ISOLATION AND PURIFICATION OF SPECIFIC ANTIGEN IN TUBERCULOSIS AND ITS USE IN IMMUNODIAGNOSIS

THESIS

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BY

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CERTIFICATE

I certify that Mr. K. Ramesh Kumar had been working in the Department of Biochemistry of Government Medical College Thrissur under my guidance and he had completed his requirements for the submission of his Ph.D thesis on "ISOLATION AND PURIFICATION OF SPECIFIC ANTIGEN IN TUBERCULOSIS AND ITS USE IN IMMUNODIAGNOSIS".

This is also to certify that this thesis is a bona fide record of research work done by the candidate during the period of study under my guidance, and it has not previously formed the basis for the award to the candidate of any degree, diploma, associateship, fellowship or other similar title.

I also certify that this thesis represents independent work of the candidate supervised by me during the course of the study.

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Abbreviations used

AbTb	-	Abdominal tuberculosis
AFB	-	Acid-fast bacilli
APS	-	Ammonium per sulphate
ATT	-	Anti tuberculosis treatment
B&J TB	-	Bone & joint tuberculosis
BCG	-	Bacille Calmette Guerin
BSA	-	Bovine serum albumin
CIC	-	Circulating immune complex
CNBr	-	Cynogen bromide activated
CSF	-	Cerebrospinal fluid
DAB	-	Diamino benzidine
DEAE	-	Diethyl amino ethyl
DOTS	-	Data on tuberculosis survey
ELISA	-	Enzyme linked immunosorbent assay
FIA	-	Freund's Incomplete adjuvant
FPLC	-	Fast-protein liquid chromatography
GPL	-	Glycopeptidolipid
HPLC	-	High pressure liquid chromatography
HRP	-	Horse radish peroxidase
LAM	-	Lipoarabinomannan
LJ	-	Lowenstein-Jensen medium
LPS	-	Lipopolysaccharide
MAC	-	Mycobacteriuim avium complex
MGIT	-	Mycobacteria growth indicator tube
MOAb	-	Monoclonal antibody
NCP	-	Nitrocellulose membrane
OT	-	Old tuberculin
PAGE	-	Polyacrylamide electrophorosis.
PCR	-	Plymerase chain reaction.
PEG	-	Poly ethylene glycol
PPD	-	Purified protein derivative
PTS	-	Pulmonary tuberculosis serum
RIA	-	Radio-immunoassay
SDS	-	Sodium dodecyl sulphate
TBLN	-	Tuberculous lymphadenopathy
TBM	-	Tubercular meningitis
TBM	-	Tuberculous meningitis
TEMED	-	N,N,N',N'-tetramethyl ethylene diamine
TPS	-	Tuberculosis patient pooled serum
TSP	-	Triton X-100 solubilised protein

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INTRODUCTION

Tuberculosis remains as one of the major infectious diseases of mankind. The International Union against Tuberculosis and Lung Diseases estimated that about 8 million new cases of tuberculosis occur annually, about one-half of these being infectious (143). Majority of these cases are believed to be due to the reactivation of latent infection. But the proportion of cases resulting from progression of new infection to reactivation of latent infection varies geographically, depending on the prevalence of infectious cases in the area. Although most cases of tuberculosis are curable, due to inadequate status of current detection and control efforts, 3 million persons die from this disease every year (54).

As per the WHO report published in March 2003 the global incidence rate of TB is growing at approximately 0.4% yearly. By the end of year 2001, 61% of the world's population lived in parts of countries providing data on tuberculosis survey (DOTS). DOTS programmes notified 2.4 million new TB cases, of which 1.2 million were smear-positive. Over 10 million patients have been diagnosed and treated in DOTS programmes since 1995 (153).

However, the 1.2 million smear-positive cases notified by DOTS programmes in 2001 represent only 32% of the estimated incidence, and the rate of progress in case finding between 2000 and 2001 was not significantly faster than the average since 1995, a mean annual increment of 137 000 cases. Globally, DOTS programmes would have to treat an extra 360 000 smear-positive patients each year to reach 70% case detection by the end of 2005. Two thirds (67%) of the additional smear-positive cases reported under DOTS in 2001 (as compared with 2000) were found in India alone (56).

Tuberculosis continues to be a major health problem in developing countries. It is estimated that approximately 3-4 million new smear-positive and another 3-4 million smear negative extra-pulmonary cases of tuberculosis develop annually (144). In India every year about 2 million develop active disease and about half a million die. The direct and indirect cost of TB to the country amounts to 12000 crore rupees per year (16).

Isolation of the tubercle bacilli, Mycobacterium tuberculosis (Mtb) by Robert Koch in 1882, marked the beginning of a new era in the history of tuberculosis. Today, the diagnosis of tuberculosis is usually established using staining and culturing techniques, and the demonstration of Mtb in clinical specimens. This is mostly done by Ziehl-Neelsen's carbolfuchsin staining of acid fast bacilli and immunoflourescence techniques. Tuberculin skin testing, using purified protein derivative (PPD) of infected persons with risk of developing disease, lacks sensitivity and specificity and requires 2-3 days for completion. Sputum smear examinations are not easy to apply under field conditions. They also lack sensitivity and specificity. Further, sputum positive cases are obtained only in pulmonary tuberculosis. In extra-pulmonary tuberculosis it is not easy to get the source of the organism. Definite diagnosis of tuberculosis requires identification of the causative organism, M. tuberculosis in culture. This usually takes 3-6 weeks for diagnosis and leads to delay in treatment.

The diagnosis of tuberculosis often poses problems, especially in children and in extra pulmonary forms of the disease. A serology test that could reliably diagnose active tuberculosis would be of immense value, not only for early diagnosis of patients but also for screening large population groups in epidemiological surveys and in detecting reactivation of disease in treated

patients. Routine use of serological tests may also provide important information regarding the pathogenesis and immune responses to tuberculosis infection.

Tuberculosis infection elicits and antibody response as in other infectious diseases. The reaction of the antigen with antibody can result in the formation of immune complexes, which can initiate an inflammatory reaction by activation of the complement pathway. Phagocytosis of immune complexes by macrophages could lead to the impairment of their capacity for further phagocytosis and also could hamper the intracellular killing mechanisms of these cells (70). Antibody formation may also modify the cell mediated immunity. Earlier reports indicated that absolute levels of immunoglobulins of IgG and IgA classes were elevated in cases of active tuberculosis. Secondary responses were observed after specific re-stimulations in individuals who have been conditioned to produce specific antibody (37, 69,133,142).

Arloing first described a technique for the serodiagnosis of tuberculosis in 1898 (5). In principle, all serology tests involve demonstration of antibodies, demonstration of mycobacterial antigen or the demonstration of immune complexes in blood or tissues of a patient. In 1976, Nassau et al (109) described the use of an enzyme-linked immunosorbent assay (ELISA) of serum antibody to mycobacterial antigen for diagnosis of tuberculosis since then, this approach has been applied by many investigators (41). ELISA is a sensitive, rapid, and reproducible technique, which can also be performed in field conditions. The measurement of IgG antibodies provides a test that has a sensitivity of approximately 50% to 60% in the areas with a low prevalence of tuberculosis and a sensitivity of

70% to 85% in areas of high prevalence. This difference in sensitivity probably relates to the extent and chronicity of tuberculosis disease at the time of diagnosis.

Serological test sensitivity and specificity depends significantly upon the antigen used in the assay. The primary approach would be to use the whole bacillus as an antigen. The results obtained with the whole bacillus as an antigen after removing the surface lipids by phenol or organic solvents were satisfactory (108) Alkali treated whole bacilli were also used in an agglutination test (12). However these tests can only detect the antigens, which are expressed on the surface of the bacillus. Concentrated sterile filtrate of autolysed, heat killed liquid cultures called "old tuberculin" (OT) and purified tuberculoprotein from OT named as "tuberculin purified protein derivative" (PPD) were also used as antigens in the serodiagnosis of tuberculosis. These preparations consist of hundred or more antigen in different stages of denaturation but many important antigens are lost due to autolysis and subsequent autoclaving of the cultures (75) various methods are followed to get a purified specific antigen from the cultures. Since protein molecules have several antigenic determinants called epitopes, it was difficult to prepare pure antigens. Some epitopes are species specific while others are shared among species and sometimes both of them may be present in the same molecule (28). This leads to the cross reactions between the different species. A single species specific purified antigen of Mtb is required to get a reliable test result in the diagnosis of disease tuberculosis.

The diagnostic usefulness of the tuberculosis antibody has to be studied on analyzing the patient serum and comparing with the available antigen

coated reagent kits. The antibodies to Mtb may be detected in healthy subjects who are tuberculin negative and in subjects who are in contact with environmental atypical mycobacteria. (11). Detection of antigen or antibody in patients especially in the smear negative or extra-pulmonary cases and in tuberculosis meningitis is useful for early diagnosis of tuberculosis.

Serodiagnosis of the tuberculosis hence requires a specific antigen or antibody in the purified form. In the present study it is envisaged to isolate one or more immunospecific antigen/s from the culture filtrate of avirulent strain of M. tuberculosis H37Ra. The isolated antigens are purified by various protein purification methods like salt fractionation, ion-exchange chromatography and molecular exclusion chromatography, and then characterized by polyacrylamide gel electrophoresis and Western blot procedures. These isolated antigens are used to raise antibodies in rabbits. The affinity purified antigen or antibodies are used to detect the serum samples or cerebrospinal fluid containing the specific antigen or antibodies. The results are compared with the polymerase chain reaction method of detecting the organism and by the existing antibody detection method. Hence it may be possible to detect the antigen or antibody in smear negative pulmonary and extra pulmonary cases especially in tuberculous meningitis patients in comparison with known smear positive pulmonary tuberculosis patients. Attempts are being made to develop an immuno diagnostic method like Enzyme-linked immunosorbent assay (ELISA) to detect antigen or antibodies in serum or cerebrospinal fluid.

The need for a purified and well-standardised specific antigen is of great importance in the study of serology of tuberculosis

infection and is necessary for accurate and early clinical diagnosis. With this object in mind the following studies were carried out.

1. Isolation and purification of specific antigen from the cultures of Mycobacterium tuberculosis.

2. Raising antibodies in rabbits and their purification by immunoaffinty chromatography and detection of immunoreactivity by Western Blot technique.

3. Detection of antibodies in serum or CSF of tuberculosis patients using purified antigen by enzyme linked immunosorbent assay techniques.

4. Comparison of the ELISA method with the existing methods.

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

Laboratory diagnosis of the tuberculosis is generally made by two methods of approach. One is the direct approach to distinguish the bacilli or its products and the other is the indirect approach to measure the humoral and cellular responses of the host against tuberculosis. Direct approach includes the microscopy to detect the acid-fast bacilli (AFB) in clinical specimens by Ziehl Neelsen's method or its modifications. The detection limit with this method requires at least few thousands of bacilli per ml of sputum. The result varies with the type of specimens, thickness of the smear, extent of decolorisation, type of counter stain used and the expertise of the personnel.

Isolation of mycobacteria from clinical samples by culture is still a gold standard. However the organism grows slowly in culture and it takes up to 4 weeks. Radiometric Bactec method is other technique to detect the mycobacterial growth using the labeled Carbon in the Palmitic acid in the selective medium. The instrument detects the presence of mycobacteria based on the metabolism rather than the visible growth. Mycobacteria growth indicator tube (MGIT), MB/BacT is the non-radiometric method to detect the metabolites of mycobacteria. Species-specific mycobacterial identification is done by various biochemical tests and molecular biological methods (62).

Serological tests are mainly possible only when there is a specific antigen, antibody or the immune complex. Mycobacteria posses' large number of antigens on the surface. These antigens of mycobacteria have been used in understanding immunological responses of mycobacterial infections. Many antigens of varying chemical composition are present in the extract of

mycobacteria. Some of the antigens may species specific, others are common to many of the species.

In 1890, Robert Koch first reported the preparation of a product named as "old tuberculin" (OT). This preparation was the sterile filtrate of autolysed, heat-killed culture fluid. Tuberculin skin testing was done using OT and later it was found nonspecific. The nonspecific reactions of OT were due to the nonpathogenic species of mycobacteria that cross-react with antigen of tubercle bacilli (12,20,21,57). Many investigators have used precipitation techniques on gels for the analysis of mycobacterial antigens. Techniques used have included single and two-dimensional immunoelectrophoresis. Many investigators used nomenclatures based upon these methods to classify mycobacterium and to identify individual mycobacterial antigens as it became available.

TUBERCULIN PURIFIED PROTEIN DERIVATIVE (PPD) AS ANTIGEN:

Seibert in 1932 (134) purified tuberculoprotein from OT by using trichloro acetic acid precipitation. This protein was called as "tuberculin purified protein derivative" (PPD). Further the PPD was purified with 50% ammonium sulphate saturation at neutral pH (135,136). PPD became the standard preparation for clinical tuberculin skin testing. Affronti (1) prepared PPD from other species of mycobacteria, and compared the cross-reactivity. PPD was also used in the laboratory investigation and studies of cell-mediated immunological responses, studies of the initiation of macrophage migration and blast transformation of lymphocytes (50).

Seibert (135) isolated antigens from mycobacteria by chemical fractionation methods. These antigens are four proteins designated as proteins A, B, C, and D and two polysaccharides I and II. Proteins A and B were capable of eliciting tuberculin reactions in man and were more potent than PPD. Polysaccharides was characterized by Birnbaum and Affronti (18) as a hetero polysaccharide consisting of arabinose, galactose and mannose. Polysaccharide II was a glucan of molecular weight 100,000 Daltons. It did not elicit delayed skin test reactions.

