. avium intercellular group, M. xenopi, M. gastri, M. Terae and M triviale.

Group IV: The rapid growers, grow within 2-7 days on ordinary media.This group includes M. fortuitum, M. cheloni, M. smegmatis and M. phlei.

Marks (1972) designed a new method of classifying mycobacteria met with in human materials, for needs of clinical bacteriology. It was based first on temperature requirement and on O_2 preference and / or pigment. Francis and Abrahams

(1983) suggested the classification of the entire family of Mycobacteria into four groups consisting of tubercle bacilli, tuberculoid bacilli, saprophytic mycobacteria and leprosy.

Bailey (1984) has recently proposed a reclassification for the atypical mycobacteria which differentiates the organisms according to clinical characteristics and response to various chemotherapeutic agents. The organisms that might be isolated from human material are divided into two classes: non-pathogens- those that are easy to treat with standard mycobacterial therapy (M. kansasi, M. xenopi, M. marinum); , those that are difficult to treat with standard mycobacterial therapy and require other approaches (M. avium intracellulare, M. scrofulaceum, M. fortuitum).

3) Saprophytic mycobacteria:

Jawetz et al. (1987) have recently included all the mycobacterial strains not associated with human illness under this group M. phlei and M. gordonae which are frequently found on plants and in soil or in water. Moreover, this group includes M. smegmatis which occurs in human sebaceous secretions and might be confused with pathogenic acid-fast organisms. On the other hand, M., paratuberculosis, producing chronic entritis in cattle but does not infect man is also included with Saprophytic mycobacteria.

4) Mycobacterial leprae and M. leprae murium:

Both species are characterized by a limited host range. M. leprae infects man, causing the leprosy and M. leprae- murium causes rat leprosy. Both are facultative intracellular parasites. Neither of them has so far been grown on non-living culture media. M. leprae murium has been successfully grown in tissue-cultures and has been found to grow extremely slowly with a generation time of 10-12 days. There is no convincing evidence that M. leprae has ever been successfully grown in tissue culture (Rao et al., 1981).

Tobly & Wilson, (1984) classified the species of Mycobacteria according to growth on culture media into the following groups:

A) The slowly growing Mycobacteria:

M. tuberculosis

M. Kansasi

M. gastri

M. marinum

M. Xenopi

M. Ulcerans

M. avium and its intracellulare variant

M. paratuberculosis (Johne's bacillus_

M. Lepraemurium

Slowly growing scotochromogens

M. Scrofulaceum

M. grodonae

M. szulgai

Slowly growing mycobacteria of uncertain taxonomic status

M. malmoense

M. simiae and M. asiaticum

M. habana

Other slowly growing species. M. nonchromogenicum, M. terrae, and miriviale

B) The rapidly growing mycobacteria :

Group I: Non-chromogenic strains with strong arylsulphatase activity

M. fortuitum

M. chelonei

Group 2: Thermophilic strains

M. semegmatis

M. phlel

M. thermoresistiblle

Group 3: Rapidly growing scotochromogenic mycobacteria with limited saccharolytic activity. M. flavescens, M. gilvum, and M. duvali.

Group 4: Other rapidly growing mycobacteria

M. diernhoferi and M. vaccae.

C) Non-cultivable mycobacteria

M. leprae.

Mycobacterium tuberculosis:

Habitat:

This species, the causative agent of tuberculosis, is an obligate pathogen, none of its several variants exists saprophytically outside the animal body *(Wilson et al., 1983).* Although the bacilli may survive in artificially contaminated environmental sites, such as shaded cool soil, for at least 6 months, there is no evidence that they replicate there. This is not surprising in view of their very slow growth, restricted growth temperature range, and sensitivity to heat *(Coper and Cohn,1933).*

Cultivation:

In the laboratory, the tubercle bacilli are not nutritionally fastidious or demanding. They adapt readily to growth on simple salt solutions with ammonium ion as a source of nitrogen and glucose as a source of **carbon** *(Redomond, 1957).*

M. tuberculosis is a strictly aerobic organism. It grows over a pH range of 4.5-8.0, with an optimum pH of about 6.8. The optimum temperature is 37C. The generation time has been reported as short as 10 hours (Harshey et al., 1977), and 15-20 hours in the study of *(Wayne, 1976, 1977).* Furthermore, a generation time of 20 to 22 hours has been reported for M. tuberculosis in the rabbit cornea *(Robson and Sullivan, 1957).* Intravenously infected mice can be used to calculate a minimal doubling time of approximately 15 hours for the bacilli in the

lung of unimmunized animals, the cell mediated immunity led to a growth plateau in which the bacilli neither replicated nor died during a period of at least 2 months *(Sever and Youmans, 1957)*. Primary cultures usually show macroscopically visible colonies in 2-5 weeks; with subcultures, colonies appear faster and may be visible even in a week *(Sever and Youmans, 1957)*.

Simple synthetic media containing glucose or glycerine and some source of nitrogen such as ammonium ion, glutamate or asparagin, can support the growth of large inocula of tubercle bacilli.Tubercle bacilli are prototrophic for all the amino acids, purine and pyrimidine bases and B-complex vitamins. The most important source of carbon is glycerol, and asparagine is the preferred nitrogen source *(Joklik et al. 1984)*.

For general use, the most satisfactory solid media for the cultivation of M. tuberculosis are the media containing egg-yolk. Of the egg media, Lowenstein-Jensen medium is the commonest one used for growing M. tuberculosis. It sustains growth of small inocula. It is relatively inexpensive and is easily prepared. Further, the green background permits early detection of growth. This medium, therefore, has found extensive application in diagnostic bacteriology *(Vestal, 1975)*.

Culture characteristics :

Cultures for the isolation of M. tuberculosis on Lowenstein-Jensen (L.J.) media should be incubated at 35°C to 36°C for a total of 6 to 8 weeks and examined at weekly intervals. Positive

culture should be reported as soon as identification has been completed *(Baily and Scott, 1983).*

Colonies of human tubercle-bacilli generally appear on egg media after 2-3 weeks at 36°C. No growth occurs at 25°C or 45°C. Growth first appears as small (1 to 3 mm), dry, friable colonies that are rough, warty, granular and buff in colour. After several weeks these increase in size (up to 8 mm); typical colonies have a flat irregular margin and a "cauliflower" centre. Because of their luxuriant growth these mycobacteria are termed eugonic. Colonies are easily detached from the medium's surface but are difficult to emulsify. After some experience one can recognize typical colonies of human-type tubercle bacilli without great difficulty *(Fregnan and Smith, 1962; Vestal, 1975).*

Mycobacterium bovis require a longer incubation period, generally 3 to 6 weeks, and appear as tiny (less than 1 mm), translucent, smooth, pyramidal colonies at 36°C. They adhere to the surface of the medium but are emulsified easily. Their growth is termed dysgonic. M. bovis will grow at 36°C. They form serpentine cords on smears from colonies on egg media *(Vestal and Kubica, 1966).*

In fluid media, on glycerol broth, mycobacteria grow as a surface pellicle. At 1-2 weeks, the pellicle is grey, translucent and veil-like. Subsequently, the growth becomes thick, white, wrinkled, and spreads up the surface of the flask. The medium

shows no turbidity, but may show a granular deposit after prolonged incubation. In fluid media containing Tween no surface pellicle is formed, instead, growth occurs diffusely in the medium, giving rise to turbidity *(Middlebrook et al., 1947).*

M. tuberculosis has been grown on the chorio-allantoic membrane and in the yolk sac of fertile hen's eggs and in tissue cultures of Hela and monkey cells *(Rao et al., 1981).*

Microscopic Examination and Morphology:

Although microscopy alone does not usually distinguish between M. tuberculosis and other mycobacteria, it is nonetheless a rapid and simple means of detecting those cases of pulmonary disease that are a source of infection. To be detected microscopically, there must be between 5,000 and 10,000 bacilli in 1 ml of sputum *(Cruikshank, 1952).*

The techniques used for staining mycobacteria are based on the resistance of the organisms to decolorization by acids after staining by an arylmethane dye. The most widely used staining technique is that bearing the names of Ziehl and Neelsen *(Ziehl, 1882 and Neelsen 1883).*

Fluorescence microscopy was introduced by *(Hangmann 1937),* who originally used berberine sulphate as the dye. *Truant et al. (1962)* used two arylmethane dyes, auramine O and rhodamine B, together. This combined staining method detected acid fast bacilli in 358 out of 3000 samples of sputum

while only 274 by the Ziehl-Neelsen method *(Somlo et al., 1969).*

Mycobacteria are usually straight or slightly curved rod-shaped organisms, with more or less parallel sides and rounded ends, usually 1-4 um x 0.3-0.6 um in size, which are often arranged in small groups. The morphology varies from species to species: cells of M. xenopi are often filamentous with occasional branching and aerial hyphae, those of M. Kansasi are elongated with a beaded appearance. While those of M. avium are very short almost coccoid *(Wilson et al., 1983).*

Electron microscopy shows that mycobacteria possess a relatively thick cell wall separated from the cell membrane by a thin electron-transparent zone *(Imaeda et al, 1968).* The cytoplasm contains a fairly well defined nuclear body, and granular and electron-transparent bodies which are probably polyphosphate and lipid storage bodies respectively. The cell membrane frequently shows infolding or mesosomes *(Asano et al., 1973).* The mesosomes are occasionally quite large and show a lamellar structure. Membranous structures resembling mesosomes are abundant in lysogenic mycobacteria *(Robbins et al., 1970).*

Electron micrographs show clearly that mycobacteria may divide by binary fission. *Chang and Anderson, (1969)* and McCarthy (1971) considered that they may display a more complicated life cycle in which bacilli fragment into a number of

coccal forms which then elongate into rods. Mattman (1970), postulated even more elaborate life cycles, with the formation of cell-wall-defective organisms that release mycoplasma-like viable forms. Much *(1907)* reported that miliary tubercles in cattle often contained few acid-fast organisms but many granules, stainable by aniline gentian violet, which he considered to be forms of tubercle bacillus. Sweany (1928), accepted that these, Much's granules, represented a stage in the life cycle of M. tuberculosis. Oerskov *(1932)* considered them to be products of degeneration, artefacts due to damage to the organisms or mitochondria (Mudd et al., 1951).

