

AIDS FOR THE EARLY DIAGNOSIS OF
TUBERCULOUS MENINGITIS (TBM)

by

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PREFACE

This study represents original work by the author and has not been submitted in any other form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Paediatrics, University of Natal, under the supervision of Professor H.M. Coovadia.

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SUMMARY

SUMMARY

Mortality and morbidity rates associated with tuberculous meningitis (TBM) are substantial. The average duration of the untreated disease from onset to death is about 17 days. The prognosis of TBM is known to correlate with the stage of the disease at the time of diagnosis and commencement of chemotherapy. Early diagnosis improves the chances of recovery without neurological sequelae. Early diagnosis is a problem because the presenting symptoms are non-specific and the onset of the disease is typically insidious.

To date no single test is available that is totally reliable and specific for TBM. I have attempted to develop a reliable and easily applicable test for the diagnosis of TBM. In fulfilling this objective, the work undertaken may be divided into three major sections:-

1. Detection of soluble *Mycobacterium tuberculosis* antigens in the cerebrospinal fluid (CSF) of patients with TBM and in control groups by using *Mycobacterium bovis* BCG antigens. The technique used was that of inhibition enzyme-linked immunosorbent assay (ELISA). The principle of this technique is illustrated in Fig. 5.
2. Detection of soluble *M. tuberculosis* antigens in the CSF of tuberculous and control groups of patients by using antibodies raised against *M. bovis* BCG. The technique used was that of the double antibody sandwich ELISA. An outline of this ELISA is given in Fig. 6.

3. Correlation of chloride levels in the blood and CSF of patients with tuberculous and other forms of meningitis. It has been established that the SERUM/CSF ratio of bromide tends towards unity in patients with TBM because the permeability of the blood-brain barrier is impaired. Since both bromide and chloride are chemically similar (both being halides), it was thought that a similar pattern may exist for BLOOD/CSF chloride ratios; and this was investigated.

The method used for the INHIBITION ELISA had to be standardized before the samples could be tested. This involved investigating the acceptability of various microtitre plates; determination of the optimal working dilutions for the coating solution and conjugate; and determination of optimal conditions for the various incubation periods, both in terms of time and temperature. A total of 70 specimens was tested. These consisted of 25 normal CSF controls; 25 pleural and ascitic fluid samples; 10 TBM samples, and 10 bacterial meningitis CSF samples. It was found that a distinction existed between the absorbance values obtained from positive TBM CSF samples (Mean $0,658 \pm 0,043$) and that from normal CSF samples (Mean $1,089 \pm 0,224$). The mean absorbance of the culture-positive bacterial CSF's also differed significantly from the other 2 groups (Tables VII; IX).

Some overlap occurred amongst the absorbance values of bacterial culture positive CSF's (Range $0,975-0,879$) and normal CSF's (Range $1,486-0,934$). The mean absorbance value for bacterial positive CSF

samples ($0,920 \pm 0,029$) differed significantly ($p < 0,01$) from those of normal CSF ($1,089 \pm 0,224$) and TBM CSF's ($0,658 \pm 0,043$). The difference between the mean values obtained with tuberculous and non-tuberculous groups of pleural and ascitic fluid was also significant ($p < 0,01$).

The method used for the DOUBLE ANTIBODY SANDWICH ELISA was that of Sada et al. (1983). Before the samples could be tested, the method had to be standardized and similar investigations to those for the INHIBITION ELISA were performed. In addition, antibodies raised against *M. bovis* BCG were conjugated to alkaline phosphatase since no commercial preparation was available. Unfortunately no distinction was recorded between negative and positive test specimens, even on repetition of the entire procedure.

Measurement of chloride was done by a fully automated procedure using the BECKMAN ASTRA-8. A total of 149 samples were tested. Of these 10 were tuberculous, 34 were viral, and the remainder were bacterial meningitis. No pattern was established that could differentiate TBM from viral or bacterial meningitis. The results obtained are tabulated in Table III and illustrated in Figures 9, 10, and 11.

In summarizing, the use of the INHIBITION ELISA technique for the accurate diagnosis of TBM seems promising. However, its validity in the clinical situation will have to be assessed further and with greater numbers of specimens before it can be adopted as a diagnostic procedure for TBM.

OBJECTIVE

To determine

1. The ability and reliability of the 'INHIBITION ELISA' technique to detect mycobacterial antigens in pleural, ascitic, and cerebrospinal fluids.
2. The accuracy and reproducibility of the double antibody sandwich ELISA in the detection of mycobacterial antigens in CSF of patients with tuberculous meningitis (TBM).
3. Whether a correlation exists between blood and CSF chloride levels in patients with tuberculous and other forms of meningitis.

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LIST OF SYMBOLS

<u>SYMBOL</u>	<u>DEFINITION</u>
Ab	antibody
ADA	adenosine deaminase activity
Ag	antigen
BCG	Bacillus Calmette-Guérin
BPT	Bromide Partition Test
^{82}Br	radioactive bromide
BSA	bovine serum albumin
Cl^-	chloride
CSF	cerebrospinal fluid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
E-rosette	erythrocyte-rosette
Ig	immunoglobulin
LJ	Lowenstein-Jensen
LPA	Latex Particle Agglutination
LD	Lactate dehydrogenase
mmol/l	millimoles per litre
nm	nanometre
PBS	phosphate-buffer saline
PPD	protein-purified derivative
SD	Standard deviation
SRBC	sheep red blood cells
TB	tuberculosis
TBM	tuberculous meningitis
TWEEN 20	polyoxyethylene sorbitan monolaurate
μl	microlitres

I. INTRODUCTION

I. INTRODUCTION

(a) Brief Historical Review of TBM

Tuberculous meningitis (TBM) was first described a little more than two hundred years ago. Robert Whytt (1714-1788), a Scotsman, gave a typical clinical picture of the disease in his "OBSERVATIONS ON THE DROPSY IN THE BRAIN" (1768). Whytt attributed the aetiology of TBM to three factors; birth trauma, tumours, and suppression of urine. (26)

Sixty-five years later in 1833; the first accurate clinical study of TBM in children was published in the United States by William Wood Gerhard (1809-1872). (24)

Another American, Ludwig Hektoen (1863-) was the first to publish work on the vascular changes associated with TBM, in 1896. (25)

The tubercle bacillus, the causative agent of TB and TBM was first discovered in Germany by Dr Robert Koch (1843-1910). Koch achieved his discovery by the use of his own method of heat-fixing bacilli to glass slides and by the use of staining techniques developed by his students Ehrlich and Weigert. He demonstrated his findings on 24 March 1882 at a meeting of the Berlin Physiological Society. (20)

Ehrlich used red fuchsin as a primary stain, decolourized with a mineral acid, and used methylene blue as a counterstain, thus showing red bacilli on a blue background as is the procedure today. Ziehl

and Neelsen made minor modifications to the stain and acquired an undeserved eponym.⁽³⁾

Koch was able to grow a pure culture of the tubercle bacilli on meat infusion which he solidified with agar-agar. He described the pure colonies as being spindle-shaped, very fine and usually 'S-shaped' ~ nowadays called serpentine cords. He also determined that the tubercle bacilli grew only in temperatures between 30°C and 41° C. By administering the pure culture to experimental animals he proved conclusively that the tubercle bacilli were the cause of TB in these animals.⁽²³⁾

One important practical application of the discovery of the tubercle bacilli was diagnostic. Isolating the bacilli from sputum and other clinical specimens was a certain method of diagnosis of all tuberculous processes.

The diagnosis of TBM in the living was impossible at this stage without cerebrospinal fluid (CSF) samples. Many unsuccessful attempts were made to remove CSF. In 1891 Henry Quincke (1842-1922) described the technique of lumbar puncture.⁽²⁴⁾

1891 was also the year in which Koch made a discovery which was the basis of one of the most valuable diagnostic investigations in all tuberculous processes, the Tuberculin Test. He showed that a local inflammatory reaction could be elicited by the subcutaneous injection of a 'glycerine extract of a pure cultivation of the tubercle bacilli' in tuberculous patients.

In 1907 Von Pirquet (1874-1929) postulated the existence of sub-clinical tuberculosis after finding that 80% of healthy 10 year-olds in Vienna were tuberculin-positive. His suspicions were confirmed by Ghon's post-mortem studies undertaken between 1908 and 1912. In 1908 Mantoux evolved the intradermal tuberculin test which is in use today to assess the status of tuberculosis in an individual.

Since Koch's discovery of the tubercle bacillus more than a century ago, many attempts have been made to develop diagnostic tests for tuberculosis and TBM. These tests include isotope-studies and numerous biochemical and immunologic studies. Many prove helpful but no single test is confirmatory as yet.

(b) Epidemiology of TBM

TBM occurs in most cases as a complication of primary infection. It usually develops within 6 months of acquiring primary tuberculosis and occurs most commonly in infancy and early childhood.

Tuberculosis is a socio-economically related disease. Its development and spread is favoured by poor housing, overcrowding, malnutrition, in fact any physical or emotional stress. The prevalence of tuberculosis in the Western World has declined sharply with increasing industrialization, hence TBM is rare in such regions as compared to Third World countries.

In South Africa the distribution of tuberculosis amongst the races is 82% Blacks; 15% Coloureds; 1,5% Asians and 1% Whites.⁽³⁾

Tuberculosis was unknown to the indigenous people of Southern Africa. It was brought to the sub-continent by White traders who had acquired resistance to the disease over many centuries of exposure to it. The Black population was very susceptible to tuberculosis and it is estimated that over 10 million Blacks are infected with the disease at present.⁽²⁸⁾

Infected people do not necessarily develop overt disease. The infection remains dormant, e.g., in the lymph nodes, in the meninges, lung apices, vertebrae. In developed countries approximately 15% of infected people undergo reactivation of the inactive bacilli. In underdeveloped countries other negative factors such as measles, malnutrition and stress result in as many as 40% of cases becoming infectious.⁽¹⁰⁾

In a recent study of TBM in children under 15 years of age in the Western Cape,⁽¹⁴⁾ the incidence per population group was as follows:-

White	0,2	} per 100 000
Coloured	5,8	
Black	25,7	

The number of cases of TBM was found to be higher in rural areas than in urban areas.

Generally, mortality rates are high for TBM. The untreated patient survives on average only 17 days from onset of the disease. Of those who are diagnosed and treated, a high proportion suffer some form of neurological or intellectual damage.

In the Western Cape over a 2 year period (1979-1981) it was found that nearly 50% of patients either died or were handicapped severely from the disease.⁽¹⁴⁾ The national case fatality rate for TBM for all ages in South Africa was 25,5% in 1980.⁽¹⁵⁾

(c) Clinical picture presented in TBM

TBM manifests itself in innumerable ways. The conventional picture of fever, headache, vomiting, photophobia, neck stiffness, and impairment of consciousness is rarely seen at the onset of the disease.⁽⁴³⁾ Clinical features differ at different stages of the disease.

Kennedy and Fallon⁽³³⁾ have staged the disease in the following manner:

(i) Stage I - Patient fully conscious and rational with signs of meningeal irritation but with no focal neurological signs or evidence of hydrocephalus. Lassitude, apathy, anorexia, constipation and slight headaches may occur at this stage.

(ii) Stage II - Patient mentally confused and/or focal neurological signs such as squints or hemiparesis present. Focal damage due to tuberculomas in the brain or cord sometimes causes fits and strokes. Meningeal inflammation may cause adhesions and infarctions and hence fits, impairment of consciousness or cranial nerve palsies may occur.

(iii) Stage III - Patient mentally inaccessible owing to the depth of stupor or delirium and/or complete hemiplegia or paraplegia

or other major neurological abnormalities present. Hydrocephalus with drowsiness, coma and moderate papilloedema may occur at this stage.

Diagnosis of TBM may not be made on the basis of clinical presentations alone; these have to be viewed in conjunction with laboratory findings. Early diagnosis is essential because the stage of the disease at which diagnosis is made is proportional to the outcome. The earlier the diagnosis, the less severe the mental and physical sequelae.

(d) Conventional diagnostic criteria for TBM

Conventionally the following criteria are used for the establishment of a diagnosis of TBM:

(i) three of the following:

- (a) CSF pleocytosis and protein level $> 0,6 \text{ g/l}$
- (b) evidence of tuberculosis, such as chest radiographic appearances, sputum or gastric washings positive on *M. tuberculosis* culture, or a positive tuberculin skin test.

- (c) CSF culture positive, bromide partition ratio $< 1,6$.

(In a study of the bromide partition ratio at King Edward VIII Hospital, Durban, Coovadia, Y.M. et al⁽¹¹⁾ found this test to be both reliable and accurate.)

or CSF adenosine deaminase activity (ADA) $> 5 \text{ U/l}$; and

- (d) Clinical course consistent with TBM; or

(ii) autopsy findings indicating TBM.

TBM is often difficult to distinguish from other causes of meningitis, therefore I will give a brief indication of diagnostic procedures for these.

(e) Diagnostic criteria for Bacterial meningitis

Lumbar puncture is the major definitive investigation in bacterial meningitis. The following criteria are used in the diagnosis of bacterial meningitis:

- (1) CSF cloudy or purulent with pleocytosis; CSF/blood glucose ratio reduced (usually $< 0,4$, g/l); and elevated protein content (usually 0,5-3,0 g/l).
- (2) evidence of bacteria in CSF from Gram stain or positive CSF or blood culture.
- (3) identification of causative organism by detecting the presence of bacterial antigen in CSF with latex agglutination or counter-immuno-electrophoresis (CIE).
- (4) clinical course consistent with bacterial meningitis; or
- (5) autopsy findings indicating bacterial meningitis.

(f) Diagnostic criteria for Viral (Aseptic) meningitis

Diagnosis is usually based on the CSF characteristics (Table I) including normal glucose and failure to grow bacteria on culture. Predominant clinical symptoms include fever, headache, vomiting, malaise, and stiff neck and back. A bromide partition ratio $> 1,6$ is indicative of viral meningitis. A precise diagnosis requires either isolation of the virus by tissue culture; or the use of paired serums to document a rise in antibodies.⁽²⁾

TABLE I : CHARACTERISTICS OF CSF IN INFECTIONS OF THE CENTRAL NERVOUS SYSTEM (after Dawood, A.A.(14)).

	Normal	Pyogenic	Aseptic	Tuberculous
Appearance	Clear	Turbid	Clear	Clear
Cells (mm ³)	0-5	5 - 2000	5 - 500	5 - 1000
	Lymphocytes	Neutrophils mainly	Lymphocytes	Lymphocytes
Protein level (g/l)	0,15 - 0,4	0,5 - 3,0	0,5 - 1,0	1,0 - 6,0
Glucose level (mmol/l)	2,2 - 3,3	Very low	Normal	Low
Other tests		Gram stain	Nil	Ziehl-Neelsen stain

II. INVESTIGATIONS AVAILABLE FOR DIAGNOSIS OF TBM

(a) Microscopy

Microscopic examination of spinal fluid is necessary in all cases of suspected meningitis. This examination includes a cell count, Gram stain, and differential staining techniques.

- (i) Cell Count - This is usually the first investigation performed since delays may cause inaccuracies as well as clot formation. An aliquot of CSF is usually diluted in a 9:1 ratio with counting fluid (crystal violet, azur A. McNeil or malachite green). Counting is done by using a Neubauer Counting Chamber.

In most cases of TBM the cell count is between 100 and 600/cu mm and is principally mononuclear.⁽²⁾ However, Parsons⁽⁴³⁾ reports that a polymorphonuclear leucocytosis of 15 000 to 23 000/mm³ is compatible with a diagnosis of TBM. Non-conforming results are frequent in TBM - some cell counts may only be slightly raised and positive cultures have been obtained from CSF samples with virtually no cellular count.^(2;23)

- (ii) Gram stain. This is performed routinely on CSF's of all suspected cases of meningitis. In cases of TBM no organisms are generally detected in a Gram stain.

- (iii) Staining techniques used in the detection of *M. Tuberculosis*. Mycobacteria are acid-fast, alcohol-fast, non-motile, non spore-forming bacilli. *M. tuberculosis* organisms are found

as slender rods (0,3 - 0,6 x 0,5 - 4,0 μ m) that occur singly, in small clusters, or in "threads" (in culture). Staining may be homogenous or granular; the latter resulting in a banded or beaded appearance.

- (a) Ordinary light microscopy - Ziehl Neelsen or Kinyoun's Acid Fast stain. The acid-fast stain is a differential stain that measures the resistance of a stained cell to decolourization by acids. The property of acid-fastness in mycobacteria is correlated with their high lipid content. Initial staining is done with hot carbol fuchsin, the bacteria are then decolorized with acid-alcohol and counter-stained with methylene blue. Mycobacteria resist decolourization and thus stain red. Non acid-fast organisms stain blue.
- (b) Fluorescence microscopy - under ultra violet light (Auramine Rhodamine stain) or under blue light (auramine-O-stain). The auramine-rhodamine stain has proved more accurate than the Ziehl-Neelsen method.⁽⁴³⁾
- (c) Oil immersion lens (900-1 000 x) - against a blue or green background (conventional acid-fast stains).