CULTURE SONICATES & SUPERNATANTS AS SOURCE OF ANTIGENS:

Sterile culture filtrates, usually or old, autolyzed cultures, have been used by a large number of workers. This source has the advantage of ready availability in large quantity. Moreover, it is easily handled in aqueous solutions and is relatively free of lipid and nucleic acid. Many investigators have worked with the extracts of whole mycobacterial cells or preparations of mycobacterial cell wall. Whole cells have been disrupted by a variety of procedures, including mechanical grinding, pressure, and sonication. The antigenic composition of filtrates of cultures grown under different conditions varied significantly. When bacilli were subjected to ultrasonic disintegration (82), the sonicates showed similar electrophoretic mobility.

Kniker and LaBorde (90) used more advanced fractionation methods to separate antigens by ion-exchange chromatography on diethyl-amino ethyl cellulose (DEAEC). Protein and polysaccharide-rich antigenic fractions are isolated and found these are impure. Gel filtration was able to isolate many antigens (7). Acrylamide gel electrophoresis was more sensitive and small amounts of Specific antigen could be isolate (110).

Two antigens, 19-kd each, were purified from Mycobacterium bovis culture filtrate protein extract by chromatofocusing. Antigen I showed 100% homology with the hypothetical protein Rv1174c. Antigen II showed a 100% homology with M. bovis MPB70/80. Antigen I was a hetero-dimer formed by a glycosylated, 10.5-kd, monomer and a non-glycosylated 8kd monomer with identical amino terminal sequences. Both antigens were recognized by the sera of PPD positive animals, but Antigen I did not cross react with sera of human PPD positive individuals. Antigen I was a weak inducer of lymphocyte proliferation and Interferon-gamma production. The results of their studies (8) show that M. bovis expresses a 19kd glycoprotein, homologue to the product of Mtb Rv1174c, which may prove useful for bovine TB diagnostic assays.

Stanford (139) demonstrated four groups of soluble antigens in ultrasonically disintegrated fraction of mycobacteria by immunodiffusion. Janicki et al (44,48,79-81) from independent laboratories used immuno electrophoresis to separate antigens from unheated culture filtrate of Mtb H37Rv. According to the relative electrophoretic mobility of each protein, it was numbered as 1-11. This numbering was made as reference standard and designated as United States-Japan reagents as this study was sponsored by United States-Japan Co-operative Medical Sciences, a Programme conducted through the National Institute of Allergy and Infectious Diseases. (27,43,45). Many investigators followed this nomenclature later.

Chaparas and Hendrik (28) used the reference antiserum to demonstrate the antigenic similarity of M. bovis-BCG strain, to M. tuberculosis and found that there was no significant change in the antigenic composition. Subsequently Chaparas (30) compared culture filtrates from 12 species of

Mycobacteria using immunoelectrophoresis and found that no antigen was specific for Mtb. Antigens 1,2,6,7 and 8 were found to be widely distributed among mycobacteria and 1,2 and 3 were characterized as polysaccharides. Subsequent studies conducted by a number of investigators suggested that antigen 5 might be species specific (38). Daniel and Anderson (47) isolated antigen 5 in a highly purified state by using immunosorbent affinity chromatography techniques. It had a molecular weight ranging from 28.5 kd to 35 kd, and appeared to be limited to M. tuberculosis and Mycobacterium bovis. The antigenic composition of culture filtrates varies depending on the culturing conditions. Age of the culture, time of filtration, medium used pH of the medium and temperature of incubation are important.

Two different excretory-secretory antigenic proteins of H37Ra namely, EST-DE1 (a 6% TCA soluble and DEAE anion exchange purified antigen) and ESAS-7 (50% ammonium sulphate solubilised and SDS-PAGE fractionated antigen) were studied in stick-indirect penicillinase ELISA for detecting tuberculous IgG antibodies in serum samples of pulmonary as well as extrapulmonary tuberculosis (tuberculous lymphadenopathy (TBLN), tuberculous meningitis (TBM), bone & joint tuberculosis (B&J TB), abdominal tuberculosis (Abtb) patients. The ESAS-7 antigen has shown comparatively better seroreactivity (90%) than that of EST-DE1 antigen in pulmonary tuberculosis cases. The overall specificity of 93.2% using ESAS-7 antigen was also found better compared to 86.4% obtained using EST-DE1 antigen. Further, in extra pulmonary tuberculosis group, using ESAS-7 antigen 84% (21/25) of histopathologically confirmed TBLN cases and 90% (9/10) clinically diagnosed and anti tuberculosis treatment (ATT) responded TBM cases showed positive reaction for tuberculous IgG

antibody. The percentage positivity using EST-DE1 antigen was however comparatively low in TBLN and TBM cases, (76% and 80% respectively). In histopathologically proven bone and joint tuberculosis and abdominal tuberculosis cases EST-DE1 antigen showed better sensitivity of 75% and 83.3% respectively in IgG antibody detection compared to that of ESAS-7 antigen (50% and 66% respectively). As per their study, the ESAS-7 antigenic fraction has a good potential in the diagnosis of pulmonary and certain extra-pulmonary tuberculosis infection. EST-DE1 was found to be better in detecting specific antibodies in bone & joint and abdominal tuberculosis (9).

PURIFICATION METHODS:

Ammonium sulphate precipitation, Gel filtration (106) and Ion exchange chromatography with DEAEC were used as the major fractionation procedures. Stepwise increase in ionic strength with decreasing pH led to the elution of several antigen containing peaks. Three major peaks and several minor peaks were recovered. Serological analysis revealed that most fractions were made up of one or more antigens. Numerous workers utilized the ion-exchange chromatography in fractionating mycobacterial extracts (94,95,112). Bennedsen (15) used saline extract of unheated culture filtrate on DEAE-Sephadex after 80% ammonium sulphate precipitation. Certain surface antigens were present in the eluted peaks.

Glencher et al (67) used pressure cell extracts for DEAEC, followed by SephadexG-25 chromatography. One fraction was eluted at lowest pH and highest salt concentration. Carboxymethyl cellulose cation-exchange chromatography was fractions contained multiple antigens. None of the ion-exchange chromatography studies showed fractions containing single antigen.

Many investigators used electrophorosis as a method of purification (44,53,99). Daniel and Ferguson (35) combined ammonium sulphate precipitation, gel filtration with high porocity p-300 acrylamide gel, DEAEC chromatography and Zonal electrophoresis on polyvinyl chloride. They were able to isolate 2 proteins. Yoneda and Fukin (156) were able to isolate 2 antigens alpha and beta by a combination of several purification methods. Single antigen has also isolated by isoelectric focusing (106) and by affinity chromatography with either concanavalin-A or specific antibody (43,46). Nagai et al (111) used two-dimensional electrophoresis to separate the proteins from culture fluid. Minden and Farr (105) developed a preparative method by gradient acrylamide gel electrophoresis to fractionate mycobacterial antigens.

Affinity chromatography on monoclonal antibodies specific to tuberculous mycobacteria was used to isolate mycobacterial antigens from ultrasonic disintegrate M. tuberculosis H37Rv. Disk-electrophoresis on polyacrylamide gel showed purified antigens to consist of polypeptides with molecular mass 16, 19, 32, 60, 27, 70 and 55kd. The antigens were tested for enzyme immunoassay of antibodies in the sera from patients with infiltrative pulmonary tuberculosis (142)

Kim et al (88) reported the extraction of a new native antigen fraction from Mycobacterium tuberculosis without massive degradation of proteins by Triton X-100. The Triton X-100 solubilised protein (TSP) antigen showed a characteristic antigen profile and reproducible extraction pattern. To characterise the nature of their composition, the TSP antigen was fractionated by Triton X-114 phase partitioning. The TSP antigen contained a variety of lipids and glycoconjugates as well as diverse proteins.

Most of the proteins were partitioned into the aqueous phase during phase fractionation, whereas non-protein molecules and lipoproteins were recovered in the detergent phase. The lymphoproliferative responses to the TSP aqueous fraction in healthy tuberculin reactors were significantly higher than those to the purified protein derivative (PPD) and unfractionated TSP. In contrast, the antibody responses to TSP aqueous fraction in tuberculosis patients showed weak reactivity. This study suggests that the TSP aqueous fraction can be used as a T-cell antigen associated with protective immunity against tuberculosis.

SEROLOGICAL TESTS:

The basis for all serological tests involves the detection of antibodies, the detection of antigens or the detection of immune complexes in the blood or tissues of the patients. Search for the serological test for diagnosis of tuberculosis was started by Arloing (5) in 1898. Then Widal and Lesourd (155) in 1901 applied a complement fixation test for diagnosis of tuberculosis. The interest in the serology of tuberculosis was revived with the use of a simple haemagglutination test by Middlebrook(104) in 1948. These workers used sheep/human group O Rh negative RBC's sensitized with water-soluble mycobacterial antigens or Old tuberculin. Large variation in the sensitivity and specificity noticed are may be due to the polysaccharides attached to untreated RBC.

Agglutination methods were also used by many workers (52,59). Tubercular antigens adsorbed on to aldehyde treated red cells and Staphylococcus aureus bearing protein A were used. The test was found to be more sensitive than the passive haemagglutination test but the specificity was not increased (64).

Nassau & Nelstrop (110) used concentrated culture filtrate of M. tuberculosis H37Rv. 31 of 38 cases of pulmonary and 6 of 9 cases of extra-pulmonary tuberculosis were found to have antibody titre more than 2 standard deviations above control sera. Tandon et al (147) reported 80%, 66%, and 4% of bacillary positive, bacillary negative and healthy control subjects respectively. Grange et al (69) used ultrasonicate of BCG partially purified by centrifugation. IgG was found in 75.2%, IgM in 31.4% and IgA in 11.8% cases.

Chandramukhi PR et al (26) studied the detection of mycobacterial antigen and antibodies in the cerebrospinal fluid of patients with tuberculous meningitis by reverse passive haemagglutination method. As per their study antigen was found in 88% of culture-positive and in 73% of culture negative TBM patients. Antibody binding was seen in 68% of all subjects with TBM and in 37% of Indian cases of pyogenic meningitis.

Zeiss et al (157) determined levels of various immunoglobulin classes in culture proved cases of tuberculosis and healthy tuberculin positive and negative individuals. Most marked difference between healthy and ill subjects was observed in levels of IgG and to lesser extent in levels of IgA.

In Children, 30% of TB cases are extra-pulmonary. In many pulmonary cases, sputum is difficult to obtain. In these cases antigen determination is difficult and indirect methods present a great importance. Serological tests for the detection of IgM, IgG and IgA is the only tool available, other than the clinical signs and symptoms. Sudha et. al (145) and Elizabeth et. al (58) evaluated different commercially available antibody detection kits and found a variation of the sensitivity from 46% to 68%.

DETECTION OF THE ANTIBODY:

Tuberculosis infection elicits an antibody response as in other infectious diseases. Secondary responses are also observed after specific re-stimulation in individuals who have been conditioned to produce specific re-stimulation. A wide variation in antibody response is seen in tuberculosis patients. Previous reports indicated that IgG and IgA classes of antibodies were elevated in active tuberculosis cases (69,83). A significant elevation in IgM levels were also noticed (2,64), and Bhatnagar et al (17) found that only IgA were significantly increased. The presence of IgE antibodies in tuberculosis was also reported (119). Thus the antibody response of patients to tuberculosis infection varies. This may be due to the genetic difference in the immune response to molecules bearing more than one antigenic determinant. Some individuals may be genetically high responders to a particular antigen, whereas others may be low responders. The response may also be due to different antigenic determinants in the same molecule. Serum antibody titre also depends on the amount of the antigen present *invivo* to elicit an immune response. In tuberculosis, if bacilli fail to multiply either as a result of natural remission or chemotherapy, antibody levels may be markedly decreases or even absent. If the bacilli are too many, the amount of antigen present may mop up many antibodies in the tissues so that the serum levels of antibodies may be considerably reduced. It was also seen that after the control of infection, antigen level decreases and antibody level increases in serum (120).

The antibody responses to Bacille Calmette Guerin (BCG) vaccination have been widely studied. Several investigators have reported persistence of antibody response for several weeks to months after the administration

of BCG (61,120,138,153). Tuberculin skin testing has been shows to produce a significant fall in antibody titre for about a week (3). It was also seen that the antibody to tuberculosis was present in healthy subjects. This may be in response to the environmental atypical mycobacteria, which may cross react with the test antigen. Some antigens of Mycobacteria were also seen in other bacterial species. Infectious diseases can lead to a polyclonal stimulation of B lymphocytes and raised levels of a wide variety of antibodies. Some serum factors may react with mycobacterial components in a non-immunological manner (34). These serum factors include C-reactive protein, some lipoproteins and sulphated polysaccharides (149) such a non-immunological reaction can also explain the ability of sera from uninfected pregnant women to agglutinate suspensions of Mtb at high titer.

The diagnostic significance of increase in antibody levels using a haemagglutination test suggested that patients with active tuberculosis had mainly IgG antibodies whereas the antibodies of normal controls were mainly IgM (59,151). Later studies also showed the presence of IgG, IgM and IgA classes of antibodies in patients with tuberculosis as well as in controls (11). The IgM response, as in other infectious diseases may be nonspecific towards polysaccharide antigens, which are widely distributed in nature, whereas IgG antibodies are supposed to be more specific for active diseases.