ANTIGENIC STRUCTURE OF M. TUBERCULOSIS

Attempts have been made to isolate individual antigenic constituents of M. tuberculosis. Chemical methods were used by **Affronti** *and coworkers (1965)* to extract polysaccharides from Mycobacteria polysaccharide I which was characterized by **Brinbaum,** *et al., (1968)* as a heteropolysaccharide containing arabinose, galactose and mannose. Its antigenicity has been attributed to contaminating protein. *Birnbaum and* **Affronti** *(1970)* reported that immuno-electrophoretic analysis has shown polysaccharide I to contain principally antigen 2, with smaller amounts of antigen I. A chemically similar material was isolated from cell walls by *Cummins 1962)* and shown to be present in numerous species of mycobacteria. *Daniel et al. (1973)* found that polysaccharide II was a glucan and immuno-electrophoresis which proved that polysaccharide II represents the antigen 3.

It should be realized that most attempts to purify mycobacterial proteins have not excluded that polysaccharides may be derived either from cell wall or cell cytoplasm and many of the so-called protein fractions have high polysaccharide content *(Daniel et al. 1978).* So, *Misaki and Azuma (1974)* reported in their study that the protein-free polysaccharides are of four principal types, arabinogalactans, arabinomannans, mannans and glucan from both mycobacterial culture filtrates and cell wall extracts.

Polysaccharides arabinogalactan and arabinomannan are excellent antigens and the antigenic determinant of arabinogalactan has been identified with a major arabinose side chain which is probably shared by all species of mycobacteria *(Affronti et al., 1965)*. However, eleven antigen fractions were reported in unheated culture filtrate of M. tuberculosis (strain H37 R) by immunoelectrophoretic studies including protein A that contains 1,2,4,5,6 antigens, protein B containing 1,2,5,6,7 antigens and protein C including 2,6,7 antigens. However, subsequent studies suggested that antigen 5 may be a specific species (Daniel et al., 1973). On the other hand, *Chaparas (1979)* referred to the difficulty in preparation of pure antigens reported in several other studies as probably being due to presence of many different antigenic determinants. It has recently been, it is well established that many constituents of mycobacterial cell are antigenic including cell wall, polysaccharides, proteins and peptides and while some of these antigens are species specific, others are shared by many species and contribute to the cross reactivity that is commonly observed with the use of various tuberculins (old tuberculin, P.P.D.) *(Joklik et al., 1980)*. However, while the earliest preparation of tuberculin by filtrates of old culture of tubercle bacilli concentrated by evaporation, and tuberculin has been widely used in studies as antigens. in recent years, many workers have liberated antigens from whole cells by freeze-pressing or by ultrasonic disintegration *(Grange, 1984)*.

Further studies of antigens in Atypical mycobacterium by *Palmer and Hopwood (1962)* reported that M. kansasii produced a great protection against a challenge with M. tuberculosis as does bacilli Calmette Guerin (BCG), while other mycobacteria were less effective. Serological studies have no place in the diagnosis of an individual case in spite of the validity of agglutination absorption that distinguishes strains within the M. avium intercellular complex. Many studies of antigens revealed reasons for non-specificity of serological tests in the diagnosis of atypical mycobacteria. *Kniker (1965)* found numerous antigens common to all mycobacteria and it was evident that there were numerous bands of identification in the sera of sensitized animal that diffuse against antigens of various types of mycobacteria. Moreover, it was evident that the term of common antigens, M. Kansasii was close to M. tuberculosis and M. avium intercellular was much further removed. However, *Stanford and Beck (1968)* used the double diffusion precipitation tests to study the antigenic structure of strains of M. fortuitum, M. kansasii, M. phlei, M. smegmatis and H37Ra of M. tuberculosis and they revealed the antigenic complexity of many of the members of these groups and the degree of cross reactions between them.

IMMUNE RESPONSE TO TUBERCULOSIS

Natural Resistance:

Man and animals are known to possess a natural resistance to tuberculosis. Different animal species display a wide difference in their resistance to tubercle bacilli. Innate resistance also occurs within any animal species and differs from one individual to another. Deficiency of this resistance may arise presumably from mutation of structure and synthesis of numerous protein products, enzymes such as lysozyme, Complement and transferrin concerned in non-specific mechanism of defence (Hill et al., 1940). Lurie, *(1955)* reported that in resistant rabbit families, the disease was slow and localized, with cavitation while in highly susceptible varieties, the disease spreads very rapidly with generalized infection.

Dold (1947) had early described anti-mycobacterial substance in normal urine and also reported a stable dialysable inhibitor of **Mycobacterium** tuberculosis in urine. Meanwhile, kidney tissue was found to contain spermine, which, when activated by spermine oxidase, has powerful bacteriostatic action on tubercle bacilli in vitro. Moreover, *Soltys (1953)* had described nearly at the same time tuberculostatic substance in liver, spleen and lymph nodes of tuberculous animals.

On the other hand, it has been established that oestrogen checks the progress of tuberculosis while chorionic

gonadotrophin enhances it. Both exert their influence through lysis of lymphocytes and fall in antibody formation. *Dienes et al. (1967)* reported that the local alveolar macrophages play a role in resistance against tubercle bacilli and in the prevention of spread of the disease from its local site.

The anti-inflammatory effect of cortisone helps to localize infection through metabolic changes in tissues and by productive effect on capillary walls *(Lurie, 1955).*

The immune response to chronic infection begins with inflammation and involves the environment of the defences of the host. In rabbits and guinea pigs, experimental tuberculous infections begin with a pouring in of polymorphonuclear leucocytes (PMNs) at the site of injection, and with subsequent ingestion of the bacilli by these cells, the bacteria laden granulocytes subsequently die, and their remnants and contents are engorged by macrophages *(Rich, 1951).* There is little or no evidence to suggest that, before dying, these PMNs have had a lethal effect on their engulfed mycobacteria *(Hanks et al., 1940 and Rich, 1951).* However, it had been early reported that during tuberculous infection, antibodies are formed *(Middlebrook et al., 1948)* and immunoglobulins reactions with M. tuberculosis had been described to be common among normal human subjects *(Freedman et al., 1963 and Bardana et al., 1973).*

Relation between immunity in tuberculosis and tuberculin hypersensitivity:

Koch in (1891), was the first worker who drew attention to the role of delayed hypersensitivity in tuberculosis when he successfully precipitated a substance from concentrated culture filtrate called tuberculin when injected, a specific tuberculin reaction is elicited. On the other hand, *Rich (1951)* proved that there is no correlation between acquired immunity to tuberculosis and tuberculin hypersensitivity. *Comstock (1964)* found great discrepancy between tuberculin hypersensitivity in human beings and immunity to tuberculosis among a BCG vaccinated group.

Neiburger and Youmans (1973) provided an evidence to support that tuberculin hypersensitivity and acquired immunity are separate immune responses directed against separate components of the mycobacterial cell. They administered rifampin, which is a potent immunosuppressive drug, to mice during the vaccination period without any effect on the immune response produced by mycobacterial RNA vaccine. Moreover, rifampin suppressed the induction of tuberculin hypersensitivity in mice vaccinated with viable H37Ra cells. So, a more reasonable view is that delayed hypersensitivity and immunity to tuberculosis are entirely separate processes that are initiated in animals in response to various components of the tubercle bacillus. Both phenomena involve a cellular type of immune response and both are mediated by thymus derived lymphocytes

Youmans (1975)., It is not clear at present whether the difference lies at the regulator or effector cell level. However, in a recent view, it is postulated that those organisms which evoke koch-like responses in vivo may trigger subclasses of T-lymphocytes which kill infected macrophages rather than activate them *(Rook et al. 1981).* Concerning the lymphokines secreted by each type of lymphocytes, *Youmans (1975)* suggested that the lymphokine which is responsible for delayed hypersensitivity is a migration inhibition factor (M.I.F.) while lymphokine responsible for acquired immunity is a mycobacterial inibiting factor (Myco. I.F.). Other lying lymphokines as chemotactic factor and mitogenic factor can be elicited by antigen of T.B. bacilli which cause cell infiltration, cell activation and cell division. *(Fishman, 1980).*

Neiburger and Youmans (1973) investigated the relationship between M.I.F. and Myco. I.F. They vaccinated separate groups of mice either with viable H37Ra cells or with mycobacteria RNA vaccine. Only spleen cells from mice vaccinated with viable H37Ra mycobacterial cells produced M.I.F., when stimulated either in vitro or in vivo with P.P.D. However, both groups of mice were equally immune to intravenous challenge with virulent tubercle bacilli. Although no sign of tuberculin hypersensitivity could be elicited with P.P.D. in animals vaccinated with RNA material, yet these animals were just as immune to infection as were the animals vaccinated with H37Ra viable cells. Both studies conducted by

Patterson & Youmans (1970) and *Klun & Youmans (1973)* confirmed the previous findings as they found that lymphocytes from mice vaccinated with both H37Ra cells and mycobacterial RNA when stimulated in vitro with H37Ra cells or mycobacterial RNA would produce Myco. I.F. However, when lymphocytes from these same animals were stimulated in vitro with P.P.D. only, the cells from mice immunized with H37Ra cells produced M.I.F. However, *Youmans (1975)* prepared partially purified highly active Myco. I.F. by gel filtration. When these preparations were tested by M.I.F. activity, they showed no inhibition of macrophage migration, that provides further support for the conclusion that mouse M.I.F. and mouse Myco. I.F. are separate substances.