The most important finding in TBM is of *Mycobacterium tuberculosis* in the CSF but the frequency with which organisms are seen varies from unit to unit. Some have success rates of 85-100% while others report that it is with difficulty that the organisms are seen or cultured even when it is known that they are present.⁽⁴³⁾ In Durban it is exceedingly uncommon to find *M. tuberculosis* in CSF specimens from patients known to have TBM by other criteria.

(b) Culture Studies

Primary mycobacterial isolation medium includes various egg yolk or whole egg preparations containing malachite green as an inhibitory agent; at least one selective medium of either egg or agar base (containing various inhibitory agents) must also be employed. Growth is very slow. Inoculated culture media is usually incubated at 35-37°C for 8 weeks with weekly inspection for growth. Most strains of *M.tuberculosis* appear within 4 weeks but they may not be visible for 8 weeks or more if they originated from patients treated with anti-tuberculous agents (Kennedy et al, 1979).⁽¹¹⁾

Cultures on Lowenstein-Jensen fresh whole egg medium show raised, rough, wrinkled, buff-coloured granular growth after 3 to 8 weeks of incubation at 37°C. PLATE 1 shows tube culture of *M.tuberculosis* in Lowenstein-Jensen medium.

Strains of *M.tuberculosis* are clearly identifiable by their lack of pigment, failure to grow at 25°C, and sensitivity to p-nitrobenzoic acid.⁽⁵³⁾

(c) Biochemical Studies

(i) CSF Adenosine Deaminase Activity

Adenosine Deaminase (ADA) was first discovered in 1939.^(8;9) It was found to be widely distributed in animal tissues including the blood of normal humans and other mammals. ADA specifically reacts with adenosine and several adenine nucleoside analogues. It has been found that raised ADA levels occur in serum, CSF, and cell lysates in a variety of diseases

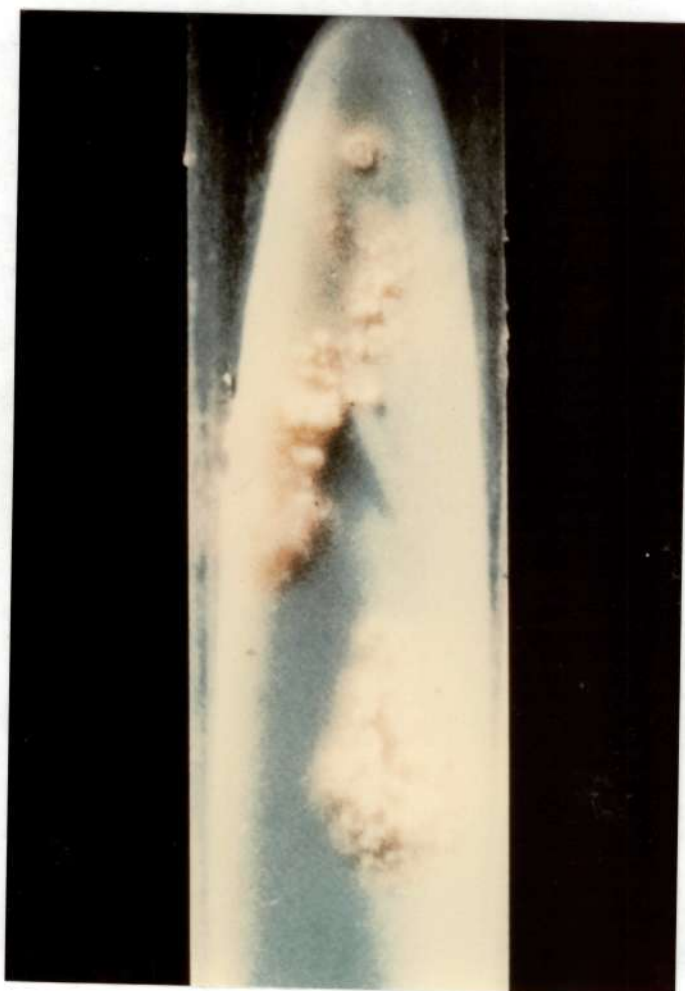


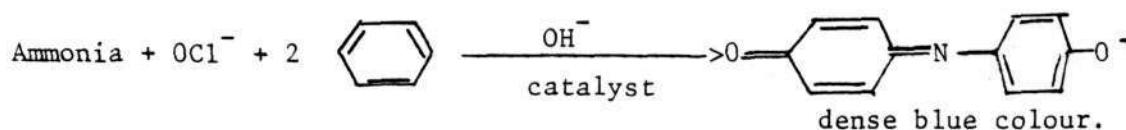
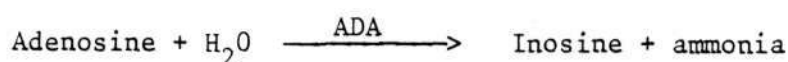
PLATE 1 : Tube culture of *M. tuberculosis*
in Lowenstein-Jensen medium

such as typhoid, malignant tumours and viral hepatitis.^(44;29;35)
 The ADA level in tuberculous pleural effusion is markedly raised.⁽⁴⁵⁾

CSF ADA levels corresponding to different types of meningitis are not well defined. Piras and Gakis,⁽⁴⁴⁾ in a study of 42 patients found that all those with TBM had ADA levels above 8U/l; all those with viral meningitis had levels below 4 U/l; and that most patients with acute bacterial meningitis had levels below 5U/l except two patients with levels above 8U/l. Pirakis and Gakis measured ADA activity repeatedly in 5 patients with TBM. They found that the ADA levels increased 15-30 days after onset of the illness and fell progressively as the patient recovered.

In a study of 58 children in whom the final diagnosis was known, Mann et al,⁽³⁸⁾ in 1982 found that 24 of 33 patients with TBM had ADA levels above 5U/l. Overall, the ADA levels were found to have an accuracy of 86%. Whilst not specific for TBM, CSF ADA levels provide valuable evidence for or against a diagnosis of TBM and should be viewed in the light of clinical and laboratory findings.

The method presently being used to measure ADA activity is based on that of Giusti⁽²⁹⁾ and is a sensitive colorimetric method. ADA breaks down adenosine to form inosine and ammonia which in turn combines with sodium hypochlorite to produce a dense blue colour which is read spectrophotometrically at 620 nm.



(ii) Gas Chromatography Studies

In 1977 Brooks et al,⁽⁴⁾ using gas chromatographs equipped with frequency-pulsed modulated electron capture (FPEC) detectors, detected the presence of a compound with amine characteristics in the CSF of 12 patients with acute untreated TBM. The compound was NOT found in the CSF chromatograph patterns of patients with aseptic and cryptococcal meningitis. In patients with TBM the compound disappeared with effective therapy. Mass spectrometry analysis identified the compound as being 3-(2'-ketoethyl) indoline. Brooks et al, found this to be valuable for differentiating between tuberculous, cryptococcal, and aseptic meningitis.

Further studies by Brooks et al,⁽⁵⁾ published in 1980 describe gas chromatography studies on 260 CSF samples from both tuberculous and non-tuberculous patients from Egypt, USA, Canada and South America. It was found that only 60% of the Egyptian tuberculous patients and none of the culture positive American specimens contained 3-(2'-ketoethyl) indoline. It was found, however, that the carboxylic acid and hydroxy acid chromatograph profiles obtained from the American and Egyptian tuberculous CSF samples were similar. Together with clinical data, these acid profiles may be used effectively in the diagnosis of TBM, even in the absence of 3-(2'-ketoethyl) indoline.

In 1983 Mardh et al,⁽³⁹⁾ reported the presence of tuberculostearic acid in the CSF of a patient with TBM. The CSF sample was taken before antibiotic treatment was instituted. Gas chromatography/

Mass spectrometry techniques were used to detect the tuberculostearic acid. This technique is very sensitive and can detect picogram amounts of the acid. The sample of CSF analysed contained 50 picograms of tuberculostearic acid, i.e., a concentration of approximately 3 ng/mg CSF. Figure 1 shows the mass chromatogram obtained. Tuberculostearic acid is a characteristic constituent of mycobacteria and the demonstration of its presence by gas chromatography is useful as a diagnostic marker for TBM.

(iii) CSF Lactate and Lactate dehydrogenase levels:
enzymatic measurement

As early as 1938 it was reported that CSF lactate and lactate dehydrogenase (LD) levels were reported to be raised in cases of TBM (Donald and Malan).⁽¹⁹⁾ Increases in lactate concentration have also been recorded in patients with bacterial meningitis,^(32;40) cerebral or subarachnoid haemorrhage,⁽⁴⁶⁾ and cerebral infarction.

The cause of increased lactate levels is not certain but is thought to be a result of decreased cerebral blood flow and hence decreased oxygen uptake in the brain. This in turn would result in the anaerobic glycolysis of cerebral tissue which involves both lactate and LD. This is illustrated in Figure 2 below.

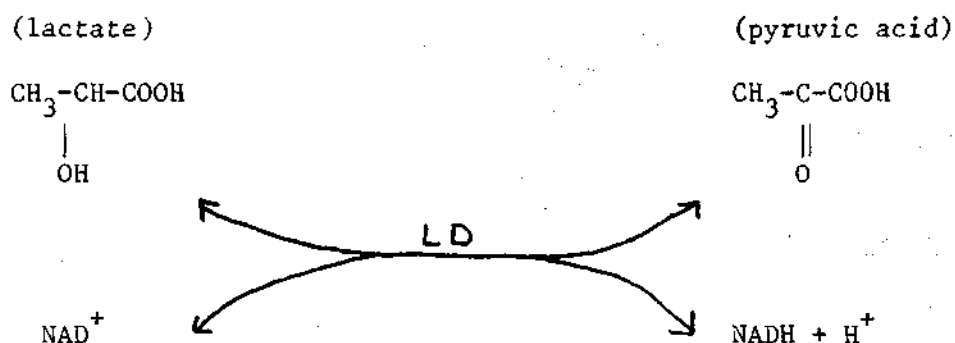


Fig. 2 - The L-Lactate Dehydrogenase Reaction

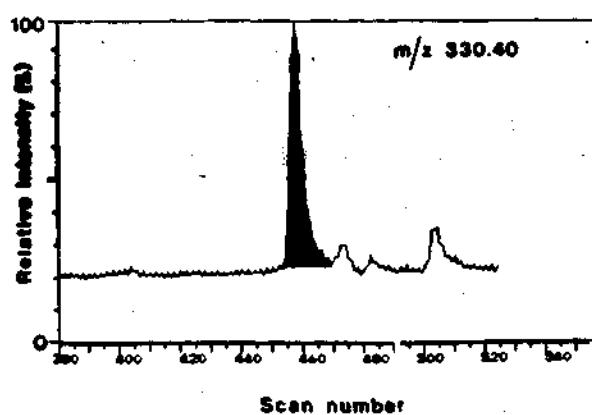


Fig. 1 : Mass chromatogram depicting tuberculostearic acid in CSF of patient with TBM

(after Mardh P.A. et al; 1983)

Donald and Malan⁽¹⁹⁾ in 1985 reported their investigation of CSF lactate and LD levels as a means of distinguishing TBM from aseptic meningitis. Lactate concentration was assayed enzymatically with a Boehringer-Mannheim kit and CSF LD was determined by an optimized standard method (Boehringer Mannheim). The results obtained from a study of 39 patients with TBM showed that although CSF lactate and LD levels were often raised in TBM, this was not always the case.

In the case of CSF lactate levels, the sensitivity of the test was only 69% in detecting TBM. In the case of LD levels, the sensitivity was only 55%.

Neither test was found to hold any marked advantages over conventional biochemical analysis of CSF in the differentiation of TBM from viral meningitis.

(iv) Tryptophan Colour Test

This is a quantitative test for all α -amino-acids, including tryptophan. It entails the oxidative decarboxylation of the amino acid by ninhydrin to produce an aldehyde, carbon dioxide, and ammonia. The reduced ninhydrin then reacts with the liberated ammonia to form a blue complex which maximally absorbs light of wavelength 570 nm.⁽⁴⁰⁾

On the basis of reports that the colour test for tryptophan was beneficial in diagnosing TBM, this test was evaluated in 1980 by Brooks et al.⁽⁵⁾ They reported that many culture positive *M. tuberculosis* specimens gave no colour. In addition a positive

colour test was obtained in CSF samples from patients with herpes, syphilis and granulomatous meningitis. In view of both the false positive and false negative results obtained and also the fact that no free tryptophan was detected by gas chromatography analysis, it was concluded that this test was of no diagnostic value for TBM.

(d) Radio Isotope Studies

Walter, in 1929 showed that the blood-brain barrier was damaged in tuberculous meningitis.⁽³⁷⁾ He was the first to measure the permeability of the blood-brain barrier by estimating the partitioning of administered bromide between blood and CSF. This was expressed as the SERUM/CSF BROMIDE RATIO. Walter observed that after a loading dose of bromide, the relative concentration of bromide in blood and CSF was between 2,9-3,5 (mean 3,1) in normal subjects. In cases of TBM this ratio was found to decrease because the permeability of the blood-brain barrier increased and the relative concentrations of bromide in blood and CSF thus tended to equalise. This is illustrated in Fig. 3 below.

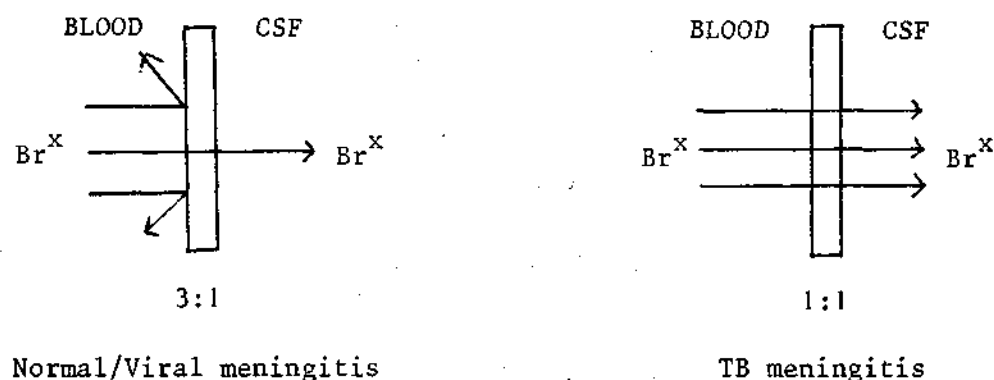


Fig. 3 : The CSF/serum bromide ratio in patients with viral and tuberculous meningitis (after Parsons, M. (1979))

Walter used the gold chloride colorimetric method for his bromide estimations, which became known as the Bromide Partition Test (BPT).

In the 1940's and 1950's several groups of workers^(41; 49; 50) modified and assessed the bromide partition test. Taylor et al⁽⁵⁰⁾ found that the bromide ratio estimated by the gold chloride method was too high as substances other than bromide in the serum were reacting with gold-chloride. He then introduced the iodometric titration method for bromide estimation.

Taylor et al in 1954⁽⁵⁶⁾ confirmed the value of the BPT in the differential diagnosis of meningitis by showing that in the great majority of tuberculous cases the ratio was $< 1,6$, whereas in cases of 'non-purulent', non-tuberculous meningitis, higher values resulted.

The chemical method of bromide estimation proved too difficult technically to be of practical value. In 1960 Crook et al,⁽¹¹⁾ advocated the use of radioisotopes to estimate the bromide ratio. The radioactive isotope used was ^{82}Br which has a half-life of 35,4 hours and emits beta rays of maximum energy 0,4 meV and gamma rays with energies in the range 0,55-1,48 meV. A scintillation counter was used to measure the radioactivity in 48 hour blood and CSF samples after the administration of sodium bromide.

Using the radioactive bromide partition test Crook et al clearly separated two patients with TBM from five patients with non-tuberculous meningitis.

In 1972 Mandal et al,⁽³⁷⁾ confirmed that the radioactive bromide partition test was a valuable procedure for the early diagnosis of TBM. Using the figure of 1,6 as the critical bromide ratio value they found the test to be 100% accurate in both tuberculous and non-tuberculous adult groups.

In a study of 91 children on whom the radioactive bromide (^{82}Br) partition test was performed, Wiggelinkhuizen and Mann (1980) found the test to be 92% accurate. They found that a low bromide partition ratio was not specific for TBM; purulent meningitis, neurosyphilis, spinal block, congenital hypothyroidism, congenital encephalo-ophthalmic dysplasia and multiple sclerosis all resulted in ratios similar to those found in TBM. The test was, however, found to be particularly useful in distinguishing early TBM from viral meningitis.

In 1982 Mann et al,⁽³⁸⁾ confirmed that the bromide partition test (BPT) provided valuable evidence for or against the diagnosis of TBM. In a comparison between the BPT and CSF ADA measurements (discussed previously under Radio-Isotope Studies and Biochemical studies) they found that the BPT proved more accurate than CSF ADA measurements (93% as compared to 86%).