Reddy JR et al (126) used the 38, 63, 64, 14, 59-kDa antigens of M. tuberculosis to develop a rapid immuno-chromatographic test kit. This study evaluated the diagnostic potential of the rapid test kit using TB positive and TB negative serum samples from various hospitals in India.

The samples were obtained from patients infected with or exposed to bacteria and viral pathogens. The results demonstrated that the combination of antigens improved the diagnostic specificity and sensitivity. The specificity of the test was 99.42% with sensitivity of 98.52% (n=241). In case of multiple infections, the specificity was 93.15% with a low sensitivity of 73.52%. Gupta et al (73) used the secretory protein ES-31 as antigen for detection the antibodies. They found that the IgG showed better sensitivity and specificity.

It is clear from these observations that the antibody response to a standard dose of M. tuberculosis varies widely and hence the level of antibodies detected may not correlate with the activity of the disease.

DETECTION OF ANTIGENS:

Performance of serological tests requires the isolation, and purification of a specific antigen, which is to be used to raise the antibody. Then specific antibodies are used to detect the antigen in tuberculosis patients. The simplest approach was the use of whole tubercle bacilli as an antigen source. Immuno-flourescence test using whole bacilli treated with phenol and organic solvents to remove the surface lipids obtained a satisfactory result. However, these tests can detect those antigens, which are expressed on the surface of the bacillus and requires a fluorescent microscope (108). Old Tuberculin (OT), and purified protein derivative (PPD) have been used extensively as antigens in the serodiagnosis of tuberculosis.

Siebert's purified protein fractions were found to contain multiple antigens by immunoelectrophoresis (39). Since protein molecules have several antigenic determinants called epitopes, it was difficult to prepare pure

antigens. Some epitopes are species while others are shared among species (29), and both of them may be present in the same molecule, thereby leading to the cross-reactions. On the other hand, one epitope may occur in a number of molecules with different physicochemical properties (110). This realization has led to the search for a single species specific antigen of M. tuberculosis, the detection of which may reliably lead to the diagnosis of tuberculosis.

The detection of mycobacterial antigens in body fluids or tissues of a patient clearly offers a better proof of infection than the antibody determination. It may be even possible to identify the infecting species of mycobacteria by using the appropriate specific reagents since the quantity of antigen present in the sample to be tested is very limited. Sensitive procedures such as radio immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) or Western blot procedures have to be used. RIA was reported to be able to detect 1000 tubercle bacilli or the equivalent of 1 ng of sonicated antigen (84,133). Antigen can be detected in a sample of sputum, which was negative on smear but turned out be culture positive.

Radhakrishnan et al (124) studied the correlation between culture of Mycobacterium tuberculosis and detection mycobacterial antigens by ELISA in cerebrospinal fluid of patients with tuberculous meningitis (TBM). An inhibition ELISA procedure was employed using crude culture filtrate antigen. They found the specificity of 100% and a sensitivity of 67.8% for the diagnosis of TBM.

38-, 30-(antigen 85B), and 16-kd native antigens of Mycobacterium tuberculosis by procedures with limited number of steps. Starting with the secreted antigens of M. tuberculosis H37Rv, the 38-kd form was purified

by preparative isoelectric focusing, followed by preparative electrophoresis. Separation of antigen (147) components was achieved by anion-exchange chromatography, followed by hydrophobic interaction chromatography. Gel-permeation chromatography was employed for the isolation of the 16-kd form, from the cytosol fraction of M. tuberculosis H37Rv. By using a minimal number of steps, considerable yields of these proteins were obtained without loss of immunological activity. The native proteins purified were characterized by analytical two-dimensional electrophoresis, HPLC, and circular dichroism studies. Conformation of the native 38-kd form purified in our laboratory was different from that of the recombinant 38-kDa form from the WHO Bank. The identities of these native antigens were established by immunoblotting with known monoclonal antibodies from the WHO Bank (137). Nair et al (113,114) isolated and characterized *invivo* 31kd antigen from tuberculosis sera and compared with culture filtrate antigen of Mtb H37Ra.

A 41kd in vivo released circulating antigen was isolated from confirmed pulmonary tuberculosis serum (PTS) and bone and joint tuberculosis serum (BJS) by trichloroacetic acid precipitation and further fractionation by fast-protein liquid chromatography (FPLC). Fractionation of PTS and BJS by gel filtration column showed six protein fractions each. PTS-G3 and BJS-G3 showed maximum antigenic activity with ELISA. Further fractionation of PTS-G3 and BJS-G3 on cation exchange FPLC gave four different fractions each, of which BJS-G3B was seroreactive similarly to in vitro released 41kd antigen (ES-41) isolated from H37Ra culture medium, whereas PTS-G3C was slightly less seroreactive. BJS-G3B could inhibit binding of in vitro released ES-41 to affinity purified antibodies in inhibition ELISA at lower concentrations than PTS-G3C (2 vs. 20 ng/ml),

showing the identical nature of the antigens. Biochemical characterisation showed that circulating antigen PTS-G3C, BJS-G3B and in vitro released ES-41 antigen were lipoproteins in nature. (10).

ELISA BASED TESTS FOR THE DETECTION OF ANTIGENS OR ANTIBODIES:

Nassau and Nelstrop (110) used concentrate culture filtrate of M. tuberculosis H37Rv as antigen. 31 out of 38 cases of pulmonary and 6 out of 9 cases of extra-pulmonary tuberculosis were found to have antibody titre more than 2 standard deviation above control sera. Tandon et al (147) reported positive results in 80%, 66% and 4% of tuberculosis positive, negative and healthy control subjects respectively.

Other workers (16,85,125,157) determined levels of various immunoglobulin classes in culture proved cases of tuberculosis and in healthy tuberculosis positive and negative individuals. Most marked difference between healthy subjects and tuberculosis patient was observed in levels of IgG and to lesser extent in levels of IgA. Antibody response to the 3 glycolipids A, B and C extracted from BCG showed considerable variations by ELISA tests (127).

Purified fractions of Antigen-5 and Antigen-6 were used by many workers (8,14,36,40,139) to diagnose the active tuberculosis. Titre up to 1:179 dilutions for patients undergoing treatment, 1:9 for the patients with inactive disease, and 1:6 in case of healthy individuals were obtained. When plasma membrane extract of M. tuberculosis was used as antigen, the results obtained in ELISA test for the relapsed tuberculosis patients and 82.5% of the new untreated cases were positive (92).

Sada et al (129) demonstrated mycobacterial antigen in CSF of patients with tuberculosis meningitis (TBM). Hernandez et al (76) evaluated the use of ELISA for rapid diagnosis of patients with TBM and without TBM. Mathai et al studied the detection of crude filtrate antigen, antigen-5 and their antibodies in CSF with TBM patients (102). They found that the sensitivity was about 70% and specificity of 100% in the diagnosis of TBM.

IgG antibodies against glycolipids and proteins were isolated from Mtb and BCG suspension. Serum and CSF samples from patients with TBM and from healthy control subjects were tested. With specificities between 90 and 94% for the antigens used, the sensitivities of 75% for Pr-ELISA, 60% for Gl-ELISA and 35% for BCG-ELISA were obtained. As specific antibodies were detected in serum and CSF, only one sample is enough to perform the test. They concluded that Pr-ELISA and Gl-ELISA could be used as a supporting test in TBM diagnosis, especially when repeated bacteriological methods failed to prove the presence of tubercle bacilli and in cases without evidence of pulmonary tuberculosis (116).

A 25kd antigen of Mycobacterium bovis had previously been identified as immunodominant during badger infections. This 25kd antigen was partially purified from sonicated M bovis bacilli by using water precipitation and ion exchange chromatography, and its purification was monitored with a mouse monoclonal antibody, MBS43, which was specific for the antigen. The partly purified antigen was used to develop an ELISA for the assay of badger sera for the presence of specific antibodies. A presumed negative badger population was used to calculate the assay's threshold of seropositivity and using this value, its sensitivity (37%) and specificity

(98%) were determined in a second population of known culture status. The results indicate that it may be possible to develop a specific and cost effective serological field assay for the diagnosis of M. bovis infection (68).

Khoemko et al (87) optimized serodiagnosis of tuberculosis by detecting mycobacterial antigens and antibodies in sera from patients with lung tuberculosis, non-related diseases and healthy controls. Mycobacterium tuberculosis H37Rv was disintegrated by pressure. Cell walls were extracted with 3 M KCL and were subjected to gel filtration in Toyopearl gel. Immune sera were prepared by immunization of rabbits with cell wall material. Anti H37Rv antibodies were purified by affinity chromatography. The reagents obtained were used to detect serum antibodies and antigens (following immune complex dissociation) using ELISA. Using fraction 6 of cell wall extract, antibodies were detected in 72.2% of TB patients; there were no positive reactions in control subjects. By use of affinity-purified antibodies, antigens were detected in 77.1% of TB patients, 10% of patients with unrelated diseases and 6.7% of healthy controls. Effective serodiagnosis of tuberculosis can be achieved only by combining detection of both circulating antibodies and antigens using highly specific purified reagents and immune complex-dissociated sera.

A rapid membrane-based antibody assay capable of diagnosing pulmonary tuberculosis within 15 min has been developed using the 38kd antigen from Mtb. To determine the specificity and sensitivity of this assay and evaluate its usefulness in a clinical setting sera from patients with active pulmonary tuberculosis were obtained from three hospitals in China. The control groups consisted of patients who were diagnosed with lung diseases

other than tuberculosis and healthy subjects. Antibody was detected in 54 of 61 (89%) sputum positive patients and 67 of 91 (74%) sputum negative patients who had been clinically diagnosed as having active pulmonary tuberculosis. Five out of 56 (9%) patients with respiratory diseases other than tuberculosis and 1 out of 30 (3%) healthy controls had a positive antibody response. The overall specificity of the assay was 93% and the positive predictive value was 95%(33).

Kitada et al (89) developed an enzyme immunoassay (EIA) for diagnosis of Mycobacteriuim avium complex (MAC) pulmonary diseases that uses glycopeptidolipid (GPL) antigens specific for MAC, and it is used for diagnosis in immunocompetent patients. The mean optical densities (+/- standard deviation) of serum immunoglobulin G antibodies to GPLs in patients with MAC disease, MAC colonization, M. kansasi disease, and tuberculosis and in healthy subjects were 0.778+/-0.784, 0.042+/-0.035, 0.059+/-0.035, 0.071+/-0.035, and 0.030+/-0.027, respectively. A significant increase in the level of anti-GPL antibodies was detected in patients with MAC disease. The level of anti-GPL antibodies reflected disease activity, because the level was decreased in culture-negative patients who had conversion of culture results. In their study when a cutoff level of seropositivity (0.119) was defined, the sensitivity of EIA for diagnosis of MAC disease was 92.3%, and the specificity was 96.7%. Measurement of serum anti-GPL antibodies is useful for both the diagnosis of and assessment of activity.

There are reports (103) of the isolation of an immunodominant lipopolysaccharide (LPS) antigen from M. tuberculosis H37Rv, which can be used for the serodiagnosis of TB. The LPS antigen was compared with

three commercially available mycobacterium-specific antigens for the detection of TB. The antigens were evaluated using serum samples obtained from 59 Indian patients (19 patients with active pulmonary TB, 20 with extrapulmonary TB, and 20 with nontuberculous pulmonary disease) and 20 healthy adults. Antigen 60 IgG (sensitivity 89%, specificity 97%) and LPS (sensitivity 84%, specificity 97%) were more sensitive and specific than 38kd antigen IgG (sensitivity 79%, specificity 97%) and Kp90 IgA (sensitivity 82%, specificity 40%). These results indicate that the LPS antigen can be used as a sensitive tool for the serodiagnosis of TB and could be utilized to develop an ELISA for the screening of patients for TB. Kawamura et. al. (86) developed a thin layer chromatography (TLC) immunostaining method to detect specific antigens for antibodies in the serum of patients with tuberculosis. The detected specific antigens, TDM and specific gylcolipid fraction, were individually purified from M. tuberculosis H37Rv by column chromatography. The two purified fractions were mixed and the mixture, termed TBGL antigen, was applied to an enzyme immunoassay suitable for the measurement of antituberculosis antibodies in serum. This EIA meets all the requirements of routine clinical assay in terms of sensitivity (detection limit: 0.125 U/ml), reproducibility (total CV: 3.3-6.0%), accuracy (recovery: 96-105%), simplicity and rapidity (< 2.5 h). Clinical validation of the assay was confirmed by the measurement of the anti-tuberculosis antibody in the serum of normal subjects and patients with pulmonary tuberculosis. The EIA tested in this study showed a high serodiagnostic discriminating power (90% sensitivity and 98% specificity).

Dogan UB & Aksu HS (55) studied the diagnostic utility of ELISA in pulmonary tuberculosis using serologically active glycolipid antigens.

Twenty-seven patients who were smear positive, and 30 patients who were smear negative, but with evidence of active pulmonary tuberculosis (sputum culture positive in 10, response to anti-TBC chemotherapy in 20) were included in the study group. Twenty cured patients who had been free of TBC for at least 1 year, 50 TBC-free persons with PPD results of 0-10 mm and more than 10 mm, and 21 patients with active inflammatory diseases other than TBC formed the control groups. Sensitivity and specificity were 96% and 91%, respectively. They concluded that serologically active glycolipid antigens were as sensitive and specific as other purified antigens, and even superior to them from the point of view of their production, their extraction was quicker and easier.