It is also of interest to point out that animals which develop delayed hypersensitivity easily are highly susceptible to tuberculosis, whereas animals that have a much lower capacity to develop delayed hypersensitivity are far more resistant. If tuberculin hypersensitivity is responsible for acquired immunity, the opposite relationship would be expected *(Youman, 1979).* In a study to confirm this relation *Ismail (1983)* reported that most tuberculin positive, normal controls, gave M.I.F. values less than 0.86, whereas the M.I.F. of most tuberculin negative controls exceeded 0.86. The results also indicated the presence of a significant difference between the mean values of normal tuberculin negative healthy controls on one hand, and patients with pulmonary tuberculosis, malignant

lung diseases (both tuberculin positive and negative) and suppurative lung diseases who are tuberculin positive on the other hand.

Humoral immunity in tuberculosis:

During tuberculous infection, antibodies are formed and immunoglobulins reactive with M. tuberculosis are reported to be common among normal human subjects *(Bardana et al., 1973)*. The importance of antibodies in recovery from tuberculosis had long been questioned some years ago *(Daniel et al., 1968)*.

B-lymphocytes are concerned with the synthesis of circulatory antibodies. The B-lymphocytes are characterized by at least three surface receptors:

1) Receptors that bind the Fc region of Ig molecules and provide a site for antibody molecules, antigen-antibody complex, or aggregated Ig *(Basten et al., 1972)*.
2) Surface Ig receptors for specific antigens *(Nossal et al., 1971)*.
3) Receptors for the C3 component of complement *(Nussenzwing et al., 1971)*.

These B-lymphocytes can be stimulated to produce the lymphokines, monocytes chemotactic factor by: Mitogen carrying multiple binding sites e.g. lipopoly-saccharides *(Feldman et al., 1971)*; antigen-antibody complexes or aggregated gamma globulin interacting with the Fc receptor

and interaction of antigen-antibody complexes at the C3 receptor. However, the bridging or cross-linking of receptors is thought by some to be the key to stimulation and such stimulation is affected by non-mitogenic as well as mitogenic molecules as long as they can cross link the receptors *(Wahl and coworkers, 1974).*

B-lymphocytes with an immunoglobulin having an affinity for a specific antigen, giving rise to a clone of plasma cells (monoclonal stimulation), are capable of synthetizing immunoglobulins of specific antibodies activities, although the B-lymphocytes prior to antigenic stimulation possess Fc and C3 receptors, the cells of clones (plasma cells) have lost or have covered up the C3 receptors *(Moller, 1974). Coutinho and associates (1974)* found that B-cells may also be generally triggered to mitosis by certain species of molecules defined as mitogens, as well as by C3 and lipopolysaccharides. These compounds, capable of polyclonal activation, have been termed polyclonal B-cell activators.

T-lymphocytes, probably with macrophages, are essential for maximal B-cell response to certain antigens. Through experiments employing T and B cells in separate chambers linked by nucleo-pore membrane, *Feldman (1972)* has demonstrated that T and B cells cooperation can occur through soluble factors, thus it appears to be no requirement for cell-to-cell contact. He also suggested that complexes of IgM and T-

cells with antigen become bound to macrophages and it is with this macrophages-bound complex that B cells interact and become stimulated to produce antibody.

Amos and his colleagues (1967) found that the sera of guinea pigs sensitized to BCG followed by P.P.D. in Freund adjuvant contained cytophilic antibodies capable of fixing to macrophages and rendering them sensitive to migration inhibition by P.P.D. This migration inhibition could be passively transferred with serum and was not to be confused with lymphokines *(David, 1973). Waksman and Namba (1976)* have pointed out that cytophilic IgG has two functions in its binding, by its Fc end to the macrophages and by its Fab end to antigen.

Recently, it has been found that humoral antibody (IgG) can induce or inhibit an in vitro-lymphocyte-mediated cytotoxicity by thymus independent-cells. In this lymphocyte-mediated killing, normal lymphoid cells are cytotoxic for particular target cells in the presence of an antibody to the latter, the Fc portion of the antibody molecule is essential for cytotoxicity *(Perlmann and associates, 1972). Maclennan (1972)* had suggested the term (cytotoxic B-cell) for the non-glass adherent nonphagocytic, mononuclear cells that kill target cells sensitized with IgG. He considered such cells as athymic in development and postulated that they do not release lymphokines. However, *Rappaport and Khalil (1976)* pointed out that subcutaneous BCG immunization in mice produced

systemic proliferation of macrophages preceded by proliferation of lymphoid cells, in the thymus and spleen (both T-and B-lymphocytes). On the other hand, *Burnet (1978)* showed that B-lymphocytes respond with enhanced or depressed levels of proliferation relative to the non-specific levels of stimulation induced by tuberculin. The type of response depends on the source of the lymphocytes and how B.C.G. has been administered. The proliferation of splenic B-lymphocytes was depressed when B.C.G. was given I.V. due to stimulation of suppressor T-cells in the spleen which depressed the splenic B-cells, and unchanged when given subcutaneously whereas the lymph node B cells response was enhanced regardless of the route of injection of B.C.G.

Complement system:

It has been defined as a group of at least 20 distinct serum proteins including 11 proteins of the classical complement system (C_{1q}, C_{1r}, C_{1s}, C_4, C_2, C_3, C_5, C_6, C_7, C_8, C_9). acting in sequence, many of whichare pro-enzymes and under certain condition are activated to enzyme *(Broom et al., 1970).*

Role of complement in defence:

1) Cytolysis: The full complement system leading to membrane damage, causes bacteriolysis in gram negative organisms by allowing lysozymes to reach plasma membrane where it destroys the macro-peptide layer *(Davis and associates, 1972).*

2) Immune C_3b adherence: This plays a major role in facilitating phagocytosis of micro-organisms after coating with antibody and complement, or after activation of alternative complement pathway. Adherence to macrophages and polymorphs may operate largely through C_3b binding. Purified C_3b has been shown to trigger extracellular release of lysosomal enzymes from macrophages and it is possible that this could damage adhering micro-organisms *(Henson and coworkers, 1972).*

3) Immunocoagulation: This may play a role by agglutinating relatively smal complexes containing bound C3 making them more susceptible to phagocytosis *(Roitt, 1977).*

4) Inflammation: The fragments produced during complement consumption (C_3a, C_5a) stimulate two helpful features of acute inflammatory responses: chemotactic factors attract phagocytic neutrophils, polymorphs to the site of complement activation. Anaphylatoxins, through histamine release increase vascular permeability and hence the flow of serum antibody and more complement to the infected area *(Giertz, 1969).*

Since the main role of the complement system is increasing phagocytosis which is the main line of defence in tuberculosis, so the complement system may cooperate with humoral and cellular immunity in tuberculosis. The information related to serum complement levels in disease often appears conflicting. During periods where the disease process is active and complement activation is presumably at maximum, the levels of

complement components would be expected at a low level in contrast to quiescent periods when normal levels might be encountered, or during a recovery phase where there might be an increase in a particular component (Kohler and Ten Bensel 1969). In clinical reports of the complement level (C_3 and C_4) in T.B. various values were recorded, including decreased levels, normal or increased levels. Much of this variation occurs because of the time of sampling during the activity of the disease process *(Gatner and* Anderson 1980).

Antibody response in tuberculosis:

When an antigen is introduced into a body for the first time, a primary response is evoked in which antibodies are detected after a lag period of 1-30 days, depending on dose, route of injection, size and solubility of antigen in addition to the type of adjuvant used. The time required to achieve maximum antibody titre and the duration of the peak level also vary with nature of the antigens and the methods of immunization. After the antibody levels in the primary response have declined, a subsequent encounter with the same antigen usually evokes a much stronger secondary response with shorter lag period, faster rate of increase, and a longer persistence of antibody titre at the peak than the primary response *(Fudenberg and associates, 1978).*

However, *Middlebrook and Dubos 1948)* using old tuberculin (O.T.) as sensitizing antigen found that 50% of

animals infected with soluble O.T. and 37% given alum precipitated P.P.D. produced antibody measurable by haem-agglulation test. This antibody was of short duration and was of IgM type exclusively. Five of eight animals responded to . O.T. in adjuvant and produced antibodies of both IgM and IgG. In these animals, there was an early production of both classes of antibodies, while IgG was predominating in late periods Moreover, Turcotte and coworkers (1963) reported that specific immunoglobulin of (IgG)type was detected in specimens obtained from patients with active pulmonary tuberculosis only, whereas non-specific IgM was detected in sera of both tuberculous and healthy individuals. During the course of a primary immunization, the antibodies produced at the early stage are predominatly IgM class. In most cases, IgM antibodies are gradually replaced by IgG antibodies after a few days. The early IgM and the late IgG antibodies often share idiotypic determinants *(Qudin and Michel, 1969).*

Studies with chickens indicated that the change takes place in the bursa of fabricus and follows the sequence IgM, some of the IgM producing cells may become IgG producers during the maturation of the B-lymphocytes *(Nossal et al., 1973).* However, *Wanner (1974)* suggested that IgM precursor cells may give rise to cells producing other classes of antibodies by separate pathways.