In summary it may be said that the bromide partition test is not specific for TBM and the results should be interpreted in the light of all clinical and laboratory findings. A ratio of below 1,6 is strongly supportive of a diagnosis of TBM and one above it is against the diagnosis. Studies are presently being done concerning the replacement of bromide by technetium and this promises to make the test more sensitive than at present.⁽⁴³⁾

(e) Immunological Studies

(i) Precipitin Test

The precipitin test for mycobacterial antigens was developed in 1929 by Doan.⁽¹⁸⁾ The antibody he used was rabbit antisera to 'phosphatide' (a plasma membrane antigen from *M. tuberculosis*). Doan tested blood sera, spinal fluids, pleural effusions, ascitic fluids and joint fluids from tuberculous infants. Only the CSF of one of 12 infants with TBM failed to give a positive precipitin reaction with the anti-phosphatide rabbit sera. Only 1 false-positive test was recorded in sixteen control spinal fluids. The blood sera of all tuberculous infants tested was strongly positive. However, the blood sera from 9 cases of Hodgkin's disease also gave positive precipitin titres.

(ii) Enumeration of T-cells in CSF by E-rosette formation

The mature T lymphocytes of humans carry receptors which bind to determinants on the surface of sheep red blood cells (SRBC). When mixed with SRBC, each T-cell binds a cluster of SRBC to its surface, forming a rosette (Figure 4). Such 'E' (erythrocyte)-rosettes provide a convenient way to count and identify human T-cells.

El-Naggar and Higashi⁽²¹⁾ reported in 1981 that they had enumerated the number of lymphocytes in the blood and CSF of tuberculous patients by using E-rosettes. They found that the proportion of T-cells in the CSF was greater than that in the peripheral blood. The number of T-cells in the blood of tuberculous patients was significantly lower than that in normal people. However, the number of T-cells in the CSF of normal

people was found to be no different from that in tuberculous patients. No reliable way was found to assay CSF T-cell functional responses to mycobacterial antigens.

Until T-cell enumeration in bacterial and viral meningitis is undertaken, little value may be placed in the ratio of CSF to blood T-cell numbers as a diagnostic marker in patients with TBM.

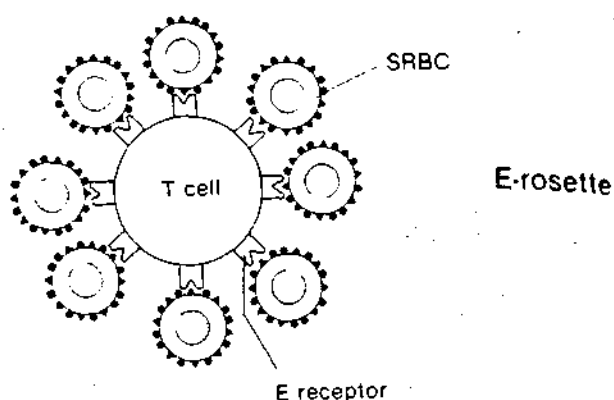


Fig. 4 : E-Rosette formation-mechanism

(iii) Enzyme-linked Immunosorbent Assay (ELISA)

(1) Detection of specific antibodies

Hernandez et al,⁽³⁰⁾ have reported 100% sensitivity and no false-positive reactions whilst using an indirect ELISA to diagnose TBM. Patients with TBM undergo a vigorous humoral immune response in the CNS (central nervous system). This results in the production of oligoclonal antibodies against *Mycobacterium tuberculosis* which also react against *Mycobacterium bovis* BCG. These antibodies are secreted into the CSF.

In the ELISA of Hernández et al, BCG antigen was covalently attached to plastic discs. CSF samples from patients with tuberculous, pyogenic and viral meningitis and from a control group were assayed for immunoglobulin G (IgG) and immunoglobulin M (IgM) activity against *M.bovis BCG*. No overlapping of values from positive and negative samples occurred and there was a marked difference in antibody activity between samples from TBM and the other groups studied. This assay may prove useful in the early diagnosis of TBM.

Kalish et al,⁽³²⁾ have shown the ability of the ELISA method to measure IgG activity against protein-purified derivative (PPD) from *M.tuberculosis* in the CSF of patients with TBM. They found that IgG could be detected in the CSF of all tuberculous patients but not in those of controls. The result of serial studies showed that IgG activity against PPD decreased as the recovery of the patients progressed. Since only three patients with tuberculous meningitis were studied, many more patients will have to be evaluated before this ELISA for IgG against PPD can be used as a means of early diagnosis of patients with suspected TBM.

(2) Detection of mycobacterial antigens

A double antibody sandwich ELISA to detect mycobacterial antigens in the CSF of patients with TBM has been developed by Sada et al.⁽⁴⁷⁾ Since *Mycobacterium bovis BCG* and *Mycobacterium tuberculosis* were found to have the same surface antigens,⁽⁶⁾ antibody against *M.bovis BCG* was used in this assay. The specificity of the test proved to be 95% and the sensitivity was 81,25%. The authors suggest the use of antibodies directed against antigen 5 and 6 of *M.tuberculosis*⁽¹³⁾ which are specific for this bacillus as a means of improving the test.

(iv) Latex Particle Agglutination

Krambovitis and co-workers⁽³⁶⁾ have used latex particle agglutination (LPA) for the detection of *M. tuberculosis* plasma membrane antigen as a means of detecting *M. tuberculosis* in CSF. Rabbit immunoglobulin raised against the plasma membrane antigen was purified and used to passively sensitize latex particles. These particles were mixed with the CSF sample and antigen was indicated if agglutination occurred within three minutes. A positive result was obtained in the initial sample from 17 of 18 children with TBM. Only one false-positive was recorded in the 134 controls tested. The authors state that the LPA test is a very simple and rapid technique requiring small quantities of CSF and is suitable for use in developing countries. Before this test can be adopted for clinical use a high degree of specificity and sensitivity will have to be obtained in trials on a larger group of patients.

(v) C reactive protein measurement by Radioimmunoassay

C reactive protein measurement in sera is measured serially by radio-immunoassay. It has been established that C reactive protein determinations in serum differentiate reliably between bacterial and viral meningitis. De Beer et al,⁽¹⁵⁾ undertook an investigation to determine whether C reactive protein measurements in patients with TBM differed appreciably from those of patients with viral and bacterial meningitis. C reactive protein measurements were made in sera from 31 children with bacterial meningitis, 15 with TBM, and 28 with viral meningitis.

It was found that C reactive protein measurements in patients with TBM were intermediate, lying between those of bacterial and viral meningitis. It was also found that C reactive protein concentrations fell rapidly after treatment began and became normal after 10 days. This occurred in all but two of the patients with TBM. This rapid decline in concentration after treatment is of diagnostic value for TBM. Although not conclusive, it remains a useful additional parameter in the diagnosis and management of tuberculous meningitis.

(f) Currently-used method for diagnosis of TBM

To date no single diagnostic test for TBM has proved to be totally confirmatory of the disease. Many of the techniques already discussed may prove helpful in the diagnosis of TBM if used in conjunction with the CSF laboratory profile and the clinical picture.

Some of the following criteria are used currently for the establishment of a diagnosis of TBM:

- (1) CSF pleocytosis and protein level $> 0,6 \text{ g/l}$
- (2) evidence of tuberculosis such as chest radiographic appearances, sputum or gastric washings positive on *M.tuberculosis* culture
- (3) positive tuberculin skin test
- (4) CSF culture positive
- (5) bromide partition ratio $< 1,6$
- (6) CSF adenosine deaminase activity (ADA) $> 5 \text{ U/l}$
- (7) CSF glucose content less than half that of simultaneously obtained blood
- (8) clinical course consistent with TBM

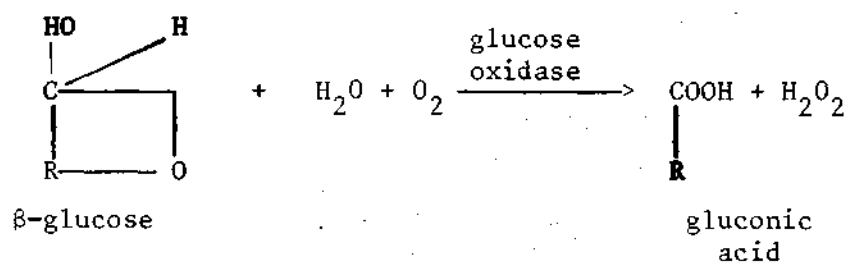
It must be stressed that 'non-conforming' bacteriological, cytological, biochemical and clinical data are frequently occurring in patients with TBM. The absence of any of the above-mentioned criteria should not be reason to dismiss the possible diagnosis of TBM in a patient. Diagnosis is strongly suspected when there is a past history of TB or of exposure to someone with it, or when demonstrable tuberculous foci are present elsewhere in the body.

1. Routine Studies on CSF

Each sample of CSF was subjected to routine cytological and biochemical tests, using standard techniques. These tests included measurement of chloride levels; total protein and globulin content; and glucose levels. In addition, direct smears, Gram stains and cell counts were made. If organisms were identified, identification was confirmed by culturing or by bacterial agglutination kits. If no organisms were visible and the CSF profile resembled that of TBM, then CSF culture for *M. tuberculosis* was undertaken.

(a) Measurement of Glucose in CSF

(Standard technique performed routinely in the laboratory using a fully automated instrument.) The instrument used was the BECKMAN ASTRA-8, Automated Stat/Routine Analyser. The method of measurement is based on an enzymatic reaction, the GLUCOSE OXIDASE REACTION.



The oxygen consumed in the oxidation of glucose to gluconic acid is measured by an oxygen electrode. The rate of oxygen consumption is proportional to the initial glucose concentration in the sample.

(b) Measurement of Globulin and Total Protein in CSF

Both total protein and globulin measurements are made using a fully automated instrument, the BECKMAN ASTRA-8 Automated Stat/Routine .

III. METHODS

Analysers. The instrument simultaneously determines total protein (by the BIURET METHOD) and makes a direct spectrophotometric determination of globulin levels. This combination of determinations allows the calculation of CSF albumin by subtracting the globulin value from the total protein.

Total globulin is measured by the reaction of the tryptophan residues of the globulin molecules with glyoxylic acid in a strongly acidic medium. The coloured product of the reaction absorbs strongly at 550 nm. Globulin is recorded as negative; trace; one, two or three plus; depending on the density of the coloured product.

The principle of total protein measurement involves the precipitation of protein by trichloroacetic acid. The degree of turbidity is measured and is related to the quantity of protein present.

(c) Method for Cell Count in CSF

(Standard technique using a light microscope and a NEUBAUER counting chamber). Since the number of blood cells found in CSF is generally very small, manual counting techniques were employed.

(d) Measurement of CSF Chloride level

Standard technique used. Fully automated procedure with BECKMAN ASTRA-8 Automated Stat/Routine Analyser. Method of measurement was colorimetric titration. Both electrode and cathode comprised of silver chloride.

(e) CSF Culture for *M. tuberculosis*

A loopful of CSF is streaked on Lowenstein-Jensen (LJ) medium and incubated at 37°C for eight weeks. Smears are prepared from growth, if any, and stained by Ziehl-Neelsen's technique.

2. Correlation of Blood and CSF Chloride levels

(i) Patients

Blood and CSF samples taken within about four hours of each other were collected from a total of 149 Black children admitted to King Edward VIII hospital during the six-month period February-August 1985. In addition, a detailed case-history and record of the clinical examination was made. Each sample was subjected to the following studies:

- (a) Routine cytological and biochemical tests were done on both the blood and CSF, using standard techniques.
- (b) Blood and CSF chloride measurements were made.
- (c) CSF was cultured for *M. tuberculosis* on LJ medium.
- (d) All patients were followed up as far as possible, to confirm or reject the initial diagnosis.

For the establishment of a diagnosis of TBM, the following criteria were used:

- (a) CSF pleocytosis; predominantly lymphocytes (5-1000 cells per mm³).
- (b) protein level > 0,6 g/l
- (c) ancilliary evidence of tuberculosis such as chest radiographic appearances, sputum or gastric washings positive on *M. tuberculosis* culture, or a positive tuberculin skin test

- (d) CSF culture positive, bromide partition ratio $< 1,6$; or
CSF ADA $> 5U/l$
- (e) Clinical course consistent with TBM
- (f) Autopsy findings indicating TBM.

The diagnostic criteria for viral meningitis were:

- (a) CSF characteristics - clear: $5-500 \text{ cells/mm}^3$, mainly lymphocytes; protein level of $0,5-1,0 \text{ g/l}$
- (b) Normal CSF glucose level
- (c) Clinical picture consistent with viral meningitis
- (d) Bromide partition ratio $> 1,6$.

The diagnostic criteria used for bacterial meningitis were:

- (a) CSF cloudy or purulent with pleocytosis; CSF/blood glucose ratio reduced (usually $< 0,4 \text{ g/l}$); and elevated protein content (usually $0,5-3,0 \text{ g/l}$)
- (b) evidence of bacteria in CSF from Gram stain or positive CSF or blood culture
- (c) detection of bacterial antigens via latex agglutination or CIE.
- (d) clinical course consistent with bacterial meningitis.
- (e) autopsy findings indicating bacterial meningitis.

(ii) Measurement of Chloride

This procedure was fully automated. The mode of measurement was via colorimetric titration, using silver chloride for both the cathode and the anode. The instrument used was the BECKMAN ASTRA-8 AUTOMATED STAT/ROUTINE ANALYSER. Measurements were made in mmol/l .

3. Inhibition of BCG-anti-BCG reaction by *M.tuberculosis* antigen using ELISA


The diagnosis of TBM on clinical grounds alone is very difficult. Many non-specific tests such as routine examination of CSF, bromide-partition test,⁽³⁷⁾ adenosine deaminase estimation⁽²⁹⁾ available so far are not very useful. The low bacterial density of CSF from patients with TBM makes bacteriological diagnosis with smears and culturing very difficult especially in the Third World. It has been established that the neurological sequelae and fatalities associated with diagnosis of TBM at an advanced stage of the disease are severe compared to that in early diagnosis.

An enzyme-linked immunosorbent assay (ELISA) introduced by Sada et al was standardized to detect the presence of mycobacterial antigens in the CSF from patients with meningitis. Pleural and ascitic fluid samples from patients with pulmonary and abdominal TB were also tested for the presence of soluble mycobacterial antigens.

Antibody directed against *M.bovis* BCG and coupled to alkaline phosphatase was used in the assay. The test sample was pre-incubated with an optimal dilution of unconjugated antibody. If antigens were present in the CSF, pleural or ascitic fluid, these would combine with the antibody to form an immune-complex. When the solid-phase antigen (BCG) was reacted with this pre-incubated mixture, no antibody would be available to complex with the solid-phase BCG antigen. Hence, the antibody-enzyme complex would have no binding site and no colour production would result.

If, on the other hand, no antigen were present in the test specimen, antibody from the pre-incubated sample would attach to the solid-phase antigen (BCG). The antibody-enzyme complex, when added, would link onto the antibody-solid phase antigen complex. When the enzyme substrate is introduced, this would be acted upon by the enzyme to produce a colour reaction product (PLATE 2). The intensity of the colour produced is proportional to the amount of hydrolysis undertaken by the enzyme and also to the amount of soluble antigen in the test sample. Fig. 5 illustrates the above.

Before the ELISA could be employed in the diagnosis of TBM, the technique had to be standardized. The variables that existed are listed below:

- (i) Types of microtitre plate
- (ii) Optimal dilutions of 
 - conjugate
 - antibody
 - solid-phase antigen
- (iii) Time (length of incubation)
- (iv) Temperature (of incubation)

Each of the above-listed criteria was investigated in detail before the ELISA was utilized to detect mycobacterial antigens in CSF.

(i) Testing of Microtitre plates for acceptability

(Voller, A., Bidwell, D.E. and Bartlett, A.)⁽⁵²⁾

Reagents: All reagents prepared under sterile conditions.

(1) Coating buffer

Carbonate-bicarbonate buffer 0,05M pH 9,5

(2) Antigen

Human IgG

100 ng/ml solution made up in coating buffer

(3) Washing buffer

0,01M phosphate-buffer saline (PBS) pH 7,4 and 0,05%

Tween 20 (polyoxyethylene sorbitan monolaurate)

(4) Conjugate

Alkaline-phosphatase conjugated (Rabbit) Anti-Human IgG

M.A. Bioproducts B30-315.

(5) Substrate

p-nitrophenylphosphate disodium salt (Merck Cat. No. 6850)

(6) Substrate Buffer

10% Diethanolamine buffer pH 9,8 containing 0,2% NaN_3

(7) Plastic Microtitre plates

(a) Titertek polyvinylchloride immunoassay plate

77-773-05. Activated.

(b) Dynatech. Removastrip Systems of Immulon[®]

polyvinylchloride immunoassay wells

(c) Nunc flat-bottomed polystyrene immunoassay plate # 442404.