A capture enzyme-linked immunosorbent assay (ELISA) was developed for detection of lipoarabinomannan (LAM) in human sputum samples. As a capture antibody used was a murine monoclonal antibody against LAM with rabbit antiserum against Mtb. The sensitivity of the capture ELISA was evaluated by using purified LAM and Mtb whole cells. They were able to detect 1 ng of purified LAM/ml and 10^4 M. tuberculosis whole cells/ml. LAM could also be detected in culture filtrate of a 3-week-old culture. The culture filtrate contained approximately 100ìg of LAM/ml. The detection limit in sputum pretreated with *N*-acetyl-L-cysteine and proteinase K was 10^4 Mtb whole cells per ml. Thirty-one (91%) of 34 sputum samples from 18 Vietnamese patients with tuberculosis (32 smear positive and 2 smear negative) were positive in the LAM detection assay. In contrast, none of the 25 sputum samples from 21 non-tuberculous patients was positive. This specific and sensitive assay for the detection of LAM in sputum is potentially useful for the diagnosis of tuberculosis (98).

A diagnostic method for the detection of M. tuberculosis by immunocapture technique has been developed using magnetic beads coated with polyclonal anti-Mtb. The detection of captured bacilli using biotinylated anti-APA monoclonal antibody (APA is a minor secreted antigen) was found more sensitive than microscopy. The results suggest that the development of a rapid strip test to detect major antigen could be a useful tool for the control of tuberculosis (122).

A dot blot ELISA test for detecting tubercular antigen in sputum samples of patients of pulmonary tuberculosis has been standardized using nitrocellulose paper. The sensitivity of the assay is 20 ng/ml. The cut-off value was 80 ng/ml. Of the 1042 patients in the study group, the percentage positivity by smear and culture was 54.51 and 57.93 per cent respectively; 68.7 per cent of the ELISA positives were confirmed by smear. The dot blot ELISA could be used as a rapid and specific test as it not only picked up 88.88 per cent of the smear positive, culture positive cases but also 81.89 per cent of the smear negative, culture positive cases. If the results of smear and dot blot ELISA are combined, 91.08 per cent of the culture positive cases were picked up as positive (50).

A rapid direct sputum antigen and antibody assay, based on immunoblotting and enzyme immunoassay is described (140). The test can detect mycobacterial antigens or antibodies in clinical specimens from pulmonary tuberculosis patients. In this study, 87 sputum, 87 serum samples and 40 paired sputum samples and serum samples were utilized from smear-positive and smear-negative, culture-positive patients. The antigen detection in sputum by dot assay has 86.1% sensitivity on active tuberculosis patients, 92.9% specificity, 91.6% positive predictive value

(PPV), 88.2% negative predictive value (NPV) and 10.3% error. The antibody assay has 83.6% sensitivity, 95.4% specificity, 94.4% positive predictive value, 85.6% negative predictive value and 11% error. The test performed on paired sputum and serum samples has a sensitivity of 93.3%, which rose to 96.1% on smear-positive and culture-positive patients, but the specificity decreased to 83% in sputum, whereas in serum it was 92%. The result of the assay in combination with other clinical data was useful in starting an earlier course of treatment for tuberculosis.

George Watt et al (66) studied rapid diagnosis of TBM by Elisa for the detection both antigen and antibody in CSF. They used BCG as the antigen found the specificity of 96% in their study using 29 CSF samples.

A target mycobacterial-circulating antigen of 55-kD molecular weight was identified in sera from confirmed Mycobacterium tuberculosis infected individuals by using Western blotting based on a specific mouse IgG anti-Mtb monoclonal antibody. The target TB antigen was isolated and characterized as a protein and it was used for the direct demonstration of the 55-kD TB antigen in serum samples of pulmonary TB patients. A simple and rapid immunoassay for the direct detection of a circulating mycobacterial antigen was developed (6).

SEROLOGICAL TESTS BASED ON THE DETECTION OF CIC

Circulating immune complex (CIC) is detected in blood when the antigen concentration relative to the antibody concentration is in excess. The significance of CIC in tuberculosis may be associated with active disease. CIC was quantitated by assaying their binding to complement C1q in a sold phase radioimmunoassay technique. Carr et al (24) detected CIC in 68% of patients with active tuberculosis. Successful cure reduced the detection rate to 15%. In several patients there was an inverse correlation between C1q binding and antimycobacterial antibody levels (101). Samuel et al (130) found elevated levels of CIC and they dissociated to determine the levels of tuberculosis antigen as well as the antibody.

MONOCLONAL ANTIBODIES (MOAb) FOR DETECTION OF ANTIGENS:

An IgM MOAb was found to react with culture filtrates of several mycobacterial species and also with several purified antigens of M. tuberculosis, including protein antigen 5 and 6. Immunoblotting had demonstrated reactivity with 4 distinct components of Mtb (42) Crossed immunoelectrophoresis technique has shown single precipitation line (74) with each MOAb. Kolk et al (91) have described methods for the production of highly specific MOAb, which can help in the diagnosis of tuberculosis infection and in identification of different mycobacteria. Olds et al (117) characterized the antigen-5 epitopes using a group of 19 MOAb. They found that the antigen-5 has two nonspecific epitopes, possibly carbohydrates in nature and one apparent specific epitope, which are shared with antigen-6.

Antigen A60 is a component of purified protein derivative (PPD) and a complex immunodominant in TB, about 8% of the antibodies produced against it are specific for MTb, the rest being inter-specific for all mycobacterial species. Elisa method using antigen A60 complex are commercially available, manufactured by M/s Anda Biologicals, France. It is a complex protein belongs to the thermostable macromolecular antigen family defined by Cocito(32) on the basis of high molecular mass and thermostability. A60 is the immuno-dominant antigenic complex in both BCG and M. tuberculosis. It is the first component against which antibodies are formed in rabbit (127). Elisa test based on the A60 complex is compared with the antigen isolated in the study.

MoAb against Mtb H37Rv culture filtrate was raised by immunizing BALB/c mice and characterization was done. Attempts are made towards identifying mycobacterial antigens in biological fluids by employing polyclonal and monoclonal antibodies specific for M. tuberculosis. Immunohistologic studies were also made using MoAb for the localization of whole or fragmented bacilli in the biopsy (93).

MOLECULAR METHODS

Molecular methods show great importance in the diagnosis of TB and other infectious diseases. The genome of every microorganism contains DNA or RNA base sequences that are specific for a particular genus, species and strain. These highly specific based sequences will bind specifically with denatured complementary single stranded DNA or RNA. Thus a probe consisting of a DNA sequence unique to Mtb can be hybridized directly to DNA from the organism present in clinical samples without the need for culture (14,71,112,137,149). Polymerase chain reaction (PCR) is a

technique that can detect and amplify even the smallest quantity of a specific DNA sequence contained within the organism by a process of enzymatic synthesis. The technique comprises 3 major steps. In the first step DNA is isolated from the given specimen. Second step isolated DNA is amplified in the thermalcycler by repeated change in the temperature and in the 3rd step the amplified sequences are detected by electrophoresis. Various modifications to the basic techniques are now available to detect Tb. Ligase chain reaction, amplified Mycobacterium direct test, Nucleic acid sequence based amplification (NASBA) are the various other methods of PCR by which the bacilli can be identified.

No single serological test confirms the diagnosis of the tuberculosis. Rapid detection of Mycobacterium tuberculosis is of vital importance for patients with tuberculous meningitis. A rapid, simple and inexpensive alterative to sputum microscopy like ELISA tests for the diagnosis of tuberculosis is to be made available as for other infectious diseases. The sensitivity and specificity of ELISA based diagnostic tests rely mainly on the purity of antigen or the antibody isolated.

METHODOLOGY

K. Ramesh kumar " Isolation and purification of specific antigen in tuberculosis and its use in immunodiagnosis" Thesis. Government Medical College Thrissur , University of Calicut, 2001

METHODOLOGY

PREPARATION OF CULTURE FILTRATE

Mycobacterium tuberculosis H37Ra strain was received from the Tuberculosis Research Centre, Madras. The single Lowenstein-Jensen (LJ) Medium slope was multiplied into 10 slopes. The 6 weeks old cultures were taken, scrubbed the upper layer with sterile scalpel in sterile atmosphere and made 2 ml suspension. Pellicle cultures were grown on Saunton's Synthetic medium (25) prepared by dissolving 4gm aminoacid Aspargine, 2gm Citric acid, 500mg dibasic potassium phosphate, 500 mg magnesium sulphate, 50mg ammonium ferric citrate, 60 ml glycerol and 5mg Tween 80. Made upto 1litre with distilled water and adjusted the pH 7.4 with aqueous ammonium hydroxide (28%). Autoclaved for 30 minutes at 120°C. After cooling, pH of the medium was adjusted to 6.9.

1000 ml of the medium in Roux culture bottles were inoculated with 2 ml of bacilli from 6 weeks old cultures grown in LJ medium, and incubated at 37°C for 6 weeks without shaking. The cultures were harvested and centrifuged at 10,000 RPM for 15 minutes and separate bacterial cells. The supernatant was passed through 0.45m Sartorious cellulose acetate membrane filter to get a completely cell free supernatant. The cell sediment was subjected to sonication using the cell sonicator at high frequency.

PURIFICATION BY SALT FRACTIONATION

Proteins in the culture filtrate and sonicates were separated on the basis of the solubility difference in the presence of neutral salts like ammonium sulphate. The dielectric constant of ammonium sulphate increases on addition and this decreases the solubility of protein fractions and hence

precipitated (106). 1000 ml culture filtrate was subjected to 20%, 40%, 60% and 80% sequential saturation by adding 115gm, 125gm, 140gm and 180gm of ammonium sulphate analytical grade respectively. After each addition, the solution was incubated at room temperature for 30 minutes and centrifuged for 15 minutes at 10,000 RPM. The precipitate obtained was dissolved in a minimum volume of sodium phosphate buffer 0.01M, pH8.0. Cell sonicate was also fractionated similarly.

PREPARATION OF TB PATIENT SERUM & CSF

Venous blood samples were drawn from the patients admitted in the wards of the Medical College Chest Hospital, Thrissur. Fasting samples were drawn by venipuncture using sterile containers. Allowed the tubes to clot. Centrifuged for 10 minutes at 3000 RPM. Serum samples were 0.2ml aliquated (5 numbers) in sterile containers. The balance serum samples were pooled in a plastic container, added 0.1% Sodium azide and 0.01% thiomersal. Filtered through the 0.45m Sartorious cellulose acetate membrane filter to get a clear contamination free serum and labeled as Tuberculosis patient pooled serum (TPS). Again the clear serum was made aliquot in storage vials and stored in -20° deep-freezer.

CSF specimens were obtained from the patients with a clinical diagnosis of TBM and suspected meningitis. Sample collection and time from onset of meningitis until collection of CSF varied from patient to patient. TBM was considered on the basis of clinical manifestation, elevated proteins and pleocytosis in CSF specimens. Under aseptic condition the CSF was centrifuged at 1,500 rpm for 15 minutes and the deposit was inoculated into LJ medium to confirm by culture for Mtb. The supernatant CSF was stored in aliquots at –70°C deep freezer.

DETECTION OF IMMUNO REACTIVITY OF EACH FRACTION:

Fractions obtained by the ammonium sulphate precipitation were desalted by dialysis using the dialysis membrane (Sigma) with sodium phosphate buffer 0.01M, pH 8.0. Two overnight changes were made at 2-8°C. Small fractions were subjected to Sephadex G25 gel filtration. Fractions were collected after the void volume, centrifuged, aliquoted and stored at -70°C deep freezer.

a. Immunodiffusion

Immunodiffusion (Ouchterlony, 118) and the precipitation in gels are based on the principle that the protein molecules diffuse in the aqueous phase supported by the gel matrix. Diffusion occurs radially from the wells and precipitation lines develop within the gel between antigen and antibody wells. Contact of the antigen with the antibody results in the formation of the precipitate. The precipitate is in the form of arc differentiated easily on the gel matrix. The antibody used was antiserum to BCG in rabbits, and TPS. Slides were made with 3ml of 1% low endosmosis agarose (Sisco) in veronal buffer 0.05 M, pH 8.0. 3% PEG was added to the mixture. Boil; add 3.0ml into the microscopic slide, cool. Wells were made using the gel puncher on the agarose. Fractions containing the antigens were added into the peripheral wells. In the central well antibody was added. Kept the slides for incubation on a humid chamber for overnight at a constant room temperature. Look for the precipitation lines or arcs in between the antigen and the antibody wells. Fixed the slide in Methanol, dry the gel slide by applying water-dampened filter paper onto the gel surface at 70°C. Stained the slide with Coomassie Brilliant Blue stain for 1 minute. The antigen that had the immunoreactivity was preserved at -20°C deepfreezer.

b. Immunoelectrophoresis

Immunoelectrophorosis is a method combines the electrophoretic migration of antigens in agarose with immunoprecipitation in gel. Electrophoretic component offers two advantages. It achieves a partial separation of mixed antigens, as the rate of the migration is controlled by their overall charge at the optimum pH and it allows negatively charged molecules to migrate rapidly into an antibody gel to accelerate the precipitation step with minimum lateral diffusion. The advantage of immunoelectrophoresis over immunodiffusion method is that the complex antigenic mixtures are separated by the resolving power due to the molecular weight and net ionic charge (123,22). 2% Agarose gel was made in the Veronal buffer 0.05M, pH8.0. 3% PEG was added to the mixture. Heat to boil, pour 2.0 ml into the microscopic slide, cool; keep in the refrigerator at 8-10°C. A 2mm central channel was made, and on each side of the channel towards one end 2 wells were punched out. Filled the antigen in the wells, electrophoresis was conducted at 150V for 45 minutes. Slide was taken after electrophoresis, antiserum was added to the central channel and the slide was kept inside a humid chamber at constant temperature. Examined and differentiated the precipitation lines after 24 hours. Slides were dried, fixed and stained with Coomassie Brilliant Blue (0.5%). By immuno-electrophoresis it will be possible to identify and differentiate the various protein antigens.