Elevated levels of serum concentration of immunoglobulins IgG, IgA and IgM were also reported among patients with tuberculosis by many authors *(Malomo et al., 1970); (Alacron and Fishbein , 1971 and Al-Tawil and Thewaini 1978).* However, other investigators demonstrated significant elevation of serum IgG, IgA but not IgM in patients with Tuberculosis *(Faulkner et al., 1967 & Gatner Anderson 1980).*

Ismail (1983) found that the comparison of immunoglobulins concentration between pulmonary tuberculosis, extrapulmonary tuberculosis, suppurative lung diseases and pulmonary malignancy revealed that the mean value of serum IgA among patients with pulmonary tuberculosis was significantly elevated if compared with those obtained in other disease categories ($P<0.05$). Although serum IgG was significantly elevated in patients with pulmonary tuberculosis when compared with extrapulmonary tuberculosis and malignancy ($P < 0.05$), but not significantly higher in comparison with values recorded among patients with suppurative lung diseases. IgM mean level was significantly elevated in pulmonary tuberculosis as compared with malignancy and suppurative lung diseases ($P < 0.05$) but not for extrapulmonary group. In another study, *Nash (1979)* reported that IgG levels were significantly increased among patients with pulmonary tuberculosis compared with COAd ($P < 0.001$) and those with bronchogenic carcinoma ($P < 0.005$).

The humoral immune function was studied by *Mohamed (1983)* as he asssessed the number of the B-lymphocytes measuring C3, C4 concentration and measuring IgG, IgM and IgA concentration. he found in the patients group (Pulmonary and extrapulmonary tuberculosis) that there was a significant rise of B-lymphocytes, IgG and IgA concentration, while there was no significant change in IgM and C3 levels, neither was the level of C4 significantly changed in pulmonary T.B. while in extrapulmonary T.B., it showed significantly lower levels.

Cellular Immunity in tuberculosis:

1) Role of macrophages:

The macrophage is a third type of cell that is intimately involved in the development and expression of humoral and cell-mediated immune response. In contrast to T cells and B cells. macrophages are neither clonally restricted nor antigen specific but function as non-specific accessory cells. Their roles as accessory effector cells both in cellular and humoral immune response *(Nelson, 1976).*

Macrophages are thought to have a supportive position in the induction of acquired immunity. Their function is solely to engulf and degrade antigen, thereby, protection of the immune apparatus from antigen overdose *(Hoffmann and Dutton 1971).* Others have reported that macrophages release nutrient factors that provide the necessary environmental conditions for optimal immune induction *(Chen & Hirsh, (1972)* However, many

investigators have shown that macrophages concentrate and maintain antigen on or near their surface and then either preserve it, or present it to lymphocytes in a manner conductive to immune induction *(Unanueand Cerottini 1970).* Cellular immunity in tuberculosis may be defined as a state in which macrophages have been activated, and proliferated and have acquired an increased capacity to destroy tubercle bacilli. Its activation increases their digestive and microbicidal capahilities by increasing levels of hydrolytic enzymes *(Mackenancess,1964). Siato and Suter (1965)* found that BCG injection elevates the acid phosphatase, B-glucuronidase and cathepsin levels and protein content of macrophages washed from peritoneal cavities of mice. Also, *Evans and Myrvik (1967)* proved that intravenous or intratracheal injection of heat killed tubercle bacilli led to an increase in alveolar macrophages, the levels of lysozyme, B-glucuronidase, acid phosphatase, B-galactosidase, a protease, a lipase hydrolyzing napthol laurats, ribonuclease and deoxyribonuclease. It also increased their hexose monophosphate shunt activity and their bactericidal ability. Moreover, *Dannenberg (1968)* found that lysozyme and acid phosphatase levels in peritoneal macrophages were higher in tuberculous animals than in controls.

The origin of these defence cells has been the subject of several investigations. *Kosuinen et al. (1963)* found that circulating monocytes and lymphocyte-like macrophages

but as chronicity is established, local proliferation becomes more important *(Spector, 1969).*

II) Role of T-lymphocytes:

T-cells or thymus-derived lymphocytes on appropriate stimulation by antigen proliferate undergo morphological changes and transform to lymphoblasts which are seen in electron microscope to have abundant free ribosomes *(Roitt, 1977).*

In the mechanism of cell-mediated immune response, two basic effector processes have been identified: cytolytic activity, of T cell from immune animals, which can be cytotoxic, killing cells that carry membrane antigens against which the host is immune *(Henney, 1973);* T-cells from immune animals in the presence of homologous antigen, synthesis and subsequently secrete a number of biologically active soluble factors called lymphokines *(Bloom, 1971).*

Lefford et al. (1973) provided additional direct evidence for the involvement of lymphocytes in immunity to tuberculosis by showing that in rats, specific immunity to tuberculosis could be adoptively transferred to non vaccinated recipient rats using thoracic duct lymphocyte suspensions that were free of macrophages. *Patterson and Youmans (1970)* also found that lymphocytes obtained from immunized mice when stimulated with specific antigen would elaborate and secrete into the tissue culture medium a substance (lymphokine) that when

added to macrophages infected with virulent tubercle bacilli would bring about inhibition of the growth of the intracellular tubercle bacilli and they called it mycobacterial growth inhibiting factor (Myco. I.F.).

DIAGNOSIS OF PULMONARY TUBERCULOSIS

(1) Clinical Features:

In summary the following are the common modes of presentation of patient suffering from pulmonary tuberculosis as outlined by *(Crofton and Douglas, 1981).*

- Symptoms free; tuberculosis discovered at routine mass miniature radiography (M.M.R.).
- Smoker's cough or persistent cough.
- Unexplained loss of weight.
- Undue tiredness.
- Failure to recover adequately from an attack of influenza.
- Haemoptysis.
- Chest pain - Dyspnea and pneumonia which turn out to tuberculosis.

The general condition in pulmonary tuberculosis may be excellent even with relatively advanced disease on X-ray, but there may be pallor, hectic flush, tachycardia in proportion to pyrexia. In the chest there are often no physical signs, the most common early abnormality consists of post tussive crepitation in the upper zones. In advanced cases there are signs of consolidation, fibrosis, localized wheeze due to tuberculous bronchitis.

(2) Roentgenographic Data:

It is seldom possible to make a completely confident diagnosis of pulmonary tuberculosis on radiological ground alone, as almost all the manifestations of tuberculosis can be mimicked by other diseases. The following characteristics of a chest radiograph favour the diagnosis of tuberculosis as shown by *(Crofton and Douglas 1981).*

- Shadows mainly in the upper zones.
- Patchy or nodular shadows.
- Presence of cavity or cavities.
- Bilateral shadows especially in the upper zones.
- Presence of calcification.
- Persistance of abnormal shadows without alteration in an X-ray repeated after several weeks.

However the the American National Tuberculosis Association (1964) classified the extent of pulmonary tuberculosis on radiological bases into:

(a) Minimal lesions, including those which are of slight to moderate density, but do not contain cavitation.

They involve a small part of one or both lungs, but the total extent, regardless of distribution, does not exceed the volume of lung on one side which is present above the second chondrosternal junction and the spine of the fourth or the body of the fifth thoracic vertebra.

(b) Moderately advanced lesions may be present in one or both lungs, but the total extent should not exceed the following limits: dissiminated lesions of slight to moderate density which may extend throughout the total volume of one lung, or the equivalent in both lungs: dense and confluent lesions which are limited in extent to one third the volume of one lung, total diameter of cavitation, if present, must be less than 4 cm.

(c) Far advanced lesions are more extensive than moderately advanced, quoted from *(Crofton and Douglas, 1981).*

Tomography, is a form of X-ray which divides the lung into laminae to localise the site and size of small cavities, also it may reveal calcification and satellite lesions of tuberculoma (Crofton & Douglas, 1981).

(3) Demonstration of Tubercle bacilli:

I- Sputum examination by direct microscopy:

Direct smear microscopy is important because the results are available immediately, but complete examination needs culture of specimen in order to determine the species of mycobacterium as direct smear only cannot indicate that acid fast bacilli are present or not.

Staining : ***(a)*** acid fast stains which include:

1- Ziehl-Neelsen stain : heating of the fixed sputum preparation on new glass slides flooded with carbol fuschin for about 3-5 minutes, then rinsed in water and decolorized with sulphuric acid 25% concentration for 2-5 minutes and 95%

alcohol for 2 minutes until no colour appear on washing, then counter stained with methylen blue for 30 seconds, finally it is rinsed and dried, and examined by light microscopy with x100 oil immersion lense and x10 eye pieces, (Bishop, 1970).