(d) Nunc flat-bottomed polystyrene immunoassay strips

469914. Modules F-16.

(8) IN NaOHProcedure: (under sterile conditions)

Microtitre plates were checked by adding 200 μl of human IgG solution (100 ng/ml) made up in coating buffer to each well and incubating overnight at 4°C.

The coating solution was aspirated and each well was washed three times with PBS-Tween. 200 μ l of conjugate (diluted $1/1000$ in PBS-Tween) was then added to each well and incubated for 18 hours at 4°C . The conjugate was removed by aspiration and the wells were again washed three times with PBS-Tween.

A fresh solution (1 mg/ml) of the substrate was made up in diethanolamine buffer and 200 μ l was added to each well and incubated for 30 minutes at room temperature.

The reaction was stopped by adding 50 μ l of 1N NaOH to each well.

Absorbance of contents of each well was read at 405 nm. The plate giving the least total variation of values was chosen for use in the test. NUNC flat-bottomed plates ~~X~~442404 were found to be most acceptable. (Table VI).

(ii) Determination of optimal test conditions

(a) Enzyme Immunoassay (EIA) for Alkaline Phosphatase conjugates

The method used to determine the working dilution of the alkaline phosphatase conjugates was supplied by Bio-Yeda Ltd. of Kiryat Weizmann/Rehovot/Israel.

Reagents: All reagents prepared under sterile conditions.

(1) Coating buffer

Bicarbonate buffer - 0,05M pH 9,5

(2) Antigen

Behring Human IgG (γ -chain).

Concentration of antigen was 2,9 g/l. Diluted 966 μ l antigen in 13034 μ l coating buffer to make a 200 μ l/ml solution.

(3) Conjugate

Alkaline-phosphatase conjugated goat anti-rabbit IgG(H+L).

Bio Yeda Code No 4471.

Dilutions of 1:100; 1:150; 1:200; 1:300; 1:800; 1:1200; 1:1600; 1:2400 and 1:3200 were made up in washing buffer under sterile conditions.

(4) Negative Control or Blank

Bovine serum albumin (BSA) from Sigma Chemical Co

No A-403. Fraction V powder 96-99% albumin.

Diluted 0,002g BSA in 5 ml PBS pH 7,4 to make a 200 µg/ml solution.

(5) Phosphate-Buffer Saline (PBS)

0,01M phosphate buffer pH 7,4 containing 0,15 M NaCl and 0,1% NaN_3 .

(6) Washing Buffer

PBS and 0,05% Tween 20 (polyoxyethylene sorbitan monolaurate).

(7) Substrate buffer

10% Diethanolamine buffer pH 9,8 containing 0,2% NaN_3 .

(8) Substrate

p-nitrophenyl phosphate disodium salt (MERCK Cat. No 6850)

Dissolved immediately prior to use in substrate buffer.

This was done by pre-warming 12 ml of substrate buffer to room temperature and then adding 12 mg of substrate to give a final concentration of 1 mg/ml.

(9) Polystyrene tubes 12 x 75 mm(10) NaOH - IN(11) H₂O - distilled waterProcedure (under Sterile Conditions)

- (1) The polystyrene tubes were coated by pipetting 1 ml of antigen solution (200 µg/ml) into each tube. The tubes were covered with parafilm and incubated overnight at room temperature.

- (2) The coating solution was aspirated and the tubes were washed three times with PBS + Tween 20 and then three times with water, aspirating each time.
 - (3) Various dilutions of the conjugate, ranging from $1/100$ to $1/3200$ were made up in washing buffer. One ml of each dilution was added to each of the polystyrene tubes. The tubes were then covered with parafilm and incubated at room temperature for two hours.
 - (4) The conjugate solution was removed by aspiration and the tubes were washed as in step (2).
 - (5) 1 ml of freshly prepared 1 mg/ml substrate solution was then added to all 12 tubes. These were incubated at room temperature for 30 minutes.
 - (6) At the end of the incubation period 0,4 ml NaOH was added to terminate the reaction.
 - (7) The absorbance of each tube was read at 405 nm using a Beckman spectrophotometer.
- A 200 μ g/ml solution of BSA was used as a blank.
- (8) The dilution of conjugate which gave an absorbance reading of approximately 1,0 was chosen as the working dilution for enzyme immunoassays. (This was found to be a 1:2000 dilution).

Fig 12, Table V.

- (b) Selection of optimum amount of the solid-phase antigen (chequer-board titration using *Mycobacterium bovis* BCG antigen and reference sera)

The methods used to determine the optimal concentrations of *M. bovis* BCG antigen for coating the microtitre plates was adapted from a method used by Voller et al, ⁽⁵²⁾ for measles antigen.

Reagents: All reagents prepared under sterile conditions.

(1) Antigen

BCG VACCINE (DRIED). Japanese strain 172. Total mass 24 mg.

Manufactured by Dept. of Health and Welfare, State Vaccine Institute, Cape Town.

Reconstitution of one vial with 0,3 ml sterile distilled water.

Resulting BCG solution 80 000 µg/ml. 10 µl of this solution mixed with 4990 µl coating buffer resulted in STOCK ANTIGEN solution (160 µg/ml BCG). Required BCG antigen solutions in the following concentrations (µg/ml): 20; 10; 5; 2; 0,5; 0,2.

Prepared as follows using STOCK ANTIGEN solution and coating buffer.

Required Ag concentration (µg/ml)	Amt. of Stock Antigen required (µl)	Amt. of Coating Buffer required (µl)
20	500	3500
10	250	3750
5	125	3875
2	50	3950
0,5	12,5	3987,5
0,2	5	3995

(2) Coating buffer

Carbonate-bicarbonate buffer 0,05 M pH 9,5

(3) Washing buffer

0,1 M phosphate-buffered saline (PBS) pH 7,4 and 0,05% Tween 20.

(4) Reference sera

POSITIVE SERUM - Rabbit IgG anti-BCG. Dakopatts Code No B124.

NEGATIVE SERUM - Normal rabbit serum. Dakopatts Code No X903

Serial dilutions ranging from $1/50$ to $1/3200$ of each reference

sera made up in washing buffer in polystyrene tubes.

(5) Conjugate

Alkaline-phosphatase conjugated goat anti-rabbit IgG (H + L).

Bio Yeda Code No 4471.

A $1/2000$ dilution was made up by mixing 5 μl conjugate with 9995 μl washing buffer under sterile conditions.

(6) Substrate

p-Nitrophenylphosphate disodium salt (Merck Cat No 6850).

Dissolved < 30 minutes prior to use in diethanolamine buffer to form a 1 mg/ml solution.

(7) Substrate buffer

10% Diethanolamine buffer pH 9,8 containing 0,2% NaN_3

(8) IN NaOH

(9) Nunc microtitre plate ~~✕~~ 442404

(10) Humidity chamber

Airtight plastic container lined with damp 'Spontex' sponges.

Procedure : Under Sterile Conditions

- (1) Various dilutions of the BCG antigen (20 $\mu\text{g}/\text{ml}$; 10 $\mu\text{g}/\text{ml}$; 5 $\mu\text{g}/\text{ml}$; 2 $\mu\text{g}/\text{ml}$; 0,5 $\mu\text{g}/\text{ml}$ and 0,2 $\mu\text{g}/\text{ml}$) were made up in coating buffer. A microtitre plate was divided into halves by a vertical line through the centre. 100 μl of each dilution was added to a vertical row of wells in each half of the microplate. The plate was incubated with a lid, overnight in a humid box at $+4^{\circ}\text{C}$. This allowed the antigen to absorb to the plastic surface.
- (2) The wells were emptied by aspiration, then washed by the addition of 250 $\mu\text{l}/\text{well}$ of PBS-Tween, leaving for a few minutes and emptying. This process was repeated 3 times and plates were then shaken dry and allowed to drain on 'Spontex' sponges.

- (3) 50 μ l of two reference sera (positive and negative for anti-bodies to BCG antigen) serially diluted in PBS Tween were added to vertical rows of wells so that each serum dilution was reacted with each antigen dilution. A row containing only PBS Tween was included. Plate was then incubated 2 hours at room temperature in a humid box.
- (4) Plates were emptied by aspiration then washed as before.
- (5) The stock conjugate was diluted in PBS-Tween and 50 μ l amounts were added to each well. The plate was then incubated for 3 hours at room temperature. Plates were emptied and washed as before.
- (6) 50 μ l of substrate solution were added to each well and incubation was allowed to proceed at room temperature for 30 minutes. The reaction was stopped by the addition of 25 μ l 1N NaOH to each well.
- (7) Absorbance of contents of each well was read at 405 nm with an EIA Reader Model EL-307, BIO-TEK INSTRUMENTS, INC.
- (8) The combination of the antigen/serum dilutions which gave maximal separation between positive and negative test samples but still gave low negative values (under 0,2) was chosen for the subsequent tests. (Table VII, Fig 13.) Results on page 76.

(c) Optimal Antibody dilution required for inhibition assay

In order to attain maximal inhibition of interaction between the solid-phase antigen (*M.bovis* BCG) and the antibody - enzyme conjugate, the inhibition assay had to be standardized.

Various dilutions of antibodies raised against BCG (rabbit IgG anti-BCG, DAKOPATTS), ranging from $1/800$ to $1/3200$ were made up in PBS containing 0,05% Tween 20 and 1% BSA. These various dilutions were mixed, in 100 μl amounts, with 100 μl of BCG standard solutions (0; 0,002; 0,02; 0,2 and 20 $\mu\text{g}/\text{ml}$) so that each antibody dilution was reacted with each BCG dilution. This was incubated overnight at 4°C . The inhibition assay was then carried out as described in the foregoing; and the absorbance was recorded.

A 1:1200 anti-BCG dilution was chosen to interact with 0,1 ml CSF in the inhibition assay (Fig 14).

(d) Sample and Conjugate Incubation

The ELISA technique described on Page 43 was carried out using sample and conjugate incubation periods ranging from 1 hour at 45°C and 2-3 hours at 37°C to overnight at 4°C in order to determine optimal incubation periods. No significant differences were obtained after incubation for 1 hour at 45°C , 2-3 hours at 37°C , or overnight at 4°C .

(iii) Patients

A total of 84 samples were studied. Of these, 59 were CSF samples and 25 were pleural and ascitic fluid samples. All samples were collected from King Edward VIII Hospital, Durban together with a case-history and details of clinical examination.

The samples were subject to the following investigations:

- (1) Routine cytological and biochemical tests were done, using standard techniques.

- (2) Culture for *M. tuberculosis* - a loopful of sample was streaked on LJ medium and incubated at 37°C for 8 weeks. Smears were prepared from growth, if any, and stained with Ziehl-Neelsen's technique.
- (3) ELISA was done on each sample as described in the foregoing.
- (4) All patients were followed up as far as possible to confirm or reject the initial diagnosis.

Of the 59 CSF samples; 3 were culture-positive *M. tuberculosis* specimens; 7 were diagnosed as being TBM on the basis of autopsy findings, or recovery on anti-tuberculous treatment. Ten CSF samples were from culture-positive bacterial meningitis patients. Twenty-five control samples were from patients who were admitted with signs of meningeal irritation but had normal CSF profiles. Fourteen samples were from patients with aseptic (viral) meningitis.

Of the 25 pleural and ascitic fluid samples; 15 were diagnosed as being from patients with pleural and abdominal tuberculosis on the basis of culturing, acid-fast staining, or recovery with anti-tuberculous treatment. Ten control samples were collected from patients with ascites caused by malignancies, bilharzia, trauma or amoebiasis.

The criteria used to diagnose TBM, viral (aseptic) and bacterial meningitis are listed on Pages 6 and 7.

(iv) Antigen Coating of Microtitre Plates

Reagents: Prepared under sterile conditions.

(1) Coating buffer

Carbonate-bicarbonate buffer 0,05 M pH 9,5

(2) Microtitre plates

NUNC flat-bottomed polystyrene immunoassay plate. ✕442404

(3) Antigen

BCG Vaccine (DRIED). Japanese strain 172. Total mass 24 mg.
Manufactured by Dept of Health and Welfare, State Vaccine
Institute, Cape Town.

Reconstitution of one vial by addition of 0,3 ml sterile
distilled water. Diluted this reconstituted vaccine in
coating buffer to produce a 10 µg/ml solution of BCG.
This was done by adding 5 µl of reconstituted vaccine
(80 000 µg/ml concentration) to 995 µl coating buffer,
hence a 400 µg/ml solution resulted. Diluted this further
in 39 ml coating buffer and stored in aliquots at -70°C.

(4) Blocking buffer

0,5% Bovine Serum Albumin (BSA). Sigma No A-403.
Dissolved in phosphate-buffer saline (PBS) 0,01 M pH 7,4

(5) Washing buffer

0,01 M PBS pH 7,4 and 0,05% TWEEN 20 and 1% BSA

(6) Humid box

Procedure: Under Sterile Conditions

- (1) Wells in a microtitre plate are sensitized by addition of 100 µl amounts of a 10 µg/ml BCG antigen solution to each well, followed by overnight incubation at 4°C in a humid box.
- (2) The wells are emptied by aspiration of coating solution.
- (3) The remaining binding sites on the surface of each well are blocked by the addition of 250 µl/well of PBS-BSA, then incubating for 1 hour at room temperature.

- (4) The wells are emptied by aspiration.

Each well is washed by the addition of 250 μl washing buffer, waiting for 5 minutes, then emptying by aspiration. This washing procedure is repeated three times.

The sensitized plate is then shaken dry and allowed to drain on 'Spontex' sponges.

- (5) Sensitized plates are sealed individually in aluminium foil and stored at -70°C for up to 6 months.

(v) Elisa Technique

(Fig. 5)

The method used was described by Voller et al, (1976)⁽⁵²⁾. The method was modified and standardized before it could be applied in the detection of mycobacterial antigens.

Reagents: Prepared under sterile conditions.

(1) Antibody

Rabbit IgG anti-Bacillus Calmette-Guérin (BCG)

DAKOPATTS Code B124

Diluted $1/1200$ in washing buffer

(2) Antibody-Enzyme Conjugate

Alkaline-phosphatase Conjugated anti-rabbit IgG (H+L)

Raised in goat. BIO YEDA code no 4471

(3) Substrate

p-Nitrophenyl phosphate disodium salt (MERCK CAT. NO 6850)

Dissolved < 30 minutes prior to use in diethanolamine buffer to form a 1 mg/ml solution.

- (4) Substrate buffer
10% Diethanolamine buffer pH 9,8 containing 0,2% NaN_3
- (5) 1N NaOH
- (6) Humid box
- (7) Micro-ELISA reader
- (8) Washing buffer
0,01 M PBS pH 7,4 and 0,05% TWEEN 20 and 1% BSA
- (9) NUNC flat-bottomed microtitre plate ~~X~~442404 coated with
BCG solution (10 $\mu\text{g/ml}$).

Procedure: Under Sterile Conditions

- (1) 100 μl amounts of both the first antibody and the sample to be tested are mixed together in an unsensitized well and incubated overnight at 4°C in a humid box.
- (2) 100 μl amounts of the contents from each unsensitized well is then transferred carefully by pipetting (without disturbing any sediments) into a corresponding well in an antigen-sensitized microtitre plate.
The sensitized plate is then covered and incubated overnight at 37°C in a humid box.
- (3) The wells are emptied by aspiration then washed by adding 250 μl amounts of washing buffer, leaving for a few minutes, then emptying. This washing procedure is repeated three times.
- (4) 100 μl amounts of conjugate (diluted $1/2000$ in washing buffer) are added to each well. The plate is then covered and incubated for 2 hours at 37°C in a humid box.

- (5) The wells are emptied by aspiration and washed thrice as before.
- (6) 100 μ l amounts of freshly prepared 1 mg/ml p-nitrophenyl phosphate solution is added to each well.
The plate is then incubated for 30 minutes at room temperature.
- (7) The reaction is stopped by the addition of 50 μ l amounts of 1N NaOH to each well.
- (8) Absorbance of contents of each well is read at 405 nm with an EIA READER MODEL EL-307; BIOTEK INSTRUMENTS, INC.

4. Double Antibody Sandwich ELISA

TBM occurs commonly in developing countries - up to 20 cases per 100 000 members have been reported in some countries.⁽⁵¹⁾ It is virtually impossible for a patient to recover spontaneously without treatment. The average duration of the untreated illness is about 17 days from onset to death. It has been established that a correlation exists between the stage of the disease at the commencement of chemotherapy and the prognosis. The earlier the diagnosis is made, the better the chances of recovery without major neurological sequelae.

The diagnosis of TBM is made presently on the basis of clinical picture, positive Tuberculin skin test, previous exposure to TB, and on the CSF profile (both cytological and biochemical). In addition, other methods such as the radioactive bromide partition ratio and the CSF adenosine deaminase activity are used in the diagnosis of TBM. These methods prove helpful but are certainly not confirmatory in the

diagnosis of TBM. Sophisticated and expensive equipment that is required is usually not available in the rural areas where the incidence of TBM is high.