ION-EXCHANGE CHROMATOGRAPHY

Proteins were bound to the ion-exchange matrix bearing the opposite electrical change. The bound proteins from the column were eluted using ionic gradient buffer (128). The ion exchange matrix used was anion

exchange resin, diethyl-aminoethyl-Sepharose (DEAE-Sepharose) Pharmacia fast flow. DEAE-Sephrose was loaded into 2x80 cm glass chromatography column. Fractionated protein solution in minimum volume was added to the column. Washed the column with starting buffer till there was no protein in the fractions collected. Then a continuous gradient using 0.01M sodium phosphate buffer and 0.3M sodium phosphate buffer, pH8.0 from a gradient mixer was connected to the column. 2ml fractions were collected, protein content was analysed by UV spectrophotometer at 280 nm. Peaks were separated, pooled and the protein content was determined by Lowry's protein estimation method (100). The purity of these peaks were analysed by immunoelectrophorosis and polyacrylamide gel electrophorosis (PAGE).

GEL FILTRATION USING SEPHADEX G100

Gel filtration is the method used to purify the protein peaks isolated from the Ion exchange resin. Separation of protein factions are based on the molecular size. Molecular exclusion chromatography also known as gel filtration, in which the mixture of proteins, dissolved in the buffer, is allowed to flow by gravity down a column packed with beads of an inert, highly hydrated polymeric material. In the column proteins of the different molecular size penetrate into the internal pores of the beads to different degrees and thus travel down the column at different rates. Very large molecule cannot enter the pores of the beads and are excluded.

Sephadex Gel of G100 pore size was immersed in water for 2 days prior to the separation. 4gm of gel was immersed and made into 75x1.5 cm column. Phosphate buffer pH 7.6 ionic strength 100 mM was used to elute the fraction. Fractions were analysed for the protein content by UV

spectrophotomter at 280 nm. Plotted and the peaks are pooled, protein content was analysed by Lowry's protein estimation method. The peaks were checked for its immunoreactivity. The immunoreactive peak is further checked for the different protein fractions by the Anode disc PAGE.

ANODE DISC POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophorosis is a useful analytical method for differentiating microbacterial antigens. The physical conditions of acrylamide gel concentration, pH and buffer and current flow are standarized to more powerful resolution. Proteins are separated by electrophoresis on polyacrylamide gel containing two pore sizes. Migration of the protein in anode disc-PAGE is towards the positive electrode. Anode disc PAGE is mainly useful for detecting the purity of the protein isolated.

The bottom ends of the glass tube of the PAGE set up were blocked with parafilm or adhesive tape. Poured the separating gel of small pore size of 7.7% polyacrylamide in 0.375 M Tris buffer pH 8.8 with N,N,N',N'-tetramethyl ethylene diamine (TEMED) and ammonium per sulphate (APS), upto the lower mark and added a few drops of isobutanol to get a flat surface. After polymerization, washed the surface with buffer and then added the stacking gel of large pore size, 4.6% gel prepared in 0.062M Tris buffer pH 6.8 with TEMED and APS upto the second mark of the tube. Applied samples in loading buffer containing glycerol, bromophenol blue in 0.01 M Tris buffer pH8.8 buffer. Electrophoresis was conducted at constant current at 100 volts at 2-4mA per gel rod (97)

After 4-5 hours, the dye bromophenol blue migrated to the bottom of the gel. Removed the tubes, fixed the gel in 10% sulphosalycylic acid for

30 mts. Protein staining was done with Coomasie blue R-250, 0.1% in 25% methanol, 10% acetic acid and 65% water. Destained with 25% methanol in 10% acetic acid and 65% water. Periodic Schiff's reagent was used to determine glycoproteins. Schiff's reagent was prepared by dissolving 1gm of basic fuchsin in 200 ml boiling water, cooled to 50°C, filtered, added 20 ml HCl, cooled to 25°C. 1gm of sodium meta-bisulphate was mixed. Stored in the dark for 12-14 hours. Gels were immersed in 7.5% acetic acid for 1 hour. Then immersed in 0.2% aqueous periodic acid at 4°C for 45 minutes. Destained with 2-3 changes of 10% acetic acid.

SDS PAGE &WESTERN BLOT ANALYSIS:

Antigens in the ammonium sulphate precipitated, and the eluted fractions and purified preparations were separated on sodium dodecyl sulphate (SDS)- polyacrylamide slab gel using Laemmli's discontinuous buffer method (97,152) After the run, protein bands were transferred to nitrocellulose paper (NCP), blotted with TPS. The specific antigen antibody complexes were detected by horse radish peroxidase conjugated with anti-human IgG. 4 Chloro-1-naphthol or Diamino benzidine (DAB) was used with hydrogen peroxide as substrate and the bluish brown colour bands due to the specific antigen was detected.

The method followed was as follows. Separating gel was prepared by 12.6% polyacrylamide in Tris-HCl 0.375 M, pH 8.8 containing 1% SDS. TEMED 25ul and APS 1% 0.25 ml were mixed with 25 ml gel, degassed and casted. Few drops of isobutanol were added above the gel to get a uniform meniscus. Stacking gel, 4% polyacrylamide in 0.125 M Tris-HCl pH 6.8with 0.1% SDS are mixed with 10ul TEMED and 50ul (100ug/ml) of APS for 10 ml gel solution. Casted above the running gel with comb and

polymerized. After polymerization, pre-run to saturate the gel and chamber for 15-20 minutes in Tris-Glycine-SDS running buffer. Mixed equal volume of sample with loading buffer containing 0.01M Tris-HCl, pH 8.0, 0.001 M EDTA, 1% SDS, 5% beta-mercaptoethanol, heated samples for 5-10mt. At 100 C. Loaded 15ul of samples, adjusted the voltage to 65V and current 25 mA for 16/16 cm slab, till the dye front moved out of the stacking gel. Then increased the voltage to 220V and 100 mA. After 6-8 hours, gel slab was taken, transferred to the NCP by using the wet blotting apparatus. Blotted for 1 hour at 50V 0.65 amp/Cm2, using the Tris-glycine-methanol buffer. NCP was dried; the unbound sites were blocked with 1% bovine serum albumin (BSA) in for overnight at 2-8°C. Washed 3 times with PBS Tween, dipped in 1-100 diluted TPS for 2 hours at 37°C. Washed 3 times with PBS Tween, then dipped in HRP-conjugated anti-human IgG for 3 hours at 37°C. Washed 3 times with PBS Tween, dipped in the substrate solution containing 40 mg 4-Chloro-1-napththol dissolved in 1ml methanol and mixed with 100ml PBS and filtered. 50ul hydrogen peroxide was added to the substrate solution. Bands are examined, washed in water.

ELISA FOR ANTIBODY- INDIRECT ELISA

Elisa measures the binding of the antibody to antigen, which is fixed onto a solid phase absorbent polystyrene microwells. The purified antigen was attached to the polystyrine microwell of the ELISA plate. The diluted serum or cerebrospinal fluid containing antibodies will bind to the antigen in the microwell. HRP-conjugated antibody is used to detect the antigen and antibody complex. A substrate like hydrogen peroxide with the chromogen Tetra methyl benzidine is used to detect the concentration of the complex. The intensity of colour formed depends on the amount of antibody present in the test serum or CSF (23,78).

Antigen 1ug/ml, was coated to microtitre ELISA plate, Dynatech Laboratories, Alexandria. 100ul containing varying concentration in carbonate-bicarbonate buffer pH 9.6, 0.05 M. Blank and antigen controls were also kept in the same plate, incubated at 4°C for overnight. Washed 3 times with PBS-Tween 20(0.05%), blocked with 200ul of 1% BSA in carbonate-bicarbonate buffer, incubated for 2 hours at 37°C, washed 3 times with PBST, added 1:100 diluted serum or 1:10 diluted CSF in 1% BSA in PBST. Incubated for 1 hour at 37°C, washed, added 100ul of 1:1000 dilution HRP conjugated anti-human IgG, incubated for 45 minutes at 37°C. Washed, added 100ul of substrate solution containing TMB and Hydrogen peroxide. After developing colour stopped the reaction using 100ul of 0.2N Sulphuric acid. Wells were read using the ELISA reader at 450nm.

RAISING ANTISERUM TO PURIFIED MYCOBACTERIAL ANTIGEN

Polyvalent antiserum to culture-filtrate and sonicate antigen to Mtb H37Ra was raised in rabbits. Rabbits are immunized with 100 mg of crude culture filtrate antigen in 1 ml PBS, and emulsified with 1ml Freund's Incomplete adjuvant (FIA). Freund's Complete Adjuvant was not used as it contains Mycobacterium species heat killed suspension. The secondary immunization was repeated on the 30th day following the primary immunization. Purified antigen of 100ug was given as booster doses after every 7^{th} day; 10-15 ml blood was collected from the marginal vein of the ear-pinna on the 7^{th} day of booster injection. Serum was separated, and gamma globulin in the serum was precipitated by ammonium sulphate at 50% saturation. The precipitate was reconstituted in the normal saline and dialysed against normal saline for 24 hours. Protein content was

estimated by Lowry's method. Sodium Merthiolate 1:10,000 dilutions was used as the preservative and stored in aliquots at -20°C (146).

PURIFICATION OF ANTIGEN BY IMMUNOAFFINITY CHROMATOGRAPHY

Gamma globulin from the rabbit serum was separated and coupled with cynogen bromide activated sepharose-4B. 1 gm of CNBr-Sepharose-4B was allowed to swell to 3.5 ml in double distilled water, washed with 20 times cold sodium bicarbonate buffer 0.1M pH 9.0. Gamma globulin 8 mg/ml was added to the CNBr-Sepharose 4B slurry, coupled for 16 hour at 4°C. Washed with sodium borate buffer 0.1M pH9.0 alternately with sodium acetate buffer 0.1M, pH 5.0 for 5 cycles starting and finishing with sodium borate buffer (124). Specific antigen from the culture supernatant and the sonicate are filtered on a 0.45u Sartorius cellulose acetate membrane filter. The clear fluid is directly added to the affinity column, incubated washed the column and eluted the antigen. Purity is checked with Anode disc page

The immunosorbent was finally suspended in PBS 0.15M, pH 7.4, poured into a glass chromatography column and equilibrated with same PBS. The column was washed with sodium bicarbonate buffer 0.15M, pH 9.0 containing 4M urea for 3 times to minimize the leaching of gamma globulin from the immunosorbent. Antigen solution was added, washed with PBS, eluted with sodium bicarbonate buffer 0.15M, pH9.0. Collected 1 ml fractions, and detected the protein by UV spectrophotometer. Pooled the fractions, lyophilized and stored.

COMPARISON OF TUBERCULOSIS ANTIBODY WITH A60 ANTIBODY

Tuberculosis patients and control subjects blood samples were drawn, allowed it to clot at room temperature. Serum separated after centrifugation and stored in aliquots -20 freezer. Cerebrospinal fluid samples (CSF) received are also stored in -20 freezer. The antigen prepared after the immuno affinity chromatography 10ug/ml concentration, 100ul was coated to the polyvinyl microwell Elisa plate using the antigen coating procedure. Washed after 2 hours, blocked the unbound sites with 1% Bovine Serum Albumin in carbonate buffer overnight. Washed with PBST 4 times.

The bacteriologically proved TB positive patient serum samples and the known negative control serum samples were diluted 1:100 with diluent buffer. CSF samples were diluted 1:10 on the diluent buffer. The diluted samples 100ul are added to the Elisa plate, incubated for 1 hour at 37 C. Washed with PBST 4 times. 100ul Horse radish peroxidase (HRP) conjugated anti-human IgM diluted 1/100 time. Incubated for 1 hour at 37 C. Washed with PBST for 4 times. TMB substrate is added. Blue colour developed is stopped with 2N Sulphuric acid. Yellow colour end product is read on the ELISA reader at 450 nm filter. The readings were recorded. Maximum number of samples, which gave positive titer, was taken as the optimum dilution for further tests.

Similarly using the HRP conjugated anti-human IgG and HRP conjugated anti- human IgA were used to detect the IgG antibody and IgA antibody respectively in patient sera.

The same samples were tested for the antibodies to Antigen 60 reagent kit manufactured by Anda biologicals, France and compared the results.

COMPARISON WITH PCR METHOD

Polymerase chain reaction method for the detection of tuberculosis DNA is done by NeoDin MTb PCR method using the insertion sequences IS6110 and IS990 (18,77). Specimen of sputum, blood or CSF collected from the patients. Pre treatment of samples was done as per the following procedure. Add 1N sodium hydroxide to the sputum. 50ul of dissolved sputum is taken in a 1.5 ml microcentrifuge tube. Centrifuge at 12,000 rpm for 3 min and discard the supernatant. Added 800ul of water to the remaining pellet. Mixed, again centrifuged and discarded the supernatant. Blood and CSF 0.3-0.5 ml is well mixed, centrifuged, discarded the supernatant, added 800ul of water to remaining pellet. Repeat this step again. DNA was extracted using the 50ul extraction buffer containing the resin. Heated at 100° C for 10 minutes centrifuged at 15,000 rpm for 3 min to precipitate resin. Collected 4ul of the supernatant for PCR reaction.