2- Kinyoun's stain : carbol fuschin is more concentrated in this stain than that in Z.N. stain, heat is not applied, malachite green is used as the counterstain, (Kinyoun, 1915).

Reading the smear:

The results of sputum examination are reported as merely positive or negative. However quantitative notation of the numbers of organisms per field or per slide is necessary.

Rizk (1981) reported that the National Tuberculosis Association recommended a simplified method of reporting smear which has since been then continued to be recommended by the American Lung. Association *(1974).* Methods of reporting positive smear according to the American Lung Association *(1974).* are shown in the following table:

If the number of bacili	It would be reported as:
0	No acid fast bacilli was found
1-2 found in the smear	Repeated examination of the same specimen is required.
3-9 are found in the whole smear	Rare or +
10 or more are present in the entire smear	Few or ++
1 or more per each oil immersion field	Numerous or +++

(a) negative result in a direct smear does not exclude tuberculosis since at least 100.000 bacilli must be present in 1 ml sputum for the reasonably ready demonstration of a positive microscopic finding *(Corper and Cohn, 1933).*

(b) phase-contrast microscopy. This is similar to Z.N. stain but with the malachite green used for counter staining.

(c) fluorescence microscopy: The tubercle bacilli are stained using the auramine 0 or Rhodamine dye and exposed to ultraviolet light. This procedure needs a special fluorescence microscope.

II- Concentration method of sputum:

The antiformin method is used if the sputum is very tenacious. The antiformin homogenizes the sputum into fluid material, so that any tubercle bacilli present can be concentrated in a centrifuged deposit. Films prepared from the deposit and stained with Z.N. may give positive results when the direct examination is negative. It must be remembered that an unexpected positive culture or smear examination in a patient whose clinical characteristics do not otherwise suggest tuberculosis may be due to a laboratory error such as contamination or a mistake in the name of a patient *(Crofton & Douglas, 1981).*

III- Culture of Mycobacterium Tuberculosis:

There are many culture media available. Currently the most popular medium makes use of the buffered egg-potato formula of Lowenstein Jensen. Other media containing egg base such as egg-yolk potato flour medium of the *(American Trudeau Society 1944)*, and Petragnani medium. Selective media containing antibiotics to inhibit non acid fast bacteria, such as Middlebrook's Oleic acid agar medium commonly known as 74-10 agar *(Dubos and Middle-brook, 1947)*. Body fluids and homogenized sterile tissues should also be cultured in liquid Tween albumin medium. The cultures should be incubated at 37°C preferably in an atmosphere of 5-10% carbon dioxide and examined at weekly intervals. Slant is not to be discarded for at least 8 weeks and they may be kept longer if facilities permit since an occasional culture becomes positive only after 8 weeks. It is recommended that after each examination all cultures with visible growth can be exposed to light for approximately one hour to allow any photochromogenic strain to develop their pigmentation. Colonies of M. tuberculosis can be noticed by an experienced technician. Colony morphology, rate of growth, pigment production, biochemical reactions can distinguish mycobacterium tuberculosis from other mycobacteria. Biochemical reactions include niacin production, catalase activity, nitrate reduction, aryl sulfatase and Tween 80 hydrolysis, *(Wayen et al., 1964)*.

Esmat and E-Maraghy (1975) recorded the results of culture as follows:

- +++ confluent growth.
- ++ innumerable colonies but growth not confluent
- + 20-100 colonies.

Count if possible the number of colonies and write it down after the symbol. The actual number of colonies if less than 20. there is

- 0 no growth.

IV- Animal inoculation:

The specimen which is, decontaminated injected subcutaneously in the thigh. of 2 guinea pigs. One is killed after 4 to 6 weeks and examined for picture of tuberculosis. The other is left alive under observation for a further period. Internal lesions which to be examined are, liver, spleen, and lymph nodes, beside microscopical examination of these organs. Guinea pigs are especially useful for sputum or urine specimens repeatedly contaminated on culture, for specimens consistently positive on microscopy but which yield negative cultures, for resected lung lesions and for pleural and spinal fluids.

V- Other investigations include:

* Gastric lavage: This gives more positive results in patients who can not collect sputum samples.

* Bronchial lavage: This give more positive results than gastric lavage, but is not recommended owing to its danger to

the operator. Aspiration at bronchoscopy, carried out for diagnosis is sometimes indicated, *(Crofton and Douglas, 1981)*, in contrast to *Kvat et al, (1979)* who reported that bronchoscopic examination and culture of bronchial washing are not the best sources for diagnosis of pulmonary tuberculosis because of the inhibitory effect of local anaesthetic upon the growth of M. tuberculosis and that culture of sputum or gastric washing are usually sufficient. They contended that this practice proved costly and the diagnostic yield was extremely low.

* Laryngeal swab is, an alternative to gastric aspiration.
* Transtracheal aspiration.
* Haematological examination includes a total and differential leucocytes and erythrocyte sedimentation rate.

Tuberculin test:

It is an allergic skin test used in the diagnosis of tuberculous infection. It is mediated by specifically sensitized small T-lymphocytes which interact with mycobacterial antigen to produce characteristic reaction. The outcome of combination with the antigen is the release of active factors called lymphokines, which result in typical cellular reaction.

Tuberculin hypersensitivity was first demonstrated in (1891) by Robert Koch. During his experiments with tuberculosis, he showed that ginea pigs infected with M, tuberculosis some two or more weeks earlier, reacted differently from uninfected ones to the subcutaneous reinjection of virulent

living tubercle bacilli, where at the site of inoculation a massive inflammatory reaction developed within two days. Extension to regional lymph nodes either delayed or did not occur. The normal animals infected with similar material developed progressive tuberculosis. He also demonstrated that these changes could be produced by dead as well as living microorganisms. A bacteria free protein fraction extract prepared from these organisms is known as (Koch old tuberculin). Later tuberculin test has been extracted in a pure state and is known as the purified protein derivative (P.P.D.) one unit of P.P.D. is equivalent to 0.1 ml of 1/10.000 dilution of the koch old tuberculin. PPD was prepared by *(Seibert and Clenn, 1941).*

Methods and results of tubercuin test:

1- The Mantoux Test, is the most accurate and commonly used by intradermal injection of 0.1 ml of a suitable dilution of the tuberculin in the forearm. A common practice is the use of 3-5 units of tuberculin, if no reaction appears, the test is repeated with 100 T.U. strength. A positive reaction results in an area of red induration not less than 9 mm in diameter which appears in two to three days after the injection. Non specific reaction may occur earlier and disappear rapidly and so the best time for reading is 72 hours after injection. Non indurated erythema is non significant.

Esmat et al., (1971) tried to find the most suitable dose of tuberculin that can detect the biggest number of infected individuals with minimal hazards of severe local reactions. They suggested that the one unit test is more suitable than the two units test for the dispensary attendants. This was because the one unit test did not produce so mueh severe reaction as that produced by the two units. It is also suggested that the two units should be available in the clinics in order to test cases who are negative with one unit and in whom tuberculosis is suspected.

Hosny and Associates (1973), in a survey project of tuberculosis in the Arab El-Mohammady, a slum East of Cairo studied the proportion of positive tuberculin reactors in different age groups in that region. In infants below the age of one year, the number of positive reactors in the non vaccinated was zero while 2.65% and 9.39% were positive reactors in the age group 1-5 and 5-10 years respectively. They concluded that BCG vaccination could be recommended up to the age of 5 years without tuberculin testing. Further, they said that above the age of 15 years, mass miniature radiography seemed to be more important in case finding than tuberculin test as above that age tuberculin positivity in non vaccinated groups ranged between 20.68 and 88.01%. They also mentioned that those who showed a high tuberculin reaction exceeding 15 mm in diameter had the highest percentage of radiological pulmonary abnormalities ranging from 38 to 52%. Below 5 years of age the intensity of

tuberculin reaction did not correspond with radiological changes. Radiological abnormalities were commoner above the age of 20, particularly in the age group of 20-60 years.

Graded test: Positive results with 3-5 T.u is regarded as an indication of recent or active focus than positive with 100 T.u. Negative results mean a very old healed focus or no infection at all. But false negative results can be obtained in a variety of cases. The following are worth mentioning.

- Pre allergic state which is 2-10 weeks after infection.
- The state of anergy, showed by some patients with severe infections as milliary, meningeal and near-terminal tuberculosis.
- Some cases with lowered cell mediated immunity as in cases of neonatal thymectomy, irradiation, antimetabolites, corticosteroids, sarcoidosis, hodjkin disease and lymphomas.
- Some cases with temporary loss of skin reactivity such as measles, mumps and influenza vaccination.
- It is also seen in patients with uraemia and alcoholic cirrhosis of the liver.

Pagel et al., (1964) mentioned that a negative reaction may occur in the following circumstances, despite the presence of definite tuberculous infection.

1- The test may be negative in a small percentage of patients with clinically active disease. These patients usually have tuberculosis in advanced form such as tuberculous meningitis as severe dissiminated disease with hepatic and splenic involvement.

2- The pre-allergic period must be kept in mind when there has been recent contact. Infection cannot be excluded unless the test is repeated at least 6 weeks after contact with a source of infection has ceased.