Various factors had to be investigated and standardized before the double antibody sandwich ELISA could be utilized to detect mycobacterial antigens in CSF. These preliminary studies are listed below:

- (i) Conjugation of the enzyme to anti-BCG.
- (ii) Testing the acceptability of various microtitre plates.
- (iii) Optimal dilutions of:
 - (a) conjugate
 - (b) solid-phase antibody
- (iv) Optimal incubation conditions of:
 - (a) time
 - (b) temperature.

The methods used to investigate the above-mentioned criteria are discussed below.

(i) Conjugation of Alkaline-phosphatase to antibodies raised against BCG

Conjugation of the enzyme to rabbit immunoglobulin G (IgG) raised against BCG antigens was done by the 'Two-step glutaraldehyde method' of Avrameas (1969).⁽¹⁾

Reagents: prepared under sterile conditions

(1) Antibody

Rabbit IgG Anti-Bacillus Calmette-Guérin (BCG)

Dakopatts ~~X~~ B124.

- (2) Enzyme
Phosphatase, alkaline from calf intestine. Cat. No. 108 138.
Boehringer Mannheim. Ammonium sulphate suspension.
- (3) Phosphate-buffer saline (PBS)
pH 6,8
- (4) TRIS buffer
0,05 M, pH 8,0
- (5) TRIS buffer
0,05 M, pH 8,0 containing 1% albumin, bovine serum (BSA) and
0,02% sodium azide (NaN_3)
- (6) Glutaraldehyde - 25% aqueous solution
- (7) Dialysis tubing

Procedure:

- (1) The protein content of the antibody and the enzyme were determined by the standard 'Lowry method' (Appendix A).
- (2) 40 μl of the IgG (protein content 10 mg/ml) is added, together with 160 μl alkaline-phosphatase (protein content 0,8 mg/161 μl), and 200 μl PBS pH 6,8, into a dialysis sac and mixed at room temperature. The mixture is then dialysed for 18 hours at 4°C against PBS (with changes of buffer) to remove ammonium sulphate.
- (3) While the solution is gently stirred, 27 μl of a 25% aqueous solution of glutaraldehyde is added to yield a final concentration of 0,2%. The mixture is allowed to react at room temperature for 2 hours; then is dialysed overnight at 4°C against PBS with several changes of buffer.

- (4) The dialysis sac containing the conjugate is transferred to 0,05 M TRIS buffer pH 8,0 and dialysed overnight at 4°C with changes of buffer.
- (5) The dialysis sac contents (conjugate) is diluted to 2,0 ml with 0,05 M TRIS buffer containing 1% BSA and 0,02% sodium azide, then is stored in the dark at 4°C.

(ii) Testing of Plates for acceptability in Double Antibody Sandwich ELISA

This was done by the same method as that used to test the acceptability of plates for the 'Inhibition ELISA'. The method is detailed on Page 32.

(iii) Determination of Optimal test conditions

(a) Working dilution of the Enzyme-Antibody conjugate

The method used to determine the optimal dilution for the alkaline-phosphatase labelled rabbit IgG anti-BCG conjugate used in the DOUBLE ANTIBODY SANDWICH ELISA was similar to that for the conjugate used in the INHIBITION ELISA. This method is detailed on Page 34. Dilutions of the conjugate ranging from 1:50 to 1:3200 were assayed to select a dilution which gave an absorbance of approximately 1,0 which was considered optimum.

- (b) Selection of optimum amount of solid-phase antibody -
(chequerboard titration using antibodies raised against
Mycobacterium bovis BCG and reference antigens)

The method used to determine the optimal concentrations of rabbit immunoglobulin G (IgG) raised against BCG required for coating the microtitre plates was adapted from that of Voller et al,⁽⁵²⁾ for measles antigen.

Reagents: Prepared under Sterile Conditions

(1) Antibody

Rabbit IgG anti-Bacillus Calmette-Guérin (BCG)

DAKOPATTS Code No. B124

Serial dilutions ranging from $1/100$ to $1/3200$ made up in coating buffer (in sterile polystyrene tubes).

(2) Coating buffer

Carbonate-bicarbonate buffer 0,05 M pH 9,5

(3) Washing buffer

0,01 M phosphate-buffer saline (PBS) pH 7,4 and 0,05% TWEEN 20

(4) Reference antigens

Negative antigen - Albumin, Bovine (BSA). Fraction V powder, 96-99% albumin. SIGMA A-4503.

Positive antigen - BCG VACCINE (Dried). Japanese strain 172.

Total mass 24 mg. Reconstituted with 0,3 ml. sterile distilled water. 5 μ l of reconstituted solution mixed with 9995 μ l washing buffer to produce 40 μ g/ml stock BCG solution.

Both negative and positive antigens are diluted with washing buffer to produce solutions with the following concentrations: 50 μ g/ml; 20 μ g/ml; 10 μ g/ml; 5 μ g/ml; 2 μ g/ml; 0,5 μ g/ml and 0,2 μ g/ml.

(5) Conjugate

Rabbit IgG anti-BCG conjugated to alkaline phosphatase.

A $1/100$ dilution of the conjugate is made up in washing buffer by mixing 0,1 ml conjugate and 9,9 ml buffer.

(6) Substrate

p-Nitrophenyl phosphate disodium salt. (Merck ~~6850~~ 6850).

Dissolved < 30 minutes prior to use in substrate buffer to produce 1 mg/ml solution.

(7) Substrate buffer

10% Diethanolamine buffer pH 9,8 containing 0,2% sodium azide.

(8) 1N NaOH(9) Micro-ELISA reader

EIA READER MODEL EL-307, BIO-TEK INSTRUMENTS, INC.

(10) NUNC flat-bottomed microtitre plate ~~X~~ 442404.Procedure: Under Sterile Conditions

- (1) Serial dilutions of the antibody (Rabbit IgG anti-BCG) ranging from $1/100$ to $1/3200$ are made up in coating buffer. 50 μ l of each dilution are added to a vertical row of wells in each half of the microplate. The plate is divided by a vertical line running down the centre.

The plate is covered and incubated overnight at 4°C in a humid box. This allows the antibody to absorb to the plastic surface.

- (2) The plate is emptied by aspiration, then washed by adding 250 μ l/well of washing buffer, leaving a few minutes, then emptying. This process is repeated three times and plate is then shaken dry.

- (3) 50 μl of two reference antigens (positive and negative for antibodies to BCG antigen), in solutions of various concentrations (refer to 'Reagents') are added to horizontal rows of wells so that each antibody dilution is reacted with each antigen dilution. A row containing only washing buffer is included. Plate is then incubated 2 hours at room temperature in a humid box.
 - (4) Plates are washed as before.
 - (5) The stock conjugate (alkaline phosphatase-rabbit IgG anti-BCG) is diluted by $1/100$ in washing buffer and 50 μl amounts are added to each well. The plate is then incubated for 3 hours at room temperature.
 - (6) Plates are washed as before.
 - (7) 50 μl amounts of substrate solution (1 mg/ml) are added to each well and incubation is allowed to proceed for 30 minutes at room temperature. The reaction is stopped by the addition of 25 μl /well of 1N NaOH.
 - (8) Absorbance at 405 nm is read in a micro-ELISA reader.
- The combination of the serum/antigen dilution which gives maximal separation between positive and negative test samples but still gives low negative values (under 0,2) is chosen for subsequent tests.

Fig 18, Table XI.

(c) Sample and Conjugate Incubation

The DOUBLE ANTIBODY SANDWICH ELISA technique was carried out using commercial BCG protein solutions for the test samples. Sample and conjugate incubation times of 1 hour at 45°C; 2-3 hours at 37°C and overnight at 45°C were experimented with.

(d) Colour development

The intensity of colour developed is controlled by the conjugate concentration and time allowed for development. Using a 30 minute incubation period at room temperature, the working dilution of the conjugate was found to be uneconomical, therefore, the following conjugate concentrations and incubation temperatures were experimented with:

Conjugate Dilution	Incubation Temperature (°C)
$\frac{1}{100}$	37°, 45°
$\frac{1}{150}$	37°, 45°
$\frac{1}{200}$	37°, 45°

(iv) Patients

A total of 20 CSF samples were collected from patients with various clinical signs of meningeal irritation. A detailed case-history and a record of the clinical examination was also collected. The CSF samples underwent the following investigations;

- (1) Routine cytological and biochemical tests were performed by using standard techniques.
- (2) ELISA was performed on all samples by the method described in the foregoing.
- (3) A follow-up of the patient was made to determine whether the initial diagnosis was accurate.

The CSF profile of all 20 patients proved to be normal in the final analysis and the majority had completely recovered within 5-7 days. The double antibody sandwich ELISA was carried out initially on the above-mentioned normal CSF samples. In addition, a row of wells containing positive commercial BCG standards was included. When it was found that no distinction could be made between the absorbance values obtained with the normal CSF's and those of the positive standards, no further testing was done on CSF samples from patients with TBM or bacterial meningitis.

(v) Antibody Coating of Plates

Reagents: Prepared under Sterile Conditions.

(1) Solid-phase Antibody

Rabbit IgG-anti-Bacillus Calmette-Guérin (BCG)

DAKOPATTS CAT. No. B124.

A $1/800$ dilution (as determined by chequerboard titration) of the antibody is made up in coating buffer by mixing 10 μl with 7990 μl coating buffer.

(2) Coating buffer

Carbonate-bicarbonate buffer 0,05 M pH 9,5

(3) Blocking buffer

0,01 M PBS pH 7,4 and 0,5% BSA

(4) Washing buffer

0,01 M PBS pH 7,4 and 0,05%. Tween 20 and 1% BSA

(5) Microtitre plates

NUNC flat-bottomed polystyrene immunoassay plate ~~X~~442404

(6) Humid box

Procedure: Under Sterile Conditions

- (1) 100 $\mu\ell$ amounts of optimally diluted anti-BCG antibodies (diluted in coating buffer) is added into the wells of a microplate. The plate is covered and incubated overnight in a humid box at 4°C. This allows the antibodies to be adsorbed onto the plastic surface.
- (2) The wells are emptied by aspirating the coating solution.
- (3) 250 $\mu\ell$ amounts of blocking buffer are added to each well. The plate is then covered and incubated at room temperature for 1 hour. This permits the remaining unblocked binding sites on the plastic surface to be filled by BSA, hence non-specific binding is reduced.
- (4) The wells are emptied by aspiration and washed by adding 250 $\mu\ell$ amounts of washing buffer, leaving for a few minutes, then emptying.

This washing procedure is repeated three times, then the plate is shaken dry and allowed to drain on 'Spontex' sponges.
- (5) Sensitized plates may be sealed individually in aluminium foil and stored at -70°C.

(iv) ELISA Technique

This method to detect soluble *M. tuberculosis* antigens in the various body fluids of tuberculous and other groups of patients was essentially that of Engvall and Perlmann.⁽²²⁾ The method was further modified by Sada et al,⁽⁴⁷⁾ and this latter version was used in our study (Fig. 6).

Reagents: Prepared under sterile conditions

(1) Antibody-Enzyme Conjugate

Alkaline-phosphatase labelled rabbit IgG anti-BCG.

A $1/150$ dilution (as determined by titration) of the conjugate is made up in washing buffer by the addition of 25 μl conjugate to 3725 μl buffer.

(2) Washing buffer

0,01 M PBS pH 7,4 and 0,05% TWEEN 20 and 1% BSA

(3) Positive Control Antigen

Commercial BCG protein standards containing 0, 2, 3, 7 and 14 ng/ml BCG are made up in washing buffer.

(4) Substrate

p-nitrophenyl phosphate disodium salt (MERCK CAT. No. 6850).

Dissolved < 30 minutes prior to use in diethanolamine buffer to form a 1 mg/ml solution.

(5) Substrate buffer

10% Diethanolamine buffer pH 9,8 containing 0,2% sodium azide.

(6) 1 N NaOH

(7) Humid box

(8) Micro-ELISA reader

Procedure: Under sterile conditions

- (1) 100 μl amounts of each sample to be tested is added to a well in a sensitized microtitre plate, (in duplicate, quantity permitting).

A row of wells containing commercial BCG standard solutions are included to serve as a positive control.

- (2) The plate is covered and incubated for 2 hours at 37°C in a humid box.
- (3) The wells are emptied and washed by the addition of 250 µl amounts of washing buffer to each well, leaving for a few minutes, then emptying. This washing procedure is repeated three times.
- (4) 100 µl amounts of optimally diluted conjugate is added to each well and incubated for 3 hours at 37°C in a humid box.
- (5) The wells are emptied and washed as before.
- (6) 100 µl amounts of p-nitrophenyl phosphate solution is added to each well. Plate is then incubated for 90 minutes at 45°C in a humid box.
- (7) The reaction is stopped by the addition of 50 µl amounts of 1N NaOH to each well.
- (8) Absorbance at 405 nm is read with an EIA READER MODEL EL-307, BIOTEK INSTRUMENTS, INC.

5. Statistical Methods

Statistical analysis of results was carried out by non-parametric tests, as no assumptions could be made on the symmetrical distribution of sample results about the mean.

The non-parametric test employed was the 'ONE-WAY ANALYSIS OF VARIANCE'. Probability values less than 0,01 and 0,05 were taken as significant, i.e., 1% and 5% points of variance (F) ratio. Methods used for a particular study are given with it.

Fig. 5 : Inhibition of BCG-anti-BCG Reaction by *M. tuberculosis* antigen using ELISA