4ul of DNA into 16ul of PCR master mixture containing the enzyme and the dNTPs and the buffer. Started the reaction using the thermal cycler set following different temperatures. 95°C/5min-1cycle, 94°C/30 sec, 72°C/ 45sec-10 cycles, 94° C/30 sec, 63°C/30 sec, 72°C/30 sec-35 cycles and finally 72°C/5min-1 cycle. Detection was done by 1.5% agarose gel electrophoresis was conducted at 100 V. Analysed the product size 181bp at PCR reaction on UV trans-illuminator. PCR results were compared with the serum antibody results of the same patient.

RESULTS AND DISCUSSIONS

K. Ramesh kumar " Isolation and purification of specific antigen in tuberculosis and its use in immunodiagnosis" Thesis. Government Medical College Thrissur , University of Calicut, 2001

RESULTS AND DISCUSSIONS

Isolation of proteins:

The culture supernatant from 6 weeks old Mycobacterium tuberculosis H37Ra (1000ml culture) and the cell sonicate in phosphate buffered saline (PBS) were individually subjected to ammonium sulphate fractionation. 20%, 40%, 60% and 80% fractions, were dissolved in minimum volume of 10mM sodium phosphate buffer pH 8.0, dialysed against the same buffer and subsequently analysed for their protein concentrations using Lowry's protein estimation method. The protein concentrations of 20%, 40% 60% and 80% fractions from the culture supernatants were 240mg, 178mg, 130mg and 112 mg respectively. The 60% fraction showed more immunoreactivity with the pooled tuberculosis patient's serum by immunodiffusion (Fig.1). These observations allowed only the 60% fractions after excluding 40% fraction in the subsequent studies.

Purification of the protein fractions:

The 60% immunoreactive fraction was subjected to ion-exchange chromatography on a DEAE- Sepharose fast flow column, equilibrated with sodium phosphate buffer 100mM, pH 8.0. The bound protein from the column was eluted with a continuous gradient up to 300mM sodium phosphate buffer pH 8.0. Fractions were analysed for the protein content on a UV Spectrophotometer at 280nm. 5 major protein peaks were obtained, of which the second major peak was found to be more immunoreactive to the serum samples from tuberculosis patients when studied by indirect ELISA. The fraction was concentrated and stored at -70 C.

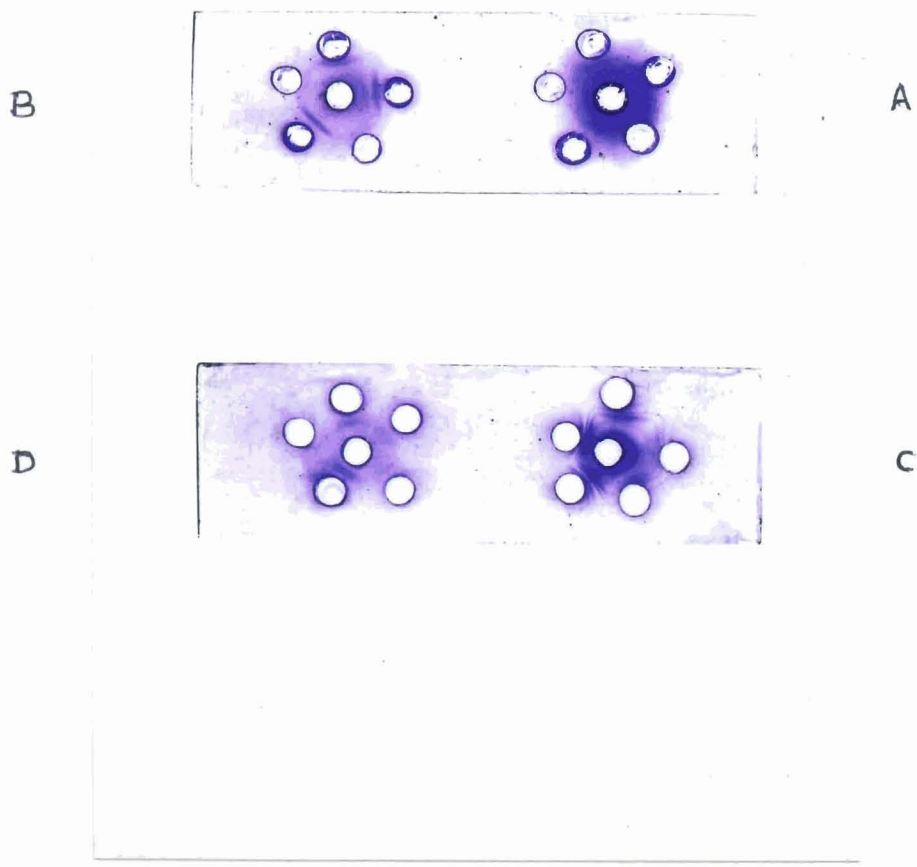


Fig 1: Immunodiffusion pattern showing the precipitation arc of The A. crude extract, B. 60% Ammonium sulphate precipitated, C.DEAE Sepharose fast flow eluted fraction and D. Sephadex Gel eluted fraction. Antibody used is Tuberculosis patient pooled serum in the central well.

Ammonium sulphate precipitation was used as a method of salt fractionation. Exclusion of the 40% precipitated protein improved the efficiency of separation of the protein from the DEAE-Sepharose column. This may be due to the excess protein load in the column, which has masked the ion exchange properties of the specific protein. Hence in this study 60% anmmonium sulphate saturation was done after removing the 40% precipitated proteins. DEAE-Sepharose column was used to separate the immunogenic protein. Gradient increase in ionic concentration of the phosphate buffer pH 8.0, 100mM to 300mM does not give a complete differentiation of protein peaks. So a stepwise increase in sodium chloride 100mM to 500 mM with an increase in 100mM at a time was used to elute the fraction (Fig.2).

Detection of Immunoreactivity:

Fractions were analysed for the protein content at 280nm on a UV spectrophotometer. Peaks were pooled and found there are 5 major peaks. All peaks are subjected to the immuno-reactivity by immunodiffusion method. Only the 2nd peak showed immunoreactivity with tuberculosis patient's pooled serum and anti BCG- rabbit serum in Oucterlony immunodiffusion method (Fig. 1).

The 2nd major peak was dialysed and the protein content was analysed by Lowry's method (100) and concentrated by lyophilization. The protein peak was immunized to the Rabbit mixed with Freund's incomplete adjuvant. Booster immunization was given after a week and second booster was given after a month. Blood samples are drawn after a month after the second booster. Serum separated and the rabbit serum is compared with the tuberculosis patient serum. The arcs obtained in the immunodiffusion

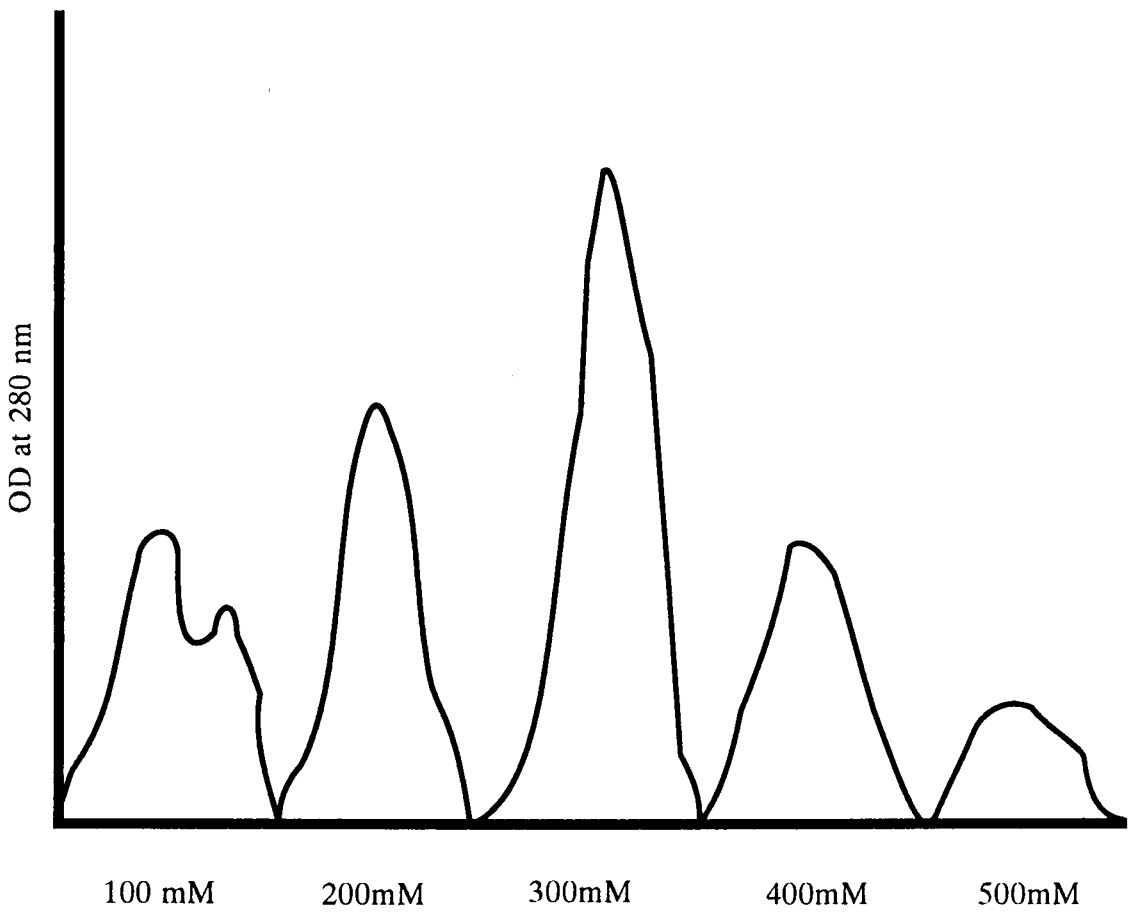


Fig. 2: DEAE Sepharose fast flow, Protein concentration Vs fractions collected. 5 major peaks were isolated and found the 2nd major peak is more immunoreactive

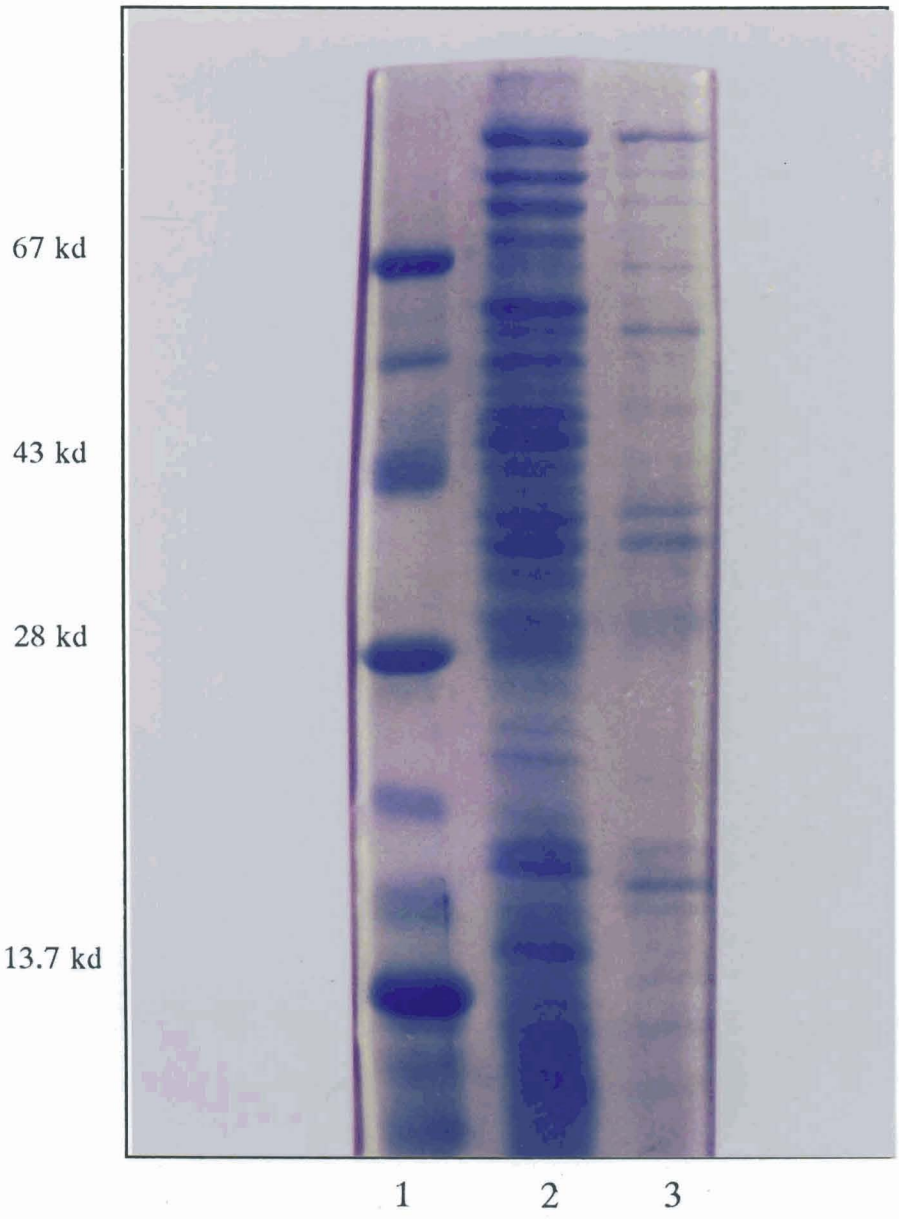


Fig.3: Polyacrylamide Gel electrophoresis with SDS : 1. Molecular weight marker.2. 60% ammonium sulphate precipitated protein and 3. DEAE-Sepharose 2nd major peak.

were comparable. This shows there is a similarity in the antibody production.