3- A number of conditions may cause non-reactivity despite the presence of a tuberculous infection. In practice, only the following need to be considered:

(i) severely cachectic patients or some with advanced generalised or meningeal tuberculosis.

(ii) Certain acute infections: measles, infectious mononucleosis, chicken pox and diphtheria.

(iii) When sarcoidosis supervenes upon tuberculosis, the tuberculin reaction may revert to negative.

4- Certain drugs may influence the response to tuberculin such as corticosteroid and ascorbic acid also decrease the cutaneous reaction to tuberculin in both tuberculous patients and in guinea pigs.

5- Healed tuberculous foci may be present in which all pathological activity has ended. Complete absence of activity is difficult to establish clinically and in patients bearing apparently healed foci, complete absence of reaction to 100 Tu is uncommon.

II- Other methods: *Baum,(1974)*include:

(a) Multiple puncture test: (Heaf test). The reactions of this test are graded as follows:

- Grade I shows at least 4 separated papules.
- Grade II papules are confluent to form a ring.
- Grade III the ring is filled in the center and induration may spread outward from the ring.

The advantage of this test, are that it is readily tolerated by young children, it requires little skill and the concentrated tuberculin keeps well, *(Crofton and douglas, 1981).*

(b) Patch test: (Vollmer's test):

It is used by pediatricians : a strip of adhesive plaster carrying a piece of gauze soaked in tuberculin and allowed to dry is applied to the skin of the back. A positive result is shown by a red papulo-vesicular eruption within 48 to 96 hours after application. It is not recommended as it gives false positive and negative results.

(c) Tuberculin test: using disposable plastic units with four teeth impregnated in dried tuberculin. It is placed on the forearm with a firm downward pressure until the prongs pierce the skin. Mantoux test is preferable to all other tuberculin tests.

Limitations of tuberculin test;

The clinical significance of tuberculin test is limited by several obstacles. The following are examples:

1- The test represents the sensitivity of the skin. So depression of the allergic state of the skin gives a negative result. The allergic fallacies in an infected individual should be excluded.

2- Fallacies encountered due to inappropriate administration and due to in accurate or small dose.

3- The time needed to pass between application of the test and return of the patient in order to assess the result allows the leak of some patients who find no time to return, in order to be assessed.

4- Mass BCG vaccination rendered the test invalid, for the conclusive diagnosis of tuberculous infection, it is only exclusive if it is negative, quoted from *(Rizk, 1981).*

SEROLOGICAL DIAGNOSIS OF PULMONARY TUBERCULOSIS

A serological test that would indicate active tuberculosis with reasonable precision wouldlbe of great value in the numerous conditions in which tuberculosis is part of the differential diagnosis and in the diagnosis of diseases of internal organs, that are difficult to assess such as the spine or the gut. *(Reggiardo et al., 1981).*

The first report of the serodiagnosis of tuberculosis was probably that of *Arloing which was published in (1898)* only 16 years after Koch's identification of the tubercle bacillus. Arloing developed an agglutination test and reported that serum from patients with pulmonary tuberculosis was positive and serum from 11% of healthy control subjects and patients with other illnesses was positive, *(Daniel & Debann 1987).*

Duboczy and White, (1969), studied the direct latex agglutination test using active tuberculoprotein antigen. The test was applied to serum specimens of 197 patients with active tuberculosis. Agglutination occurred with the antigen in 96.9% Among 84 cases of inactive tuberculosis, antibodies were demonstrated in 38.5%, and in 23.7% of 72 patients with cutaneous reactions to tuberculin and no demonstrable disease. Among 33 patients with no reaction to tuberculin and no manifest pulmonary disease, one serum gave an agglutination with a titre of 8. In progressive tuberculous disease, the agglutination titre usually increased and gradually decreased

with improvement of the disease. When the inactive status was reached, the agglutination titre returned to normal in approximately one third of the patients. The test contributes additional data of aid in the differentiation of active and inactive tuberculosis.

A soluble antigen fluorescent antibody test was used for serodiagnosis of tuberculosis in both human and non human primates. This test, when used with various serologically active isolates from mycobacteria, showed promise in detecting and measuring antibody concentration in serum. The test might be superior to currently used intradermal tuberculin skin test. With further purification of the antigen , the soluble antigen fluorescent antibody test might be useful for identifying the specific stage of the disease and for appraising the efficacy of therapy, *(Affronti et al., 1973).*

Fluorescence microscopy method can be used to facilitate and enhance rapid examination of smear for mycobacterium tuberculosis while still maintaining a high degree of accuracy. The marked contrast of selfluminous bacteria against the dark background makes it possible to see the bacteria with less magnification than required by bright methods. Low power objectives are used, permitting inspection of a large area in a short period of time. Other advantages are better contrast, minimal eye strain. Micropist fluorescence microscopy method

could be used in routine laboratory work for examination of direct smears negative by Z.N stain.

The only disadvantage of this method is higher cost *(El-Sayed, 1982).*

Nicholls, (1975) described a simple whole cell agglutination test for tuberculosis in which H37 Ra was used as antigen. The test gave positive results in 59 of 63 cases of mycobacterium tuberculosis, and in 4 of 11 cases due to other mycobacteria. Negative results were recorded in 168 of 171 control subject. A positive result was recorded when the agglutinin titre was 1/125 or more.

Winter and Cox, (1981) used Mycobacterial antigens derived from whole cells and cell walls of M. tuberculosis and M. bovis (BCG) and soluble PPD in a solid phase radioimmunoassay to measure the amount of reactive IgG antibody in serum from patients with active tuberculosis, patients with inactive tuberculosis, healthy subjects who were skin test positive to PPD and healthy persons who were PPD skin test negative. The patients with active tuberculosis had statistically larger amount of IgG antibody to M. tuberculosis whole cells, cell walls PPD and to BCG whole cells and cell wall when compared with the amount of antibody in serum from healthy subjects who were PPD skin test negative. However, no significant differences were detected in the mean antibody response between patients with active disease and those in clinical remission. Moreover,

significant amounts of antibody were detected in 7 to 20% of healthy tuberculin-reactive subjects. On the basis of these results it is unlikely that antibody assays alone will prove useful in the diagnosis of this disease.

Reggiardo, et al., (1981) studied the haemaglutination test using three serologically active mycobacterial glycolipids Al, B1, and C1,2,3 as antigens on serum from patients with pulmonary tuberculosis and from healthy contacts of patients with tuberculosis. A positive response to any of the three antigens was found in 82.5% of patients with newly diagnosed disease and 21% of contacts. The higher proportion of positive results in the contacts than in other groups of healthy subjects was previously reported. Serial positive titre during one year of chemotherapy showed an initial slight increase during the first month and then a slow decrease, although conversion from serologically positive results to negative results was uncommon. The occurrence of widely variable patterns of response to the three antigens in different patients emphasizs the importance of using a battery of tests, each with a separate antigen.

SERODIAGNOSIS OF PULMONARY TUBERCULOSIS BY ENZYME-LINKED IMMUNOSORBENT ASSAY "ELISA"

Rapid diagnosis of active tuberculosis is essential for the management and control of this disease. Clinical diagnosis of tuberculosis relies mostly on bacteriological examination and poses serious problems in a patient in whom presence of acid fast bacilli cannot be demonstrated. *(Bhattacharya et al., 1985).*

In (1972), Engvall and Perlman described the highly sensitive and reproducible technique of Enzyme linked immunosorbent assay. This technique does not require sophisticated instrumentation and the reagents that it employs are inexpensive. It has been widely used for the serodiagnosis of infectious diseases.

Enzyme linked immunosorbent assay is a sensitive, reproducible and reliable test for diagnosis of infectious diseases as it measures the binding of antibody to antigen which is fixed on to a solid phase absorbent, often the inner surface of a plastic tube or of a plastic microtiter plate well, *(Daniel and Debanne, 1987).*

The introduction of enzyme linked immunosorbent assay technique has resulted in a resurgence of interest in the development of serodiagnostic tests for active tuberculosis. It has been particularly interested in using ELISA in developing

countries with high tuberculosis prevalence and has also found ELISA to be sensitive and very specific in studies performed in Argentina and China using mycobacterium tuberculosis antigen 5 prepared by immunosorbent affinity chromatography *(Daniel et al., 1986).*

The final step in all ELISA procedures is one in which an enzyme, the quantity of which is determined by the amount of antibody present in the system, is allowed to react with its substrate with development of colour. All such reactions are time and temperature dependent. Thus there is introduced into every ELISA reaction a large opportunity for unintentional observer bias resulting from variation in the conditions of the final reaction. Additional error may be introduced by variation in washing technique. All evaluation of proposed ELISA tests must be done blinded with both positive and negative serum samples simultaneously. Furthermore every microtiter plate should contain appropriate positive and negative controls. *(Daniel & Debanne, 1987).*

Nassau et al., (1976) used ELISA for detection of antibodies to mycobacterial culture filtrate in patients with tuberculosis and control subjects. There was considerable overlap between patients and control values. The reproducibility of the assay was good.

Grange et al, (1980) reported high ELISA IgG antibody titres in tuberculous patients, but there was insufficient

discrimination between patients and control subjects. They used crude sonicates of M. bovis (BCG) strain, M. Kansasii and M. Smegmatis as antigens.