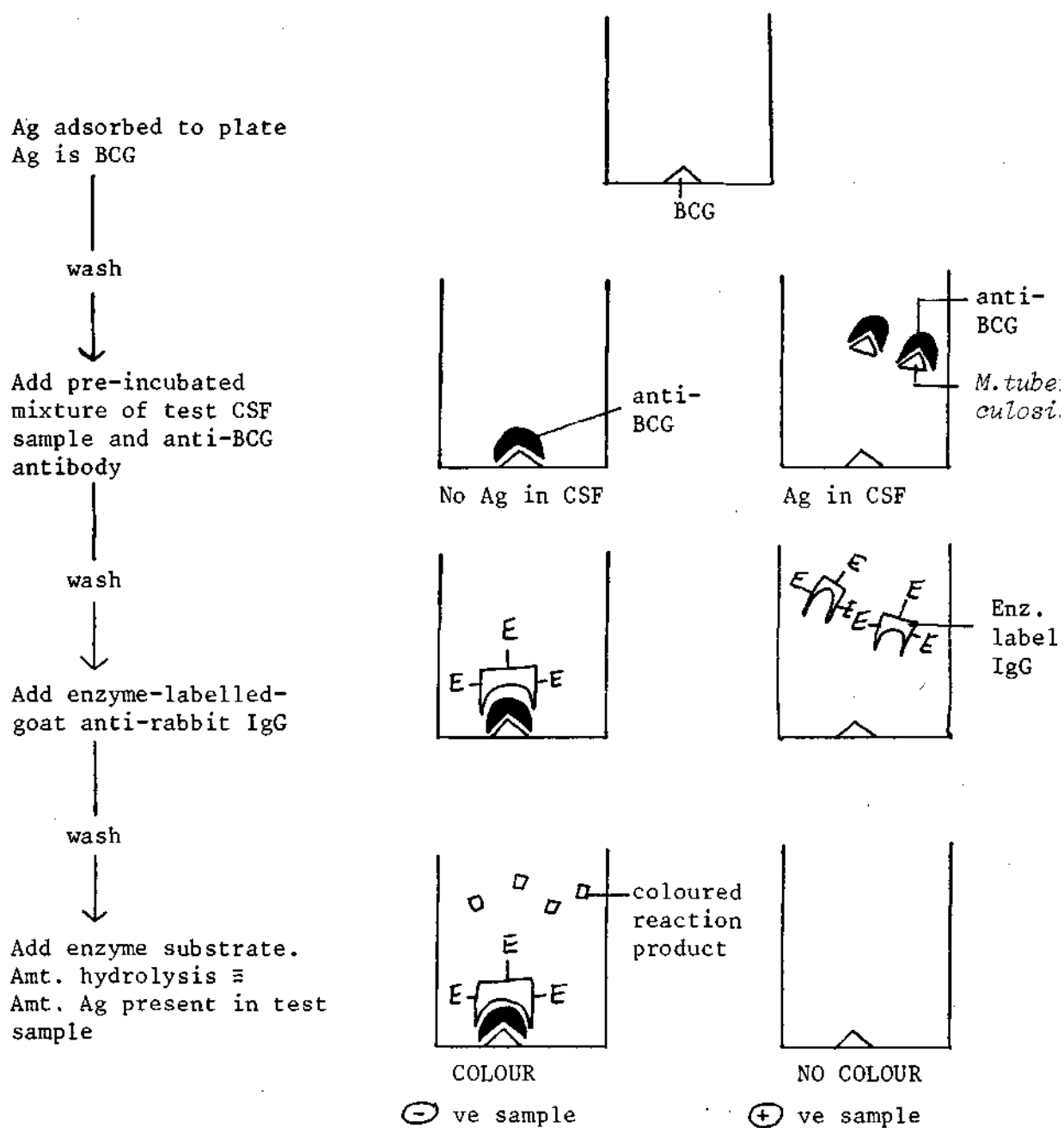
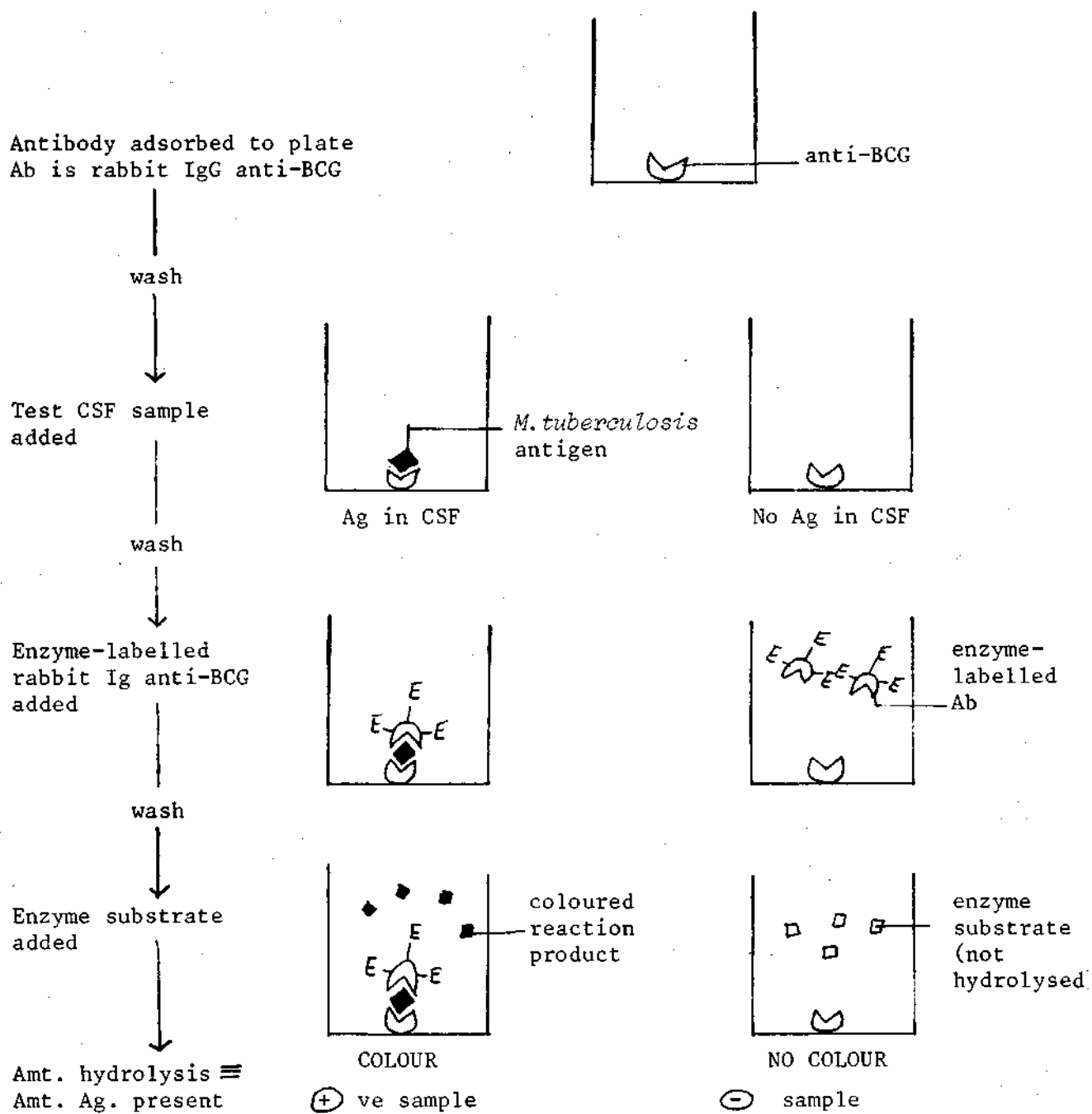


Fig. 6 : DOUBLE ANTIBODY SANDWICH ELISA for measuring antigen



IV RESULTS

1. CORRELATION BETWEEN CHLORIDE LEVELS IN BLOOD AND CSF OF PATIENTS WITH TUBERCULOUS AND OTHER FORMS OF MENINGITIS

(i) Objectives

- (a) To determine whether any correlation exists between the levels of chloride in the blood and CSF of patients with tuberculous and other forms of meningitis.
- (b) To determine the BLOOD/CSF chloride ratio in patients with bacterial, viral, and tuberculous meningitis.

(ii) Patients

Blood and CSF samples were obtained from a total of 149 Black children admitted to King Edward VIII Hospital, Durban during the six month period, February-August 1985. On the basis of clinical examination, routine cytological and biochemical tests on CSF, and culture studies, the children were found to have various types of meningitis. The children ranged in age from 1 day to 11 years. A detailed analysis of the patients studied is recorded in Table II and in Figures 7 and 8.

(iii) Method

Chloride levels in both blood and CSF were measured with a fully automated instrument, the BECKMAN ASTRA-8 AUTOMATED STAT/ROUTINE ANALYSER. This instrument utilizes the colorimetric titration method of measurement. Both the electrode and the cathode consisted of silver chloride. Readings were given in mmol/l.

**TABLE II : CHILDREN WITH MENINGITIS AT KING EDWARD VIII HOSPITAL DURING THE 6-MONTH PERIOD
FEBRUARY-AUGUST 1985**

Type of meningitis	Total no. of cases	Total no. of males	Total no. of females	Mean Age (months)	Age Range
TBM	10	5	5	35 \pm 36,2	3 mth - 10 yrs
Viral	34	25	9	20 \pm 29,1	2 d - 9 yrs
<i>S.pneumonia</i>	34	18	16	23 \pm 38,9	2 d - 11 yrs
<i>H.influenzae</i>	40	22	18	6,8 \pm 4,4	5 d - 2 yrs
<i>N.meningitidis</i>	9	6	3	29 \pm 41,6	2 mth - 7 yrs
<i>Klebsiella sp.</i>	9	3	6	1 \pm 1,5	1 d - 4 mths
<i>Acinetobacter</i>	1	0	1	2 \pm 0	-
<i>Escherichia coli</i>	8	6	2	3,5 \pm 2,1	4 d - 7 mths
<i>Proteus sp.</i>	3	2	1	88,1 \pm 62,1	5 d - 11 yrs
Cryptococcal	1	1	0	72 \pm 0	-
TOTAL	149	88	61		
% TOTAL	100	59,1	40,9		

(iv) Results(a) TBM

The mean blood chloride (Cl^-) level obtained ($98,0 \text{ mmol/l} \pm 13,2 \text{ S.D.}$) fell within the normal range of values ($95-105 \text{ mmol/l}$). The mean CSF level recorded ($107,0 \pm 9,7 \text{ (S.D.)}$) was significantly lower than that of the normal CSF Cl^- range ($120-130 \text{ mmol/l}$). The mean BLOOD/CSF Cl^- ratio was found to be $0,943 \pm 0,032 \text{ (S.D.)}$.

(b) Viral meningitis

The mean blood Cl^- level ($115,0 \pm 8,7 \text{ S.D.}$) was found to be raised substantially in this group of patients. The mean CSF chloride level obtained ($122,0 \pm 7,4$) fell within the lower limit of normal. The mean BLOOD/CSF Cl^- ratio was found to be $0,940 \pm 0,003$, a value very similar to that obtained for patients with TBM.

(c) Bacterial meningitis

The mean blood Cl^- levels for meningitis caused by *Streptococcus pneumonia*, *Haemophilus influenzae* and *Neisseria meningitides* were all within the normal range. The mean CSF Cl^- levels, however, fell below the lower limit of normal for all three of the above-mentioned bacterial meningitis.

The mean BLOOD/CSF ratio for *S.pneumonia* was $0,922 \pm 0,059 \text{ (S.D.)}$; that for *H.influenzae* $0,899 \pm 0,217$; and that for *N.meningitis* was $0,914 \pm 0,038$.

A detailed analysis of the results obtained is recorded in Table III and in Figures 9, 10, and 11.

Fig. 7 : Incidence of meningitis in males and females (children) at King Edward VIII Hospital

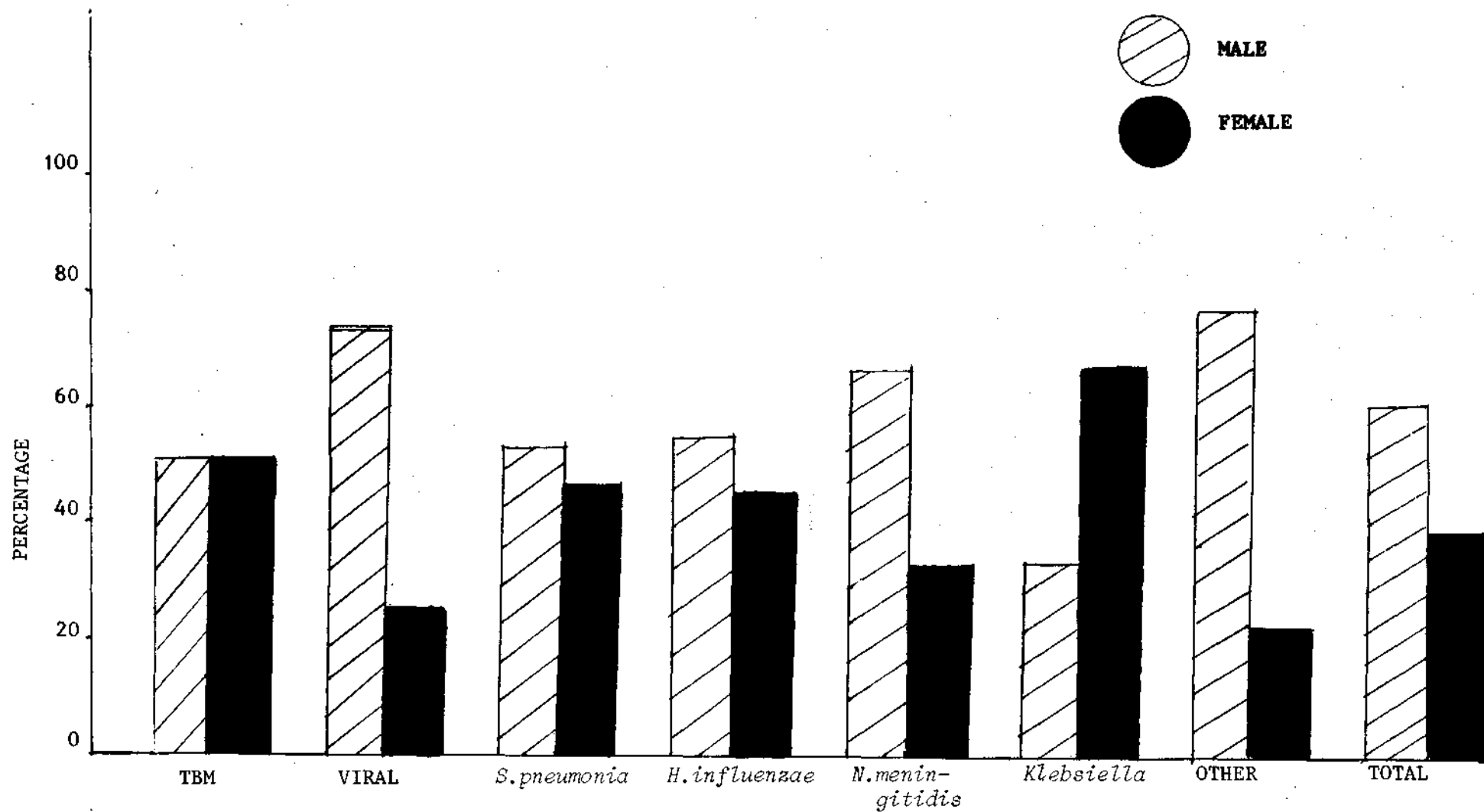
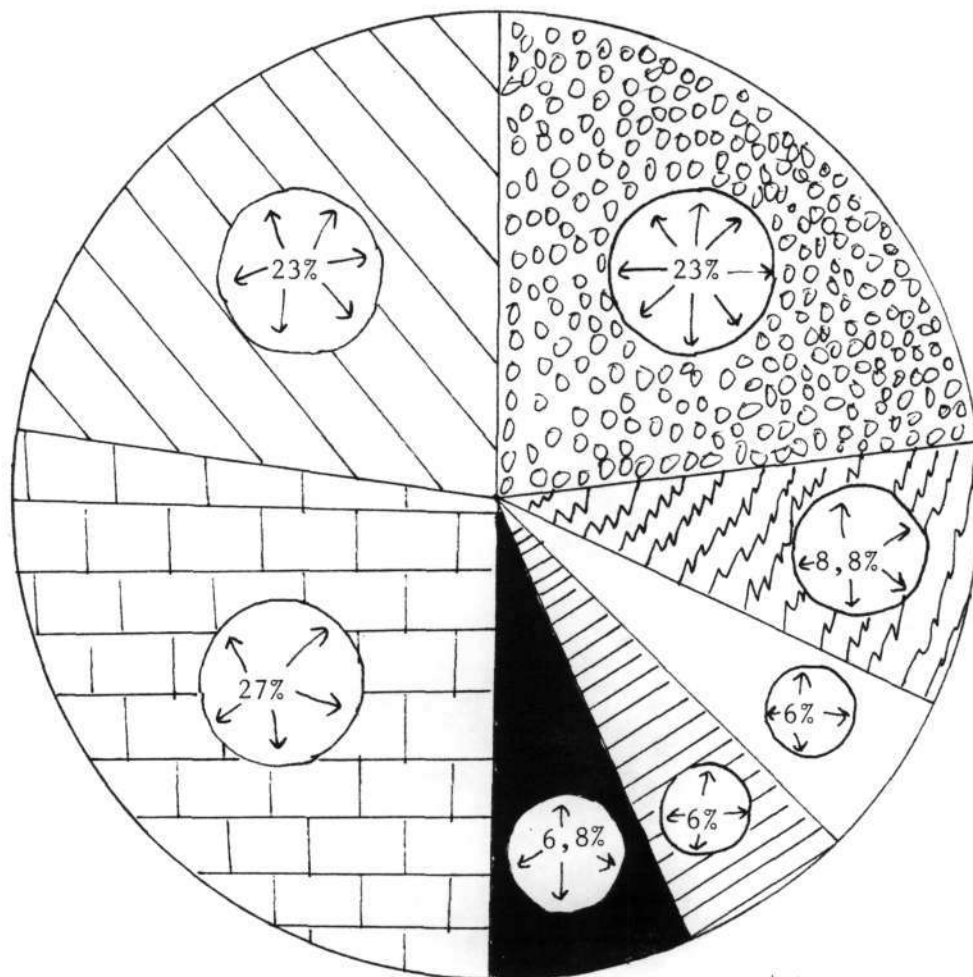


Fig. 8 : Causative agents of meningitis in children at King Edward VIII Hospital during the six-month period (February-August 1985)



-  *S. pneumoniae*
-  *N. meningitidis*
-  *Klebsiella sp.*
-  *H. influenzae*
-  VIRAL
-  TBM
-  Other - *Cryptococcus*
E. coli
Acinetobacter

TABLE III : BLOOD AND CSF Cl⁻ LEVELS AND BLOOD/CSF Cl⁻ RATIO IN BACTERIAL, VIRAL AND TUBERCULOUS GROUPS OF PATIENTS

TYPE OF MENINGITIS	Mean blood Cl ⁻ level \pm S.D. (100)*	Range of Blood Cl ⁻ levels (95-105)*	Mean CSF Cl ⁻ level \pm S.D. (125)*	Range of CSF Cl ⁻ levels (120-130)*	Blood/CSF Cl ⁻ ratio \pm S.D. (0,8)*	Range of Blood/CSF Cl ⁻ ratio (0,731-0,875)*
TBM	98,0 \pm 13,2	82-120	107,0 \pm 9,7	94-125	0,943 \pm 0,032	0,896 - 0,979
Viral	115,0 \pm 8,7	105-136	122,0 \pm 7,4	112-135	0,940 \pm 0,003	0,781 - 1,088
<i>S.pneumonia</i>	106,0 \pm 8,5	92-120	113,0 \pm 6,1	99-122	0,922 \pm 0,059	0,818 - 1,034
<i>H.influenzae</i>	104,1 \pm 4,9	92-110	116,4 \pm 9,4	90-139	0,899 \pm 0,217	0,794 - 1,020
<i>N.meningitidis</i>	104,3 \pm 4,9	96-109	114 \pm 5,8	105-122	0,914 \pm 0,038	0,880 - 0,965
<i>Klebsiella</i>	ND	ND	117 \pm 7,7	108-127	ND	ND