The protein peak was further subjected to PAGE. About 20 major protein bands are seen (Fig.3). Further the peak is subjected to Gel filtration using SephadexG100. Fractions were collected, the protein peaks are identified and the peaks are pooled. 14 peaks (Fig. 4) were obtained and each peak was checked for the immuno-reactivity. The immuno-reactive peak was seen at the 4th peak. Immuno-electrophoresis was conducted and found a single arc. (Fig.5). The 4th peak was then run for Anode disc PAGE and there is only one band of about 70kd molecular weight in the electrophoresis. The 4th peak was again subjected to SDS-PAGE and showed 2 bands. The Molecular weight obtained is 38kd and 32kd bands. This shows the 70kd protein isolated was a dipeptide of 38kd and 32kd proteins.

Gel run along with the SDS-PAGE is subjected to Western blot. The Nitrocellulose membrane after the transfer was incubated with diluted TPS. The immunoreactivity of the bands were assessed by the Western blot procedure and found that both the bands are immuno-reactive (Fig. 6). Since both the protein band showed immuno-reactivity and in Anode disc PAGE it was seen as a single protein of 70kd, further studies were carried with this single protein. The 70kd protein from the SephadexG-100 column was concentrated by lyophilization.

Antibody production:

70kd protein isolated was immunized in rabbits to get its antibody. Extracted the rabbit serum after the booster injection. Antibody was separated by ammonium sulphate precipitation. The immunoglobulin

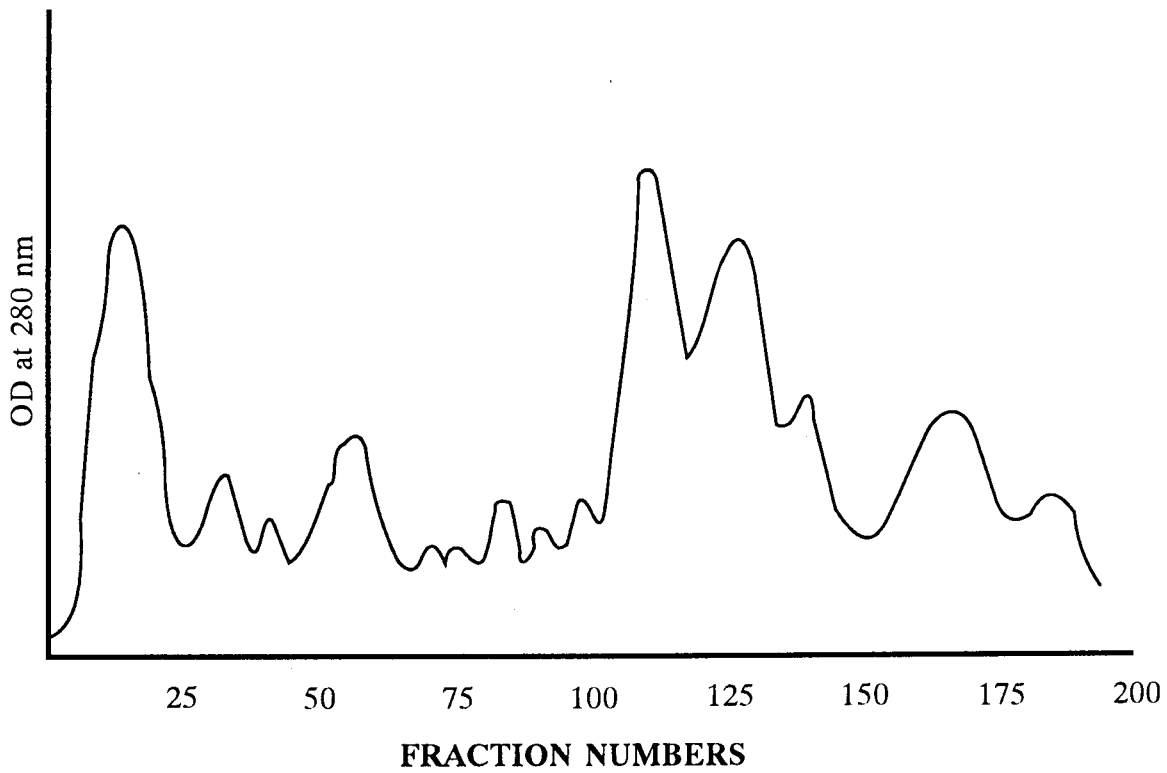
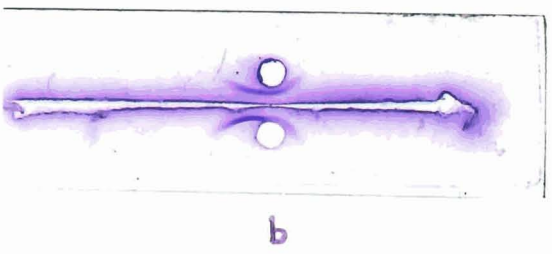


Fig.4: Sephadex G-100 gel filtration Protein concentration Vs Fractions collected. 14 peaks were identified and the 4th peak is found to be more immunoreactive.



a: Affinity Purified Antigen

b: SephadexG-100

Fig.5: Immunoelectrophorosis pattern of the eluted Fraction from Affinity Purified Antigen & SephadexG-100

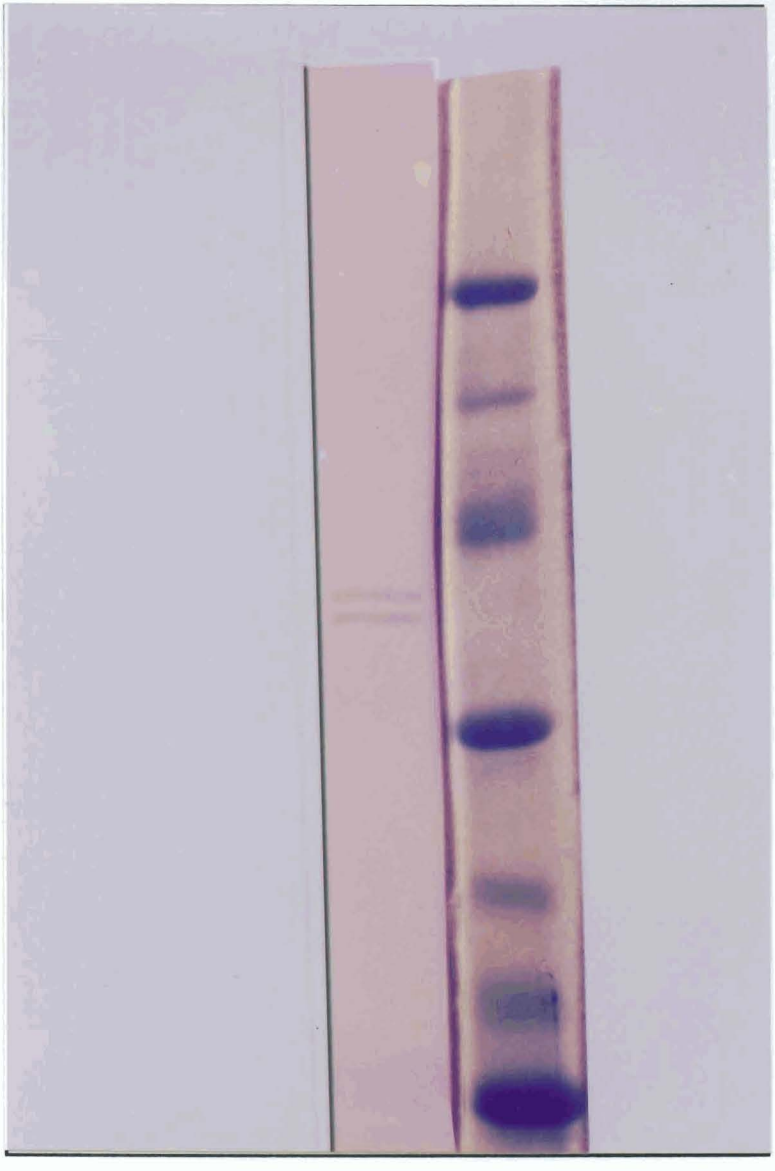


Fig.6: Western Blot test to find out the Immunoreactivity in the 38kd and 32kd position of SephadexG-100 eluted fraction.

1. Nitrocellulose membrane showing the two bands.

2. Two immuno-reactive bands are seen in between 28 kd and 43 kd bands.

isolated from the rabbit serum was coupled with CNBr-Sepharose for the affinity chromatography. More of antigen was isolated by the affinity chromatography.

INDIRECT ELISA

Elisa test for the detection of antibody was conducted by the indirect Elisa method. Serum samples were diluted 1:100 in PBS Tween containing 1% BSA. 181 blood samples of known TB patients were collected. 58 healthy individuals samples were also drawn and these values are used for the Cutoff value calculation. Cutoff values for the healthy individuals were estimated to be 0.22 optical density (OD) at 450nm on the Stat Fax Elisa reader, Awareness technology, USA. More than 0.22 OD were taken as positive and less than 0.22 OD were taken as negative. Out of this 128 were pulmonary tuberculosis patients and 53 were extra-pulmonary tuberculosis. All these patients were confirmed by bacteriological methods and all the patients were responded to anti-tuberculosis treatment. IgM, IgG and IgA antibodies to tuberculosis are tested using the isolated 70kd antigen.

Out of 128 pulmonary tuberculosis patient samples 110 were positive to IgM, 116 were positive to IgG and 93 were positive to IgA antibodies. Analysis of specificity and sensitivity were done as per the following criteria. Sensitivity of a test means its capacity to correctly identify in a population those individuals who have the disease. In mathematical terms, it is the number of persons with a positive test who have the disease divided by the sum total of persons with the disease. Specificity of a test denotes its capacity to correctly identify those who are free from the disease. It is the number of persons with a negative test who do not have the disease divided by the sum total of persons without the disease.

DETECTION OF SENSITIVITY:

Percentages of positivity or the sensitivity in case of pulmonary tuberculosis were seen to be IgM-85.9%, IgG-90.6% and IgA-72.6%. Average sensitivity calculated was 83.0%. Out of 53 extra pulmonary cases 48 were positive to IgM, 49 were positive to IgG and 39 were positive to IgA. Percentages of positivity or the sensitivity calculated was IgM-90.6%, IgG-92.5% and IgA-73.6%. Average sensitivity is 85.6% (Table No.1).

Comparative study was conducted using the commercially available A60 complex reagent kit. The above serum samples were tested with the A60 antigen kit for detecting the different antibodies. The numbers of samples positive for each antibody and the percentages were shown in the Table No. 1. and in the Fig No. 7. Considerable variation in the percentage of positivity is seen amongst these protein antigens. Percentage of sensitivity for IgG was more when compare to IgM and IgA antibody.

Table No. 1: Showing the antibody sensitivities of the isolated antigen 70 kd and the antigen 60 kd proteins. Pulmonary tuberculosis and the extrapulmonary tuberculosis samples are separately compared.

PULMONARY-128, EXTRA- PULMONARY, 53	IGM+	IGM%	IGG+	IGG%	IGA+	IGA%
Pulmonary TB Antigen 70	110	85.9	116	90.6	93	72.6
Extra Pulmonary Antigen 70	48	90.6	49	92.5	39	73.6
Pulmonary TB Antigen 60	95	74.2	98	76.5	73	57.0
Extra Pulmonary Antigen 60	40	75.5	44	83.0	25	47.2

The isolated antigen 70kd protein in this study was shown more sensitivity than the antigen 60kd both in the pulmonary and extra-pulmonary tuberculosis patient serum samples.

Tubercular meningitis:

Diagnosis of TBM depend upon the demonstration of causative agent Mtb in CSF specimens either directly or by culturing technique. Mtb isolation in CSF specimen by conventional culture techniques is time consuming and most of the time inconsistent. Detection of Tb antigen or antibody in CSF is useful for early diagnosis and treatment. In this study 85 CSF samples were received from various meningitis cases. All the samples were analysed for the specific antibody IgM, IgG and IgA to the isolated antigen. Dilutions of the CSF samples were 1/10 instead of 1/100 dilution for serum. Out of this 35 patients were confirmed to TBM. 28(87.5%) of 32 samples were detected for IgG antibody to the isolated antigen. IgA positivity and IgM positivity were detected to be 9.3% and 6.2% respectively. IgG antibody to the isolated antigen finds more specific for the detection of TBM.

Table 2: Showing the CSF antibody positivity in tuberculosis meningitis and other meningitis. IgG antibody is more positive than IgM and IgA.

	No. of Samples Tested	IgM+	IgG+	IgA+
TB Meningitis	35	2	28	3
Positive %		6.2	87.5	9.3
Other Meningitis	53	0	3	0
Positive%		0	5.7	0

53 CSF samples were other Central nervous system infections of bacterial, fungal or viral etiology. IgG antibody was detected in only 3(5.7%) of the samples in these cases. In case of TBM samples IgG antibody is detected in 87.5% cases and found more useful than the other antibodies hence IgM and IgA antibody detection may not be useful the detection TBM. 5.7% CSF IgG positive in other infectious diseases again due to the cross reacting antibodies (Table. 2).