Zeiss and his Colleagues (1982) measured IgG antibody directed against M. tuberculosis PPD by both radioimmunoassay and ELISA in patients with active pulmonary tuberculosis and healthy controls with known tuberculin skin test reaction. There was a sharp delineation between patients and skin test positive and negative values. The correlation between radioimmunoassay and ELISA results was excellent. The study was not prospective, but the serum samples were coded. In subsequent studies Kalish et al. *(1983)* found that the IgG antibody was significantly greater in patients with active pulmonary tuberculosis than in patients with other pulmonary diseases or with inactive tuberculosis.

Benjamin and Daniel (1982) studied the serological response to purified mycobacterial antigen 5 using an enzyme linked immunosorbent assay in 75 patients with pulmonary tuberculosis and 150 control subjects. The sera from the patients group had significantly higher IgG antibody concentration than infected control subjects with good specificity. In patients living in low prevalence areas where the results of skin tests in the majority of the population are negative, an antibody titre greater than or equal to 21:40 would have a 95.8% specificity for active disease. For patients living in

high prevalence areas where the results of skin tests in the majority of the population are positive, an antibody titre greater than or equal to 1:40 would have a 79.9% specificity for active disease, serum from patients with active non tuberculous mycobacterial infection had intermediate titres.

Faves et al., (1966), noted a decrease in IgG antibody titres to PPD throughout the period of treatment of tuberculosis.

Kaplan and Chase (1980) demonstrated increases in antibody levels after the initiation of chemotherapy for tuberculosis. In contrast *to Kalish et al.,(1983)* studies, sera from patients with active tuberculosis was obtained before the initiation of therapy; antibody levels were not determined after the initiation of therapy.

Daniel et al., (1981) reported higher antibody titres to four mycobacterial antigens in patients who received more than four weeks of therapy for tuberculosis as compared to those in patients treated for a shorter period of time. Antibody titres increased during the first 3 months of chemotherapy, *(Daniel and Debanne, 1987).*

Kalish et al. (1983) reported that sera from patients with active pulmonary tuberculosis and pulmonary diseases frequently mimicking tuberculosis were assayed for IgG antibody activity to purified protein derivative by an enzyme linked immunosorbent assay. Patients with active pulmonary tuberculosis had a

significantly greater mean level of antibody than had patients with a typical tuberculosis ($P < 0.005$), sarcoidosis ($P < 0.0001$), histoplasmosis ($P < 0.004$), blastomycosis ($P < 0.008$) or cryptococcosis ($P<0.017$), patients who had received bacille calmette Guerin vaccination ($P < 0.003$) or who had a history of treated tuberculosis ($P < 0.003$) and PPD skin test positive and skin test negative control subjects ($P< 0.001$). This may have a potential use as a rapid diagnostic aid in evaluating patients with suspected pulmonary tuberculosis. The results of this study demonstrate the ability of the ELISA to distinguish patients with active pulmonary tuberculosis from patients with other pulmonary diseases, from patients with a history of treated tuberculosis from PPD skin test positive or negative and from patients who have received BCG vaccination. BCG vaccination does not elevate the level of IgG antibody to PPD as measured by ELISA. This technique may be useful in evaluating patients previously vaccinated with BCG who are suspected of harboring active tuberculosis.

Kalish et al., (1983) used PPD as the antigen because of the favorable results. Other investigators have previously used PPD as it is readily available. In contrast to detecting antibody to specific antigenic components of M. tuberculosis, they expected that the multiple antigens present in PPD would more likely to detect the presence of antibody and thereby make the ELISA more useful as a rapid screening test. The results of *Kalish et al., (1983)* indicated that the measurement of antibody to PPD

antigen by ELISA technique detected a high percentage of patients with active pulmonary tuberculosis. However a few infected patients have negative or indeterminate "ODI" values. There are several explanations which account for the failure of ELISA to detect antibody. Antibody may be bound in circulating immune complexes which have recently been demonstrated in many patients with tuberculosis. An Alternative explanation may be in the increased percentage of suppressor T-lymphocytes that has been demonstrated in patients with acute tuberculosis *(Katz et al., 1979)* which decreases the activity of B-cells and partially accounts for low levels of antibody observed in some patients with active tuberculosis.

Participation of humoral immunity in tuberculosis has been widely recognized and utilized in different serologic diagnostic procedures for tuberculosis. *Viljanin. et al., (1982)* measured IgG, IgM, and IgA antibody to PPD in sera from 44 patients with tuberculosis and 35 healthy controls. Their results were presented only as group mean.

The levels of both IgG and IgM antibody were significantly higher in patients than in controls. The level of antibody was correlated with the extent of disease and significant elevation of IgA antibody was found only in the sera of patients with extensive disease. *Kardjito et al.,(1982)* found both IgG and IgM but not IgA antibody to be detected in patients with

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* Optical density index.

tuberculosis. However the addition of IgM antibody determination to that of IgG added only 2 extra positive results among 107 patients. In this study, a sonicate of BCG was used as antigen. While the test was specific with respect to tuberculin negative control subjects, many tuberclin positive healthy persons had antibody level similar to those found in tuberculosis patients. Prior BCG vaccination and size of tuberculin reaction showed no correlation with ELISA results.

Zeiss et al., (1984) studied the diagnostic utility of an enzyme-linked immunosorbent assay "ELISA" in hospitalized patients with suspected pulmonary tuberculosis. A positive culture for. M. tuberculosis identified active disease and 3 negative cultures and smears defined the negative group. IgG antibody activity was determined by adding a 1:1000 dilution of serum to plates coated with PPD antigen. Alkaline phosphatase labeled anti-IgG was added, colour developed, and an optical density index (ODI) was determined. The pulmonary tuberculosis group had a mean "ODI" of 0.77 which was higher than in patients without pulmonary tuberculosis. For patients with pulmonary tuberculosis group, ELISA had a sensitivity of 67% and a specificity of 79%.

Balestrino et al. (1984) studied IgG antibody to M. tuberculosis antigen 5 and PPD was measured by enzyme linked immunosorbent assay in serum samples from patients with active pulmonary tuberculosis and non tuberculous control

subjects. The mean titre for the tuberculosis patients was 74.6 with antigen 5 and 99.5 with PPD. In control subjects the mean titre were 3.6 and 15.6 respectively. Titres were not related to tuberculin reactor status or prior to BCG vaccination. At a serum dilution of 1:40, ELISA with antigen 5 had a sensitivity of 81.4% and a specificity of 9.34% for tuberculosis. At 1:40, ELISA with PPD showed a sensitivity of 82.6% and a specificity of 54.9% for tuberculosis. ELISA using antigen 5 would correctly classify 93.2% of persons and ELISA with PPD 55.5%. At a serum dilution of 1:80 accuracy is increased to 99.3% with antigen 5 and 83.3% with PPD, but sensitivity decreases to 64.0% with antigen 5 and 72.1% with PPD. Thus antigen 5 is more accurate than PPD for the diagnosis of tuberculosis using ELISA. In this study antigen 5 and PPD were used because the antigen 5 is a highly purified and well characterized cytoplasmic protein, and the other PPD is readily available and has been used by other investigators in ELISA serodiagnostic test for tuberculosis *(Balestrino et al., 1984).*

Daniel et al. (1986), reported that an enzyme linked immunosorbent assay evaluated as a serodiagnostic test for active tuberculosis. ELISA was compared with sputum smear in persons presenting to the. Instituto de Torax and was used for screening in 1,458 personnel. The test was performed under field conditions on 4.ul samples of capillary blood obtained by finger prick. ELISA was found to have a sensitivity of 69% and a specificity of 88%. Smear had a sensitivity of 79% and a

specificity of 100%. ELISA was found to have an undiminished sensitivity and specificity in patients who were sputum negative. ELISA led to the diagnosis of tuberculosis in 5 of 1,458 soldiers tested in the screening program.

Among patients with pleurisy and effusion ELISA was positive in 4 out of 7 patients with tuberculosis and in 1 out of 4 patients in whom a specific diagnosis could not be made. These results, do not differ significantly from those obtained in the larger study population of patients with pulmonary tuberculosis. *(Daniel et al., 1986).*

Antigens used in ELISA:

*M. tuberculosis antigen 5 was prepared by immunosorbent affinity chromatography (Daniel and Anderson, 1978). This preparation of antigen 5 gave a similar immunoelectrophoresis pattern to other batches and was found to be homogeneous protein of high purity. It was used for sensitization of ELISA microtitration plates and a concentration of 5 mg/litre in distilled water. *Balestrino et al.,(1984)* used antigen 5 in their study because of its a highly purified and well characterized cytoplasmic protein.