*normal values

1. Cl⁻ levels in mmol/l
2. ND = not done.

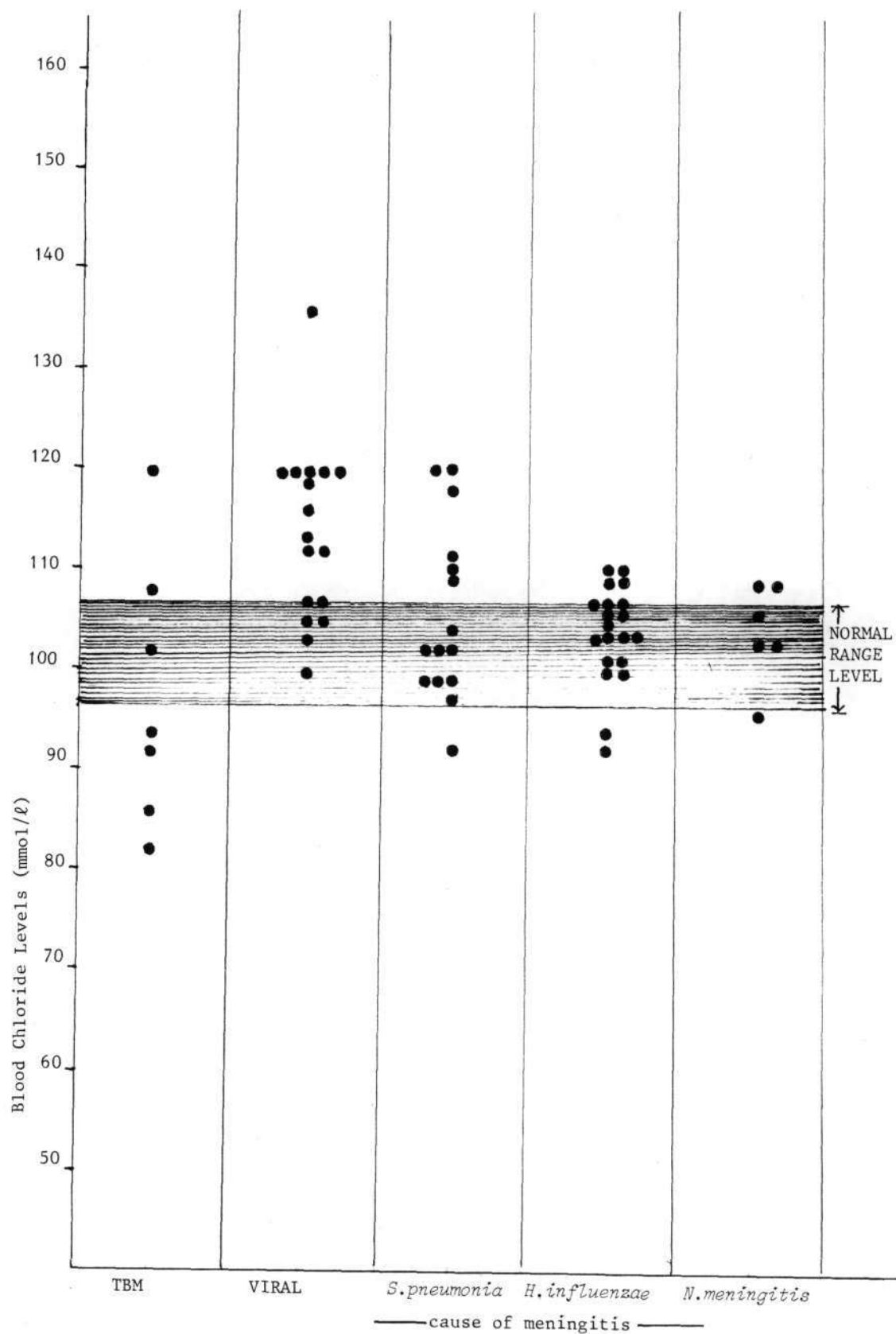


Fig. 9 : Distribution of blood chloride levels in children with meningitis

Fig. 10 : Distribution of CSF chloride levels in children with meningitis

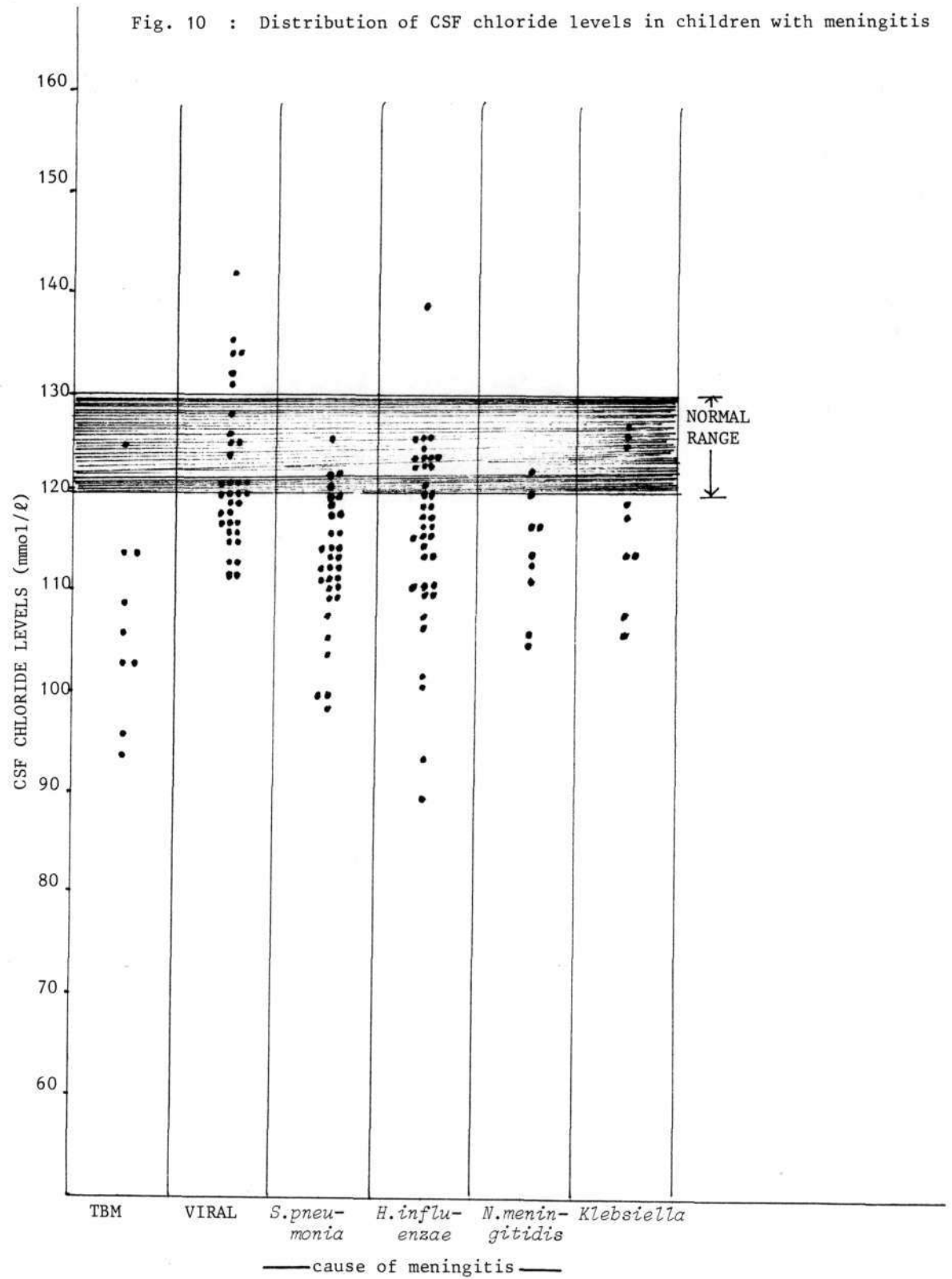
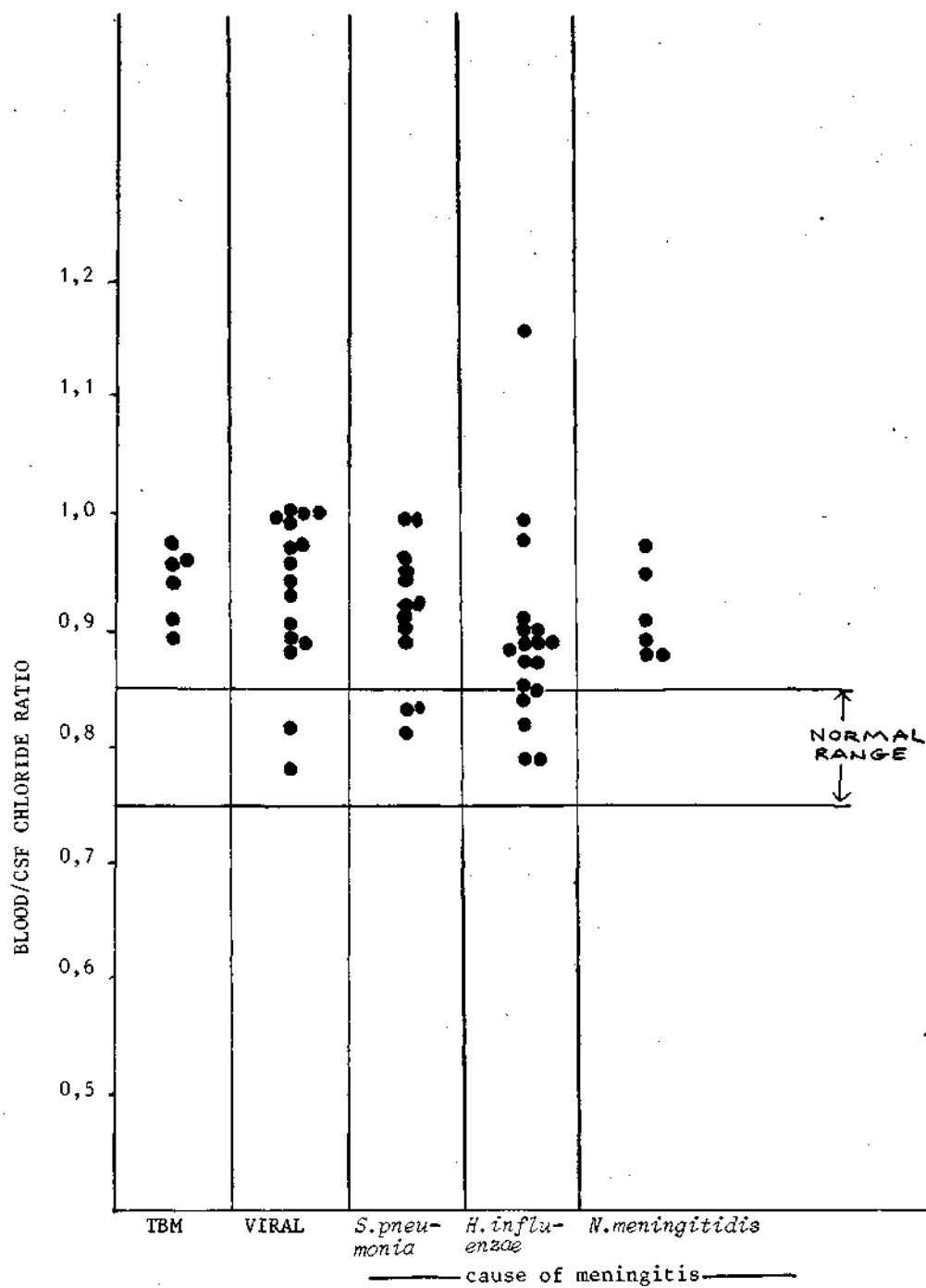


Fig. 11 : Distribution of BLOOD/CSF chloride ratio in children with meningitis



(v) Discussion

Chloride and bromide are chemically similar. They share physical and chemical properties (Table IX) and are hence grouped together as 'halides'. Studies by Mandal et al (1972), Mann et al (1982)^(37;38) and others have shown that after a loading dose, the relative concentrations of bromide in the blood and CSF are normally in a ratio of about 3:1. When the blood-brain barrier is damaged, as in the case of tuberculous but not in viral meningitis, the bromide ratio in blood and CSF tends to equalise.

It was proposed that a similar situation may exist concerning chloride levels in CSF and blood of patients with tuberculous and viral meningitis since chloride and bromide were similar chemically. Hence, the blood and CSF of 149 patients with various types of meningitis were measured for chloride content. A total of 10 patients had TBM; 34 had viral meningitis; and the rest had bacterial or cryptococcal meningitis. The distribution of blood and CSF Cl^- levels that were recorded are presented graphically in Figure 9 and 10. Unfortunately both the CSF and blood Cl^- levels were not measured in all patients. In those where both CSF and blood Cl^- measurements were made, the BLOOD/CSF Cl^- ratio was computed. This is recorded graphically in Figure 11.

The value of CSF chloride levels as a diagnostic pointer in cases of TBM is questionable. Parsons⁽⁴³⁾ deems the chloride level in the CSF as 'no longer thought to be important'. Jaffe,⁽³¹⁾ however, states that a very low CSF chloride (e.g., under 100 mmol/l) is strongly

suggestive of TBM provided that the serum chloride is not also very low. Our studies have proven contrary to that of Jaffe. Only two patients with TBM had chloride levels less than 100 mmol/l. The chloride levels in tuberculous patients was found to range from 95 mmol/l to normal values. Those patients with very low chloride values (< 100) had serum chloride levels that were equally low. The CSF chloride levels in tuberculous meningitis were found to be on the low side; no patient had a chloride level greater than that of the upper limit of normal (130 mmol/l). All but one patient had CSF chloride levels below that of the lower limit of normal (120 mmol/l). Depressed CSF chloride levels thus seem indicative of TBM.

The CSF chloride levels in patients with bacterial and viral meningitis is usually normal. Our investigations revealed that in the patients with viral meningitis, the majority of CSF fell within or on the periphery of the normal range. No patients were found to have greatly depressed CSF chloride levels, i.e., < 110 mmol/l. In patients with bacterial meningitis it was found that the vast majority of CSF chloride values fell below the lower limit of normal, i.e., below 120 mmol/l. A substantial number of these patients had normal CSF chloride levels and only one had a chloride level that was raised (> 130 mmol/l). Since many patients with bacterial meningitis had CSF chloride levels similar or identical to those with TBM, CSF chloride levels prove to be unhelpful in the differential diagnosis of meningitis.

Blood chloride levels in patients with bacterial, viral, or tuberculous meningitis are stated as remaining normal in the literature.

Our studies showed that in patients with TBM, blood chloride levels were either depressed, elevated, or normal. In patients with viral meningitis no blood chloride levels were depressed; on the contrary, the vast majority were elevated. Patients with bacterial meningitis had blood chloride levels which largely fell within the normal range (95-105 mmol/l), although some were elevated and some were depressed. Bacterial, viral, or tuberculous meningitis could not be distinguished on the basis of blood chloride levels.

The BLOOD/CSF chloride ratio is normally in the range 0,75-0,85. In all patients with TBM this ratio was found to be elevated (all ratios calculated were $> 0,89$). Patients with bacterial and viral meningitis had ratios which were largely raised. A few had normal ratios. Patients could not be distinguished as having bacterial, viral, or TBM on the basis of the BLOOD/CSF chloride ratio.

(vi) Conclusion

On the basis of our investigations of 149 children with tuberculous, viral, and bacterial meningitis, the following conclusions have been reached:

- (a) CSF chloride values are of little value in discriminating between bacterial, viral and tuberculous meningitis. However, once the diagnosis of bacterial meningitis has been eliminated, uncommonly low CSF chloride values may be associated with TBM but not with viral meningitis.
- (b) Blood chloride values of patients with bacterial, viral or tuberculous meningitis do not provide a marker by which the patients may be separated into their respective aetiological groups.

- (c) The BLOOD/CSF chloride ratio in patients with bacterial, viral, and TBM is elevated as compared to that of normal individuals. The normal BLOOD/CSF chloride ratio is generally accepted as falling in the range 0,75-0,85, hence a control group of patients with normal CSF was not included in the study.
- (d) The BLOOD/CSF chloride ratio does not provide a basis for distinguishing bacterial, viral or tuberculous meningitis patients as such.

TABLE IV : PHYSICAL PROPERTIES OF THE HALIDES
(after Sissler, et al) (48)

PROPERTY	F	Cl	Br	I	At
X^- ion radius (\AA)	1.36	1.81	1.95	2.16
Atomic radius (\AA)	0.72	1.00	1.14	1.35
1st ionization energy (ev)	17.4	13.0	11.8	10.4	9.5
Electron affinity (ev)	3.62	3.82	3.54	3.24
Electronegativity (Pauling scale)	4.0	3.0	2.8	2.5	2.2
Heat of hydration of X^- (kcal/mole)	-122	-89	-81	-72
$E^0, X_2 + 2e^- = 2X^-$ (volts)	+2.87	+1.36	+1.07	+0.54	+0.3
Bond energy (kcal/mole)	37.8	58.0	46.1	36.1	27.7
Melting point ($^{\circ}\text{C}$)	-223	-102.4	-7.3	113.7
Boiling point ($^{\circ}\text{C}$)	-188	-34.0	57.8	184.5
Density (g/cc)	1.1 (-188 $^{\circ}$)	1.57 (-34 $^{\circ}$)	3.14 (25 $^{\circ}$)	4.94 (25 $^{\circ}$)
Color	pale yellow	yellow- green	red- brown	violet- black	black

2. INHIBITION OF BCG-ANTI-BCG REACTION BY *M. tuberculosis* ANTIGEN USING ELISA

(i) Objective

To ascertain whether the rapid and accurate diagnosis of TBM may be made by using an inhibition ELISA to detect soluble *M. tuberculosis* antigens in CSF.