Detection of Crossreactivity:

175 serum samples from 9 different infectious diseases which are prevalent in this area at the time of conducting this work were carried out to check the cross reactivity of the antigen with other infectious diseases. Number of positive samples in each antibody is tabulated in the Table 3. Maximum cross reactivity obtained is for dengue infection is 3 out of 22 samples amounting to 13.6 % in case of IgG and 4 IgA positive out of 27 amounting to 14.8% in case of leptospirosis. The mean cross reactivity with these infectious diseases was IgM-3.4%, IgG-4.6% and IgA-6.3%. The cross-reactivity observed may be due to the nonspecific binding of the antibodies produced during these various infectious diseases. The cross reactivity obtained for these infectious diseases may be due to similarities in the antibody binding sites or may be due to previous encounter of these patients with tuberculosis earlier.

Patients with HIV infection was not included in the study to see the cross reactivity.

Table No. 3: Samples tested showing the cross reactivity with other infectious diseases samples

Samples tested from Patients with	Number of samples tested	IgM +	IgG+	IgA+
Malaria	33	0	1	0
Mycoplasma	20	1	0	2
Toxoplasma	26	0	2	1
Syphilis	12	0	0	0
Amoebiasis	9	1	1	0
Hepatitis B	22	2	0	3
Leptospirosis	27	0	1	4
Dengue	22	2	3	1
Sarcoidosis	4	0	0	0
Total samples	175	6	8	11

Table No. 4: Showing the comparison of the specificity of the antigens when tested with normal healthy samples.

Antigen	IgM	IgG	IgA
Antigen 70 +	3	5	10
Antigen 70 %	97.2	95.4	90.7
Antigen 60+	7	9	10
Antigen 60%	94.5	91.7	88.9

108 healthy individuals serum samples were tested for the antibody levels. Out of this 3 were positive for IgM, 5 were positive for IgG and 10 were positive for IgA. Specificity was calculated and found to be 97.2% for IgM, 95.4% for IgG and 90.7% for IgA. Specificity obtained was shown in the Table 4. and in the Fig. 8.

The result of the serum antibody detection against the isolated antigen shows that the antigen is more sensitive and specific than the A60 antigen. Hence the antigen isolated may be useful for the detection of tuberculosis in this population. As the tuberculosis strains vary in different place and the immune suppression of patients due to tuberculosis infection, the antibody response varies from patient to patient and the interpretation of the antibody result may be correlated with other clinical findings.

COMPARISON OF SENSITIVITY

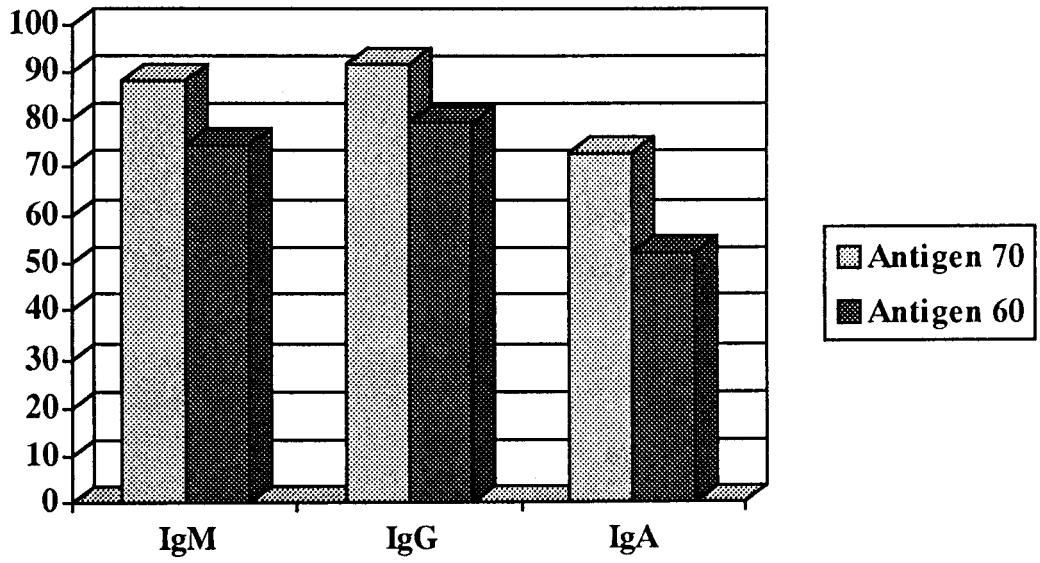


Fig. 7: Sensitivity of antibodies with isolated Antigen 70(IgM-87.3%, IgG-91.2, IgA-72.9) with Antigen 60 (IgM-79.5%, IgG-85.1%, IgA-71.8%)

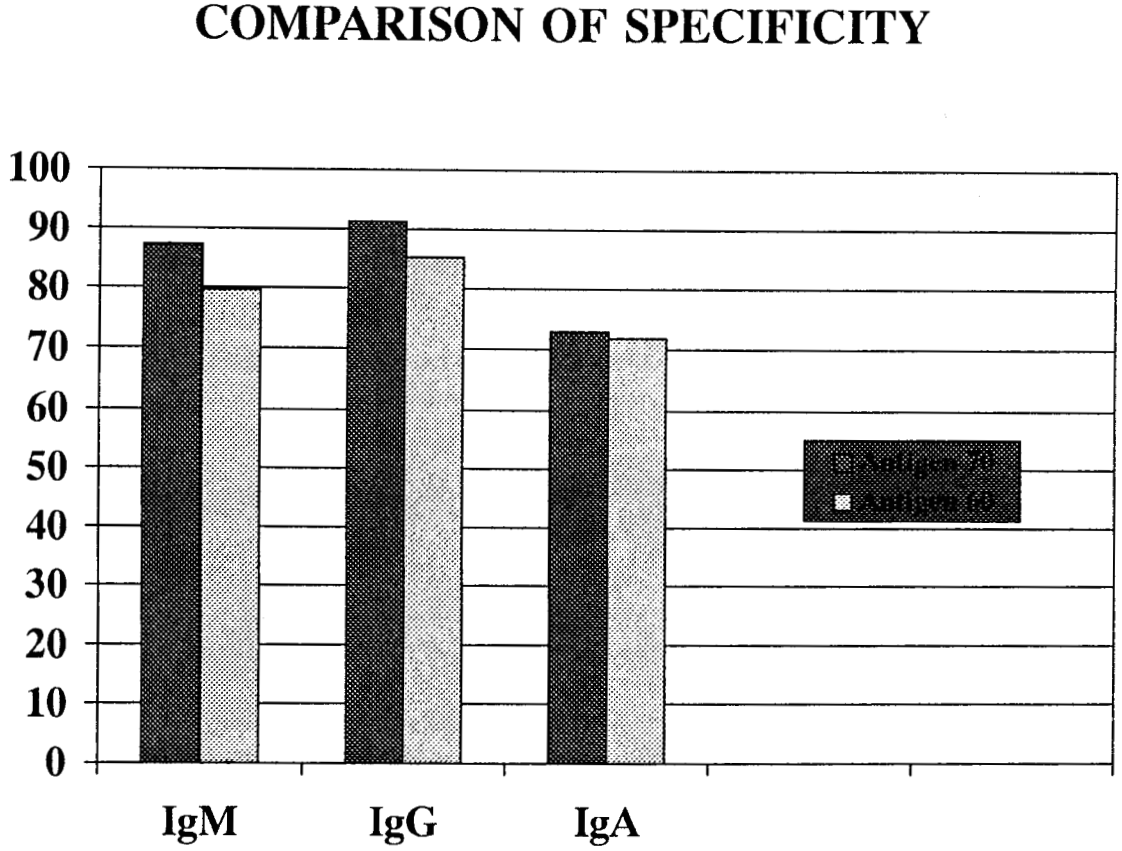


Fig. 8: Diagram showing the specificity comparison of the antibody results with Antigen 70kd and Antigen 60kd

Comparison of ELISA with PCR

Antibody test results were compared to the PCR results (Table 5). 117 PCR positive patient various types of samples comprising 38 sputum, 18 CSF, 23 Urine, 16 PUS samples and 12 blood samples.

Table.5: Comparison of the ELISA results with PCR results.

Samples	PCR +	IgM+	IgM%	IgG+	IgG%	IgA+	IgA%
Sputum	38	16	42.1	21	55.3	13	34.2
CSF	18	8	44.4	12	66.6	6	33.3
Urine	23	12	52.2	15	65.2	11	47.8
PUS	16	10	62.5	12	75	10	62.5
Blood	12	6	50	9	75	7	58.3

The percentage positive antibody results vary widely with different antibodies. IgM 42.1% to 62.5%, IgG 55.3% to 75% and IgA 34.2% to 62.5% were obtained with positive PCR patient blood samples. The low sensitivity may be due the high sensitivity of PCR method as it detects only the DNA molecule as against the antibody, which takes some time to develop in the body. The positive antibody results obtained for the 89 patients were correlated clinically with TB. These patient samples were taken for PCR tests and found only 21 samples were positive for PCR. This comparative study shows that the antibody results and PCR results vary widely for the same patient. This may due to the nature of TB infection, which is localized, and formation of the antibody takes some time depending on the immune response of the individual and type of the tuberculosis.

SUMMARY AND CONCLUSION

K. Ramesh kumar " Isolation and purification of specific antigen in tuberculosis and its use in immunodiagnosis" Thesis. Government Medical College Thrissur , University of Calicut, 2001

SUMMARY & CONCLUSION

Diagnosis of tuberculosis largely depends on the clinical examination radiological examination and bacteriological examination of the sputum smear by AFB staining and culture methods. Though the AFB culture is the gold standard, it is always not possible to get a cent percent conclusion, especially in extra-pulmonary tuberculosis. Sputum examination had 75% sensitivity and 98% specificity but in clinical practice only 30% sensitivity is achieved. Serology based identification for the antigen or the antibody finds more useful for the diagnosis of any infectious diseases including tuberculosis. Serodiagnosis of the tuberculosis requires a specific antigen or a specific antibody in the purified form. In this study it was possible to isolate a comparatively more specific and sensitive antigen.

Immunoreactive protein from the Mtb H37Ra culture medium was isolated by ammonium sulphate salt fractionation, purified by DEAE-Sepharose fast flow anion exchanger and Sephadex G100 gel permeation chromatography. Protein free liquid medium to avoid the protein interference while the antigen isolation procedure.

Tuberculosis patient pooled serum (TPS) was used as the source of the antibody. The immunoreactive fractions are identified using TPS at each stage of isolation and purification using radial immunodiffusion, immunoelectrophorosis, anode disc polyacrylamide electrophorosis, SDS-PAGE. Western Blot method for the isolated immunoreactive protein was conducted and found the protein was a dipeptide of 32kd and 38kd molecular weight protein of a single 70kd protein. Further studies were conducted with 70kd protein. 70kd protein was immunized to rabbit to get the specific antibody. This isolated antibody is used for purification of the

antigen by affinity chromatography using Cynogen bromide activated Sepharose-4B.

The 70kd protein was coated into the micro well Elisa plate. Individual tuberculosis patient serum in dilutions was used to detect IgM, IgG and IgA antibodies Elisa tests were carried as per the ELISA protocol. The results of the patients were clinically correlated. Also these samples were run with the commercially available TB Elisa kit manufactured by M/s Anda Biologicals, France. The sensitivity and specificity of the Elisa test were compared. In the comparative study conducted by indirect Elisa methods for the detection of antibody, IgG antibody was 90.6% sensitive in pulmonary tuberculosis and 92.5% in extrapulmonary tuberculosis, more specific than the IgM and IgA antibodies. The sensitivity obtained for the isolated antigen was more than the commercially available antigen 60kd kit. In other studies conducted by most of the workers using different antigen IgM antibody positivity is very low and the reliable measurement is difficult. Unlike any other viral or bacterial infection, tuberculosis infection one cannot predict the antibody response is primary or secondary as the antibody response differ from patient to patient.

Tuberculosis patient serum was used as a source of antibody instead of Anti-BCG serum in studied by many workers. How ever Anti BCG was also used as source of antibody for comparing the tuberculosis patient serum samples in the immunodiffusion studies. The purification studies were all based on the immunological binding with Elisa and immunoelectrophorosis using the tuberculosis patient serum. The yield of isolated protein antigen of 70kd after all the purification steps like salt fractionation, DEAE-Sepharose fast flow column and Sephadex gel filtrations is 8 mg from the 1000 ml culture.

Immunisation and the antibody separation for affinity chromatography were also conducted to isolate more antibodies. Production of monoclonal antibodies and developing a direct Elisa test may help in detection of antigen from the samples as the antigen is detected immediately after the infection and hence the diagnosis will be faster specially in case of TBM. More characterization and structure identification by aminoacid sequencing will be helpful in synthesizing a protein by chemical synthesis or by recombinant synthesis.

In tuberculosis meningitis, Tb specific IgG antibody was more sensitive. A sensitivity of 87.5% was observed. IgM and IgA antibody detection is not useful. A high specificity of the CSF Elisa detection for the IgG antibody makes the antigen suitable for use as a method for including patients in clinical trials of TBM.

Cross reactivity with other infectious diseases patient serum samples were also tested. Antibody results are compared with PCR methods and found poor correlation with PCR results. Elisa for tuberculosis using the isolated antigen is therefore practical and more sensitive than direct staining or by culturing or PCR methods. The results were compared and found poor correlation with PCR results.

To conclude the studies, isolation of a specific antigen and its use in Elisa for the detection of antibody is more specific and more sensitive than the other available antibody detection methods. Hence this antigen may be more useful for the laboratory diagnosis of the tuberculosis infection specially in this geographical area.

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