* P.P.D was prepared using the ammonium sulphate method of *(Seibert & Glenn 1941)* from culture filtrates of M. tuberculosis H37 Ra, grown on a synthetic medium. Sterile filtrates were autoclaved at 120°C for 30 minutes and then clarified by filtration. Ammonium sulphate was added at 4°C in

quantity of 297 g/litre. The mixture was centrifuged and the supernatant decanted. The precipitate was centrifuged and the supernatant decanted. The precipitate was then taken up in sodium chloride. This precipitation was repeated 5 more times and the final product was dialysed against distilled water to a concentration of 10 mg/litre for sensitization of microtitration plates. *Balestrino et al.,(1984)* used PPD antigen because it is readily available and has been used by other investigators in ELISA serodiagnostic tests for tuberculosis. The antigen 5 is more accurate than PPD for the diagnosis of tuberculosis using ELISA. PPD contains substantial amounts of mycobacterial polysaccharide wich may contribute to species non specificity in imunologic reaction. It is however more readily available than more highly purified materials and it has been studied extensively as an antigen in ELISA serodiagnostic tests, *(Daniel and Debanne, 1987).*

* M. tuberculosis antigen 6 has been used in ELISA. It has the advantage of being relatively stable under conditions of storage and shipping. This antigen has an additional importance because it can be used to examine patients with extrapulmonary tuberculosis in whom the diagnosis by bacteriological means is often difficult, *(Stoebel et al., 1982).*

* *Reggiardo and Colleagues (1980)* developed ELISA using serologically active mycobacterial glycolipids (SAG) A1 B1 and complement. When these ELISA tests were compared with

haemoglutination tests using the same antigens, specificity was very high to all 3 antigens. Sensitivity was also high reaching 0.913 with SAGA1. There was excellent agreement with results in the same serum tested with passive haemaglutination using the same antigens.

* *Krambovitis (1986)* established an ELISA detecting IgG antibody to an antigen extracted from mycobacterial cells with buffer containing a detergent and considered to be a plasma membrane antigen. This extract may have contained several soluble mycobacterial antigens.

ELISA sensitivity appears to be highest when used in studies conducted in areas of high tuberculosis prevalence, tuberculosis prevalence is highest in less developed areas of the world and in those regions where patients may have presented for diagnosis with more advanced disease than typically seen in areas where there is less tuberculosis. The sensitivity of ELISA may prove to be greatest in third world countries with high tuberculosis prevalence, because of its low cost. ELISA may have a role in such countries, but in this situation the diagnosis of tuberculosis is often readily made by sputum smear. Conversely, ELISA may be least sensitive for diagnosis in patients with minimal disease in areas of low prevalence, patients in whom the diagnostic problem is often great *(Daniel and Debanne, 1987).*

MATERIAL AND METHODS

MATERIALS AND METHODS

Cases:

Three groups of patients were included in this study

Group I:

Included 50 adult patients of active pulmonary tuberculosis proved by constitutional symptoms, clinical, radiological and laboratory findings of pulmonary tuberculosis. All of those patients had positive sputum for acid fast bacilli by direct microscopy using Ziehl- Neelsen stain and culture techniques.

Group II:

Included 20 adult patients of suspected pulmonary tuberculosis with clinical and radiological findings compatable with pulmonary tuberculosis but had a repeatedly negative sputum for acid fast bacilli by direct microscopy and culture techniques.

Group III:

Included 20 normal persons of age matched apparantly healthy individuals as a control group were similarly investigated but had no clinical radiological and laboratory evidences of pulmonary tuberculosis. This group included 6 positive tubericulin reactors and 14 negative tuberculin reactors.

All the cases included in this study both in the tuberculous groups and the control group were either admitted in the chest department of Tanta TB center or they were attending the

Out Patient Chest Clinic during the period between July 1991 and August 1992.

The age groups are matching in the control subjects (20) and the different groups of patients with pulmonary tuberculosis(50+20) and they were 58 males and 32 females.

Sera:

For every case of patients and control group, about 5 ml venous blood was drawn under complete aseptic technique and serum was separated and stored at - 20°C till assayed for the presence of Immunoglobulin G specific antibody to purified protein derivatives of Mycobacterium tuberculosis using the Enzyme Linked. Immunosorbent Assay (ELISA).

MATERIALS

(A) Ingradients:

1) Antigen ;

Purified protein derivative (P.P.D) (Rt23 stock solution containing 2 T.U. /0.1 ml obtained from Viterinary Institute of Vaccines and Researches. Abbassia , Cairo, was used as an antigen for ELISA test.

2) Carbonate buffer:

Consists of 1.59 gm Na $2CO_3$ and 3.93 Na HCO_2 in one litre of distilled water with 1% sodium azide as a preservative. The solution has a pH of 9.6

3) Phosphate buffered saline (PBS):

Consists of Na Cl (8.00 g/L), K_2 HPO_4 (1.21 g/l) and KH_2 PO_4 (0.34 g/L). The solution has a pH of 7.3 and also provides potassium and phosphate ions. It is a useful general diluent and suspending fluid.

4) 5% Bovine serum albumin (BSA):

To prevent non-specific binding It consists of 5 gm BSA in 100 ml PBS (Rinderalbumin, Biotest- Serum Institute Gmbh, Frankfurt/M., W. Germany).

5) Alkaline phosphatase:

Conjugated goat antiserum to human IgG and IgM.

6) Substrate tablets:

Consist of p-nitrophenyl phosphate.

7) Substrate buffer:

Contains 100 ml of diethanolamine and 102 mg of $MgCl_2.6H_20$ (0.5 m mol) per liter of distilled water. PH adjusted to 9.8.

8) Dilution buffer:

Consists of sterile phosphate buffer pH 7.2 with the addition of 40 ml of tween 20,0.2 gm of sodium azide and 50 ul bovine protein per liter.

9) Washing solution :(concentrate)

Consists of sterile, 20 (concentrated) phosphate buffer ph 7.4 mixed with 200 ml of Tween 20 per liter.

10) Stopping solution : consists of 3 N NaOH.

The reagents number 2,3 and reagents from (5-10) were obtained from Behring WerkAG, Marburg, W. Germany.

(B) Equipment:

1) Flat bottom polyethylene

Plastic microtiter plates (Titrerik, cat No. 76-200-05).

2) ELISA Behring Processor II.

3) Automatic micro- pipettes and its tips.

METHODS

Technique of ELISA test: *(Radin et al, 1983)*

(A) **Coating of the wells:**

1- A working dilution of P.P.D. solution was prepared by adding 1 ml of stock P.P.D. solution (2 mg/ml), to 9.0 ml of carbonate buffer, pH 9.6 to obtain P.P.D. concentration of 200 µg/ml.

2- To each well, 0.2 ml of working P.P.D. solution (containing 40 ug of P.P.D.) was added and incubated for one hour at room temperature and left overnight at refrigerator at 4°C.

3- The following day, the plates were emptied completely by inversion, flooded with PBS and left for 3 minutes then emptied, this washing step was repeated 3 times.

Blocking of the plates: The plates were blocked to prevent nonspecific binding by flooding with 5% bovine serum albumin (BSA) in phosphate buffer saline (PBS) for one hour at room temperature. The bovine serum albumin was discarded and the wells were dried by shaking and they are ready for immediate use or stored in sealed bags at 4°C for several weeks until used.

(B) **Serum dilution:**

For assay of IgG antibody activity the serum samples were thawn to reach the room temperature, then heat inactivated (by heating the samples at 56°C for 30 minutes) and diluted 1:1000 in 5% BSA.

(C) Test procedure:

1- The plates were properly labelled to contain 2 wells for substrate control and 2 wells for conjugate control and duplicate wells for each tested serum sample.

2- 200 µl of 1:1000 serum dilution for each serum sample were added to duplicate wells and 200 µl of PSA in PBS were added for 2 wells of substrate control and 2 wells of conjugate control. The plates were incubated at room temperature for 2 hours.

3- The wells were evacuated and washed 3 times using washing buffer.

4- 200 µl of alkaline phosphatase conjugated antihuman IgG that had been diluted 1:100 in 5% BSA & PBS were added to each well except for substrate control. 200 µl of 5% BSA were added.

5- The plates were incubated at room temperature for 2 hours, then emptied and washed three times using washing buffer.

6- Colour reaction of alkaline phosphatase enzymatic activity was assayed by adding 200 µl of substrate solution to each well. The substrate solution was prepared by dissolving 2 substrate tablets (P-nitrophenyl phosphate) in 10 ml of substrate buffer (sufficient for one plate), just before use. The plate was incubated for 30 minutes at 37°C then the

reaction was stopped by the addition of 50 µl stopping reagent (3 N NaOH) to each well. the optical density (O.D.) was determined by ELISA Behring processor at wave length 405 against air.

(D) Reading of the test:

Serum sample was considered positive for IgG specific for P.P.D. if the mean optical density (O.D.) of the two wells was equal to or more than the cut off value of 20 serum samples of the control group.

- Cut off value determined by calculating the mean (O.D) of control subjects plus 0.050.

STATISTICAL ANALYSIS OF THE DATA

Statistical analysis of the result was carried out according to the conventional standard statistical procedures:

1) ***The mean value (X)*** is the sum of all observations divided by the number of observation.

$$X = \frac{\Sigma x}{n}$$

Where :

X = sum of individual observations.

n = number of observations.

2) ***The standard deviation (SD)*** was calculated from the formula:

$$SD = \sqrt{\frac{\Sigma(x-x)^2}{n-1}}$$

3) ***The standard error (SE)*** was calculated according to the formula

$$SE = \frac{SD}{\sqrt{n}}$$

4) ***"t"*** test was used to test the significance of the difference between two means. The following formula was used

$$T = \frac{X_1 - X_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

t value was compared with its tabulated probability value at 0.05 to determine the degree of difference that two sets of observation show in term of a p value.

The table of the t distribution is entered at (n-1) degree of freedom (number of pairs minus 1).