(ii) Patients

A total of 84 samples of CSF, pleural and ascitic fluid were collected from Black patients treated at King Edward VIII Hospital, Durban. The samples consisted of 59 CSF's and 25 pleural and ascitic fluid specimens.

Of the 59 CSF specimens, 10 were from culture-positive bacterial meningitis patients, 10 were from patients with a definite or highly probable diagnosis of TBM; 14 were from patients with viral (aseptic) meningitis; and 25 were from patients who had complaints other than meningitis but who nevertheless required lumbar punctures. The diagnosis of TBM, viral and bacterial meningitis were made on the criteria listed previously in the 'Introduction' and under 'Methods'.

Of the 25 pleural and ascitic fluid samples, 15 were diagnosed as being from patients with pleural and abdominal tuberculosis on the

basis of acid-fast staining, culturing, or recovery with anti-tuberculous treatment. Ten control samples were collected from patients with ascites caused by malignancies (2 samples - exudates); bilharzia (3 samples - transudates); trauma (3 samples - transudates), and amoebiasis (2 samples - exudates).

(1) Definite or Highly probable diagnosis of TBM - CSF samples of 10 cases showing clinical features and CSF profiles of TBM were investigated for the presence of mycobacterial antigens and cultured on Lowenstein-Jensen (LJ) medium. Of these, the diagnosis of TBM could be made in all cases by various methods. In three, pathogenic, slow-growing, acid fast bacilli could be isolated on LJ medium. Two others showed evidence of TBM at autopsy. Five patients showed distinct clinical improvement with anti-tuberculous treatment and the CSF pictures showed a trend to normal after 4-6 weeks.

(2) Normal CSF samples - There were 25 patients in this group. These were admitted for a variety of complaints ranging from fever, convulsions, signs of meningeal irritation, coma, and pseudobulbar palsy, all of which necessitate lumbar puncture. The CSF picture was normal. All the patients recovered completely within 7-10 days.

(3) Definite or highly probable diagnosis of bacterial meningitis - CSF samples of ten acutely ill patients with clinical signs of bacterial meningitis were included in the study. These samples were confirmed to be from patients with bacterial meningitis when cultures of *S.pneumonia*, *E.coli*, *N.meningitidis*, *H.influenzae* & *Klebsiella* sp. were identified. Of the ten patients under study, two died whilst the other eight recovered with routine antibiotic treatment in about 2-4 weeks (Table VIII(a)).

(4) Highly probable diagnosis of viral (aseptic) meningitis -

CSF samples from 14 patients with a clinical picture of viral meningitis were included in the study. Diagnosis in all cases was based on the CSF characteristics, including normal glucose and failure to grow bacteria on culture. In all cases recovery was rapid and complete with no sequelae.

(5) Definite or highly probable diagnosis of abdominal tuberculosis -

Ascitic fluid samples of 10 cases showing clinical features of abdominal tuberculosis were included in the study. Diagnosis in all 10 cases was confirmed by either isolation and culture of the tubercle bacillus, histology of a biopsy specimen, or prompt response to anti-tuberculosis chemotherapy (Table VIII(b)).

(6) Definite or highly probable diagnosis of pleural tuberculosis -

Five pleural fluid samples from patients with a clinical picture of pleural TB and mononuclear pleocytosis were included in the study. Diagnosis was later confirmed in all 5 cases by either culture or direct smear of pleural fluid, histologic examination of pleural biopsy specimen, or prompt response to anti-tuberculous therapy.

(7) Non-tuberculous Ascitic fluid samples - There were 10 patients in this group. These were admitted with a variety of complaints ranging from amoebiasis, bilharzia, malignancy and trauma.

(iii) Method

The ELISA had to be standardized before mycobacterial antigens could be detected in pleural, ascitic, or cerebrospinal fluid. Optimal conditions of temperature, incubation time, conjugate, coating antigen and antibody concentrations were determined for each step of the assay.

(iv) Results

(a) Standardization of ELISA

Selection of optimum dilution of the antibody-enzyme conjugate - Absorbance of approximately 1,0 given by 1:2000 dilution of the conjugate was considered optimum and was selected for the next step. (Fig. 12 and Table V).

Selection of the Solid-Phase - NUNC flat-bottomed polystyrene immunoassay plates ~~X~~ 442404 were found to be most acceptable for use in the ELISA (Standard deviation from the mean was $\pm 0,047$ as compared to 0,056; 0,049; and 0,072 for the other plates. (Table VI).

Selection of optimum amount of the solid-phase antigen - The absorbance recorded with 0,1 ml of 10 $\mu\text{g/ml}$ BCG as the solid-phase antigen was found to be optimum and hence was selected for the next step (Fig. 13; Table VII).

Standardization of Inhibition assay using anti-BCG antibody -

A 1:1200 dilution of anti-BCG produced approximately 50% inhibition of interaction between the solid-phase (BCG) and the antibody-enzyme conjugate, i.e., when using a conjugate dilution of 1:2000 and a 1:1200 dilution of anti-BCG, the absorbance value was decreased from 1,5 (0,2 $\mu\text{g/ml}$ BCG) to 0,87 (20 $\mu\text{g/ml}$ BCG), a drop of approximately 50%. The 1:1200 anti-BCG dilution was thus chosen to interact with 0,1 ml of CSF in the inhibition assay. (Fig. 14).

Sample and Conjugation Incubation - No significant differences were obtained after incubation for 1 hour at 45°C, 2-3 hours at 37°C or overnight at 4°C.

(b) ELISA results:

CSF samples from patients with TBM could be clearly distinguished from those with bacterial and viral meningitis and from those of control groups on the basis of Inhibition ELISA results: The mean absorbance of the positive TBM samples was $0,658 \pm 0,043$ as compared to $1,089 \pm 0,224$ for normal CSF samples, $0,920 \pm 0,029$ for bacterial CSF samples, and $0,903 \pm 0,104$ for viral CSF samples. This difference in the means is statistically significant. Using the 'One Way Hypothesis of Analysis of Variance' it was found that the Variance ratio (F) was greater than both 1° and 5° points of variance-ratio (F) distribution, i.e., when $P < 0,01$ and when $P < 0,05$, the variance between the mean absorbance values remained significant. (Table IX(a)). The distribution of absorbance values obtained when the various CSF samples were tested is illustrated in Fig. 16. The ELISA on TBM samples proved to be almost 100% sensitive and specific, i.e., no false-negative and only one false-positive result was recorded in the case of all 59 CSF samples tested.

In the case of the pleural and ascitic fluids, the absorbance values obtained (Table VIII) showed a similarity in the case of tuberculous samples. The mean absorbance for pleural and ascitic fluid from tuberculous patients was significantly lower than that of the mean absorbance value for the control ascitic fluid samples. On application of the 'One Way Analysis of Variance' test this difference between the normal and tuberculous groups was confirmed ($p < 0,05$ and $p < 0,01$). (Table IX(b)). The distribution of the absorbance values obtained whilst testing the pleural and ascitic fluid samples is illustrated in Fig. 15.

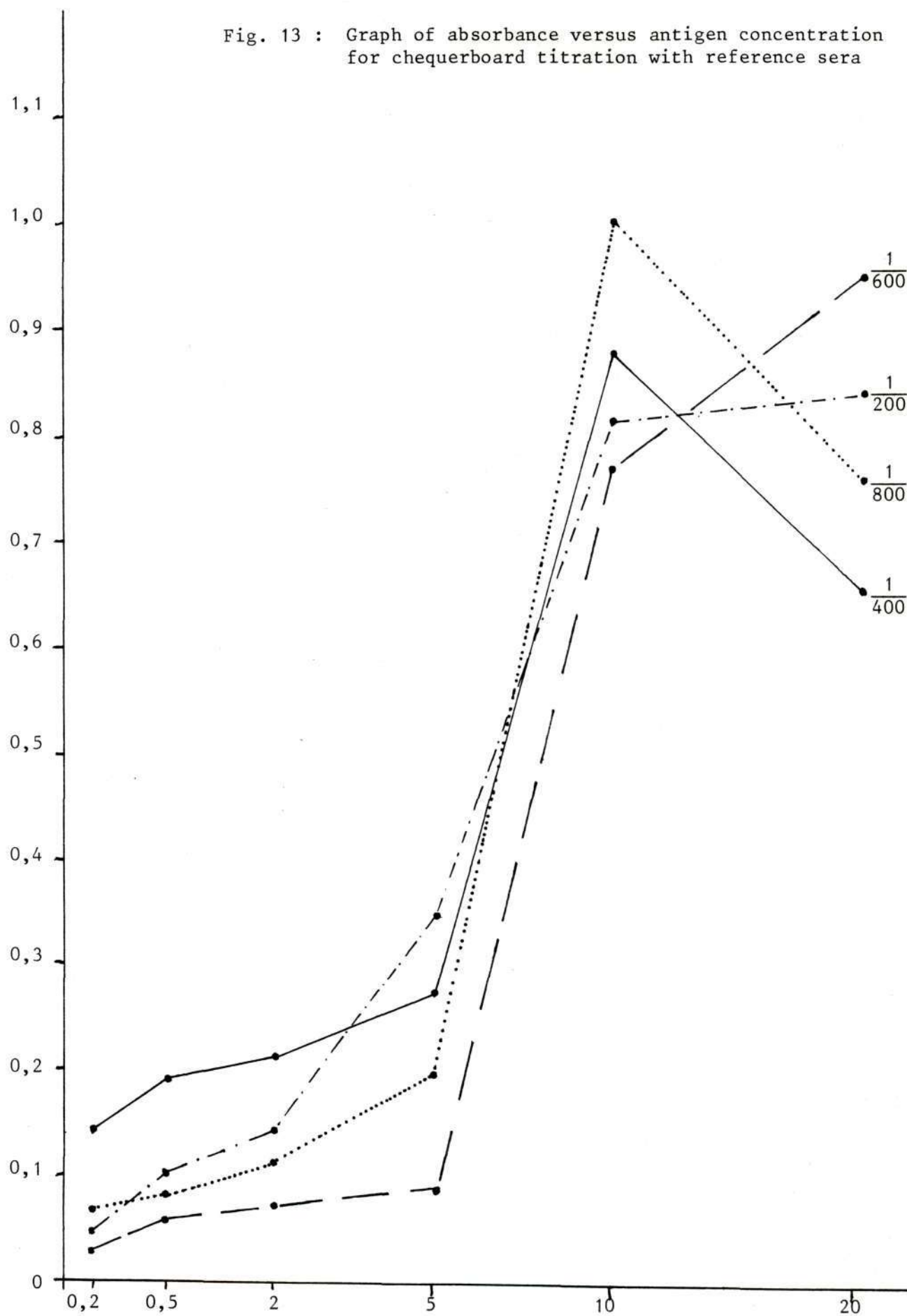
TABLE VII : RESULTS OF CHEQUERBOARD TITRATION USING BCG ANTIGEN AND REFERENCE SERA
(THE VALUES REPRESENT SPECTROPHOTOMETER READINGS (E405))

POSITIVE SERUM DILUTED	ANTIGEN DILUTION ($\mu\text{g/ml}$)						ANTIGEN DILUTION ($\mu\text{g/ml}$)						NEGATIVE SERUM DILUTED
	20	10	5	2	0,5	0,2	20	10	5	2	0,5	0,2	
$\frac{1}{200}$	1,100	0,983	0,435	0,261	0,303	0,163	0,245	0,163	0,085	0,119	0,203	0,116	$\frac{1}{200}$
$\frac{1}{400}$	1,120	1,036	0,367	0,359	0,275	0,243	0,443	0,151	0,090	0,142	0,085	0,095	$\frac{1}{400}$
$\frac{1}{800}$	0,906	1,120	0,258	0,207	0,165	0,132	0,137	0,113	0,066	0,090	0,080	0,071	$\frac{1}{800}$
$\frac{1}{1600}$	1,061	0,855	0,147	0,153	0,119	0,105	0,100	0,080	0,063	0,064	0,059	0,069	$\frac{1}{1600}$
$\frac{1}{3200}$	0,966	0,796	0,168	0,139	0,121	0,080	0,092	0,076	0,064	0,067	0,067	0,065	$\frac{1}{3200}$

The antigen dilutions on the left-hand side of the microtitre plate were reacted with each of the POSITIVE serum dilutions listed above.

The antigen dilutions on the right-hand side of the micro-titre plate were reacted with each of the NEGATIVE serum dilutions listed above.

Fig. 13 : Graph of absorbance versus antigen concentration for chequerboard titration with reference sera



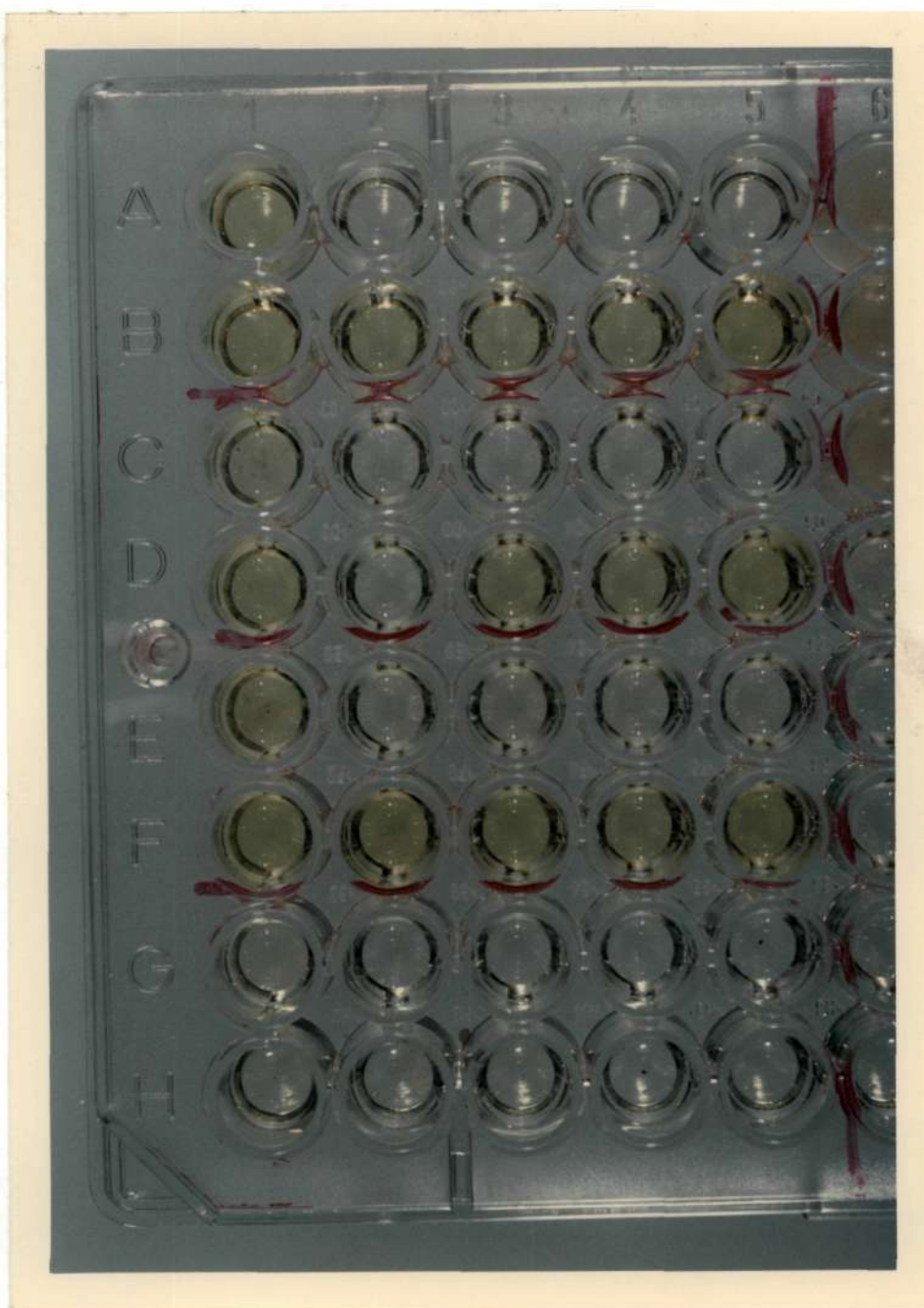


PLATE 2 : Depiction of the yellow-coloured product (p-nitrophenol) produced by the enzymatic hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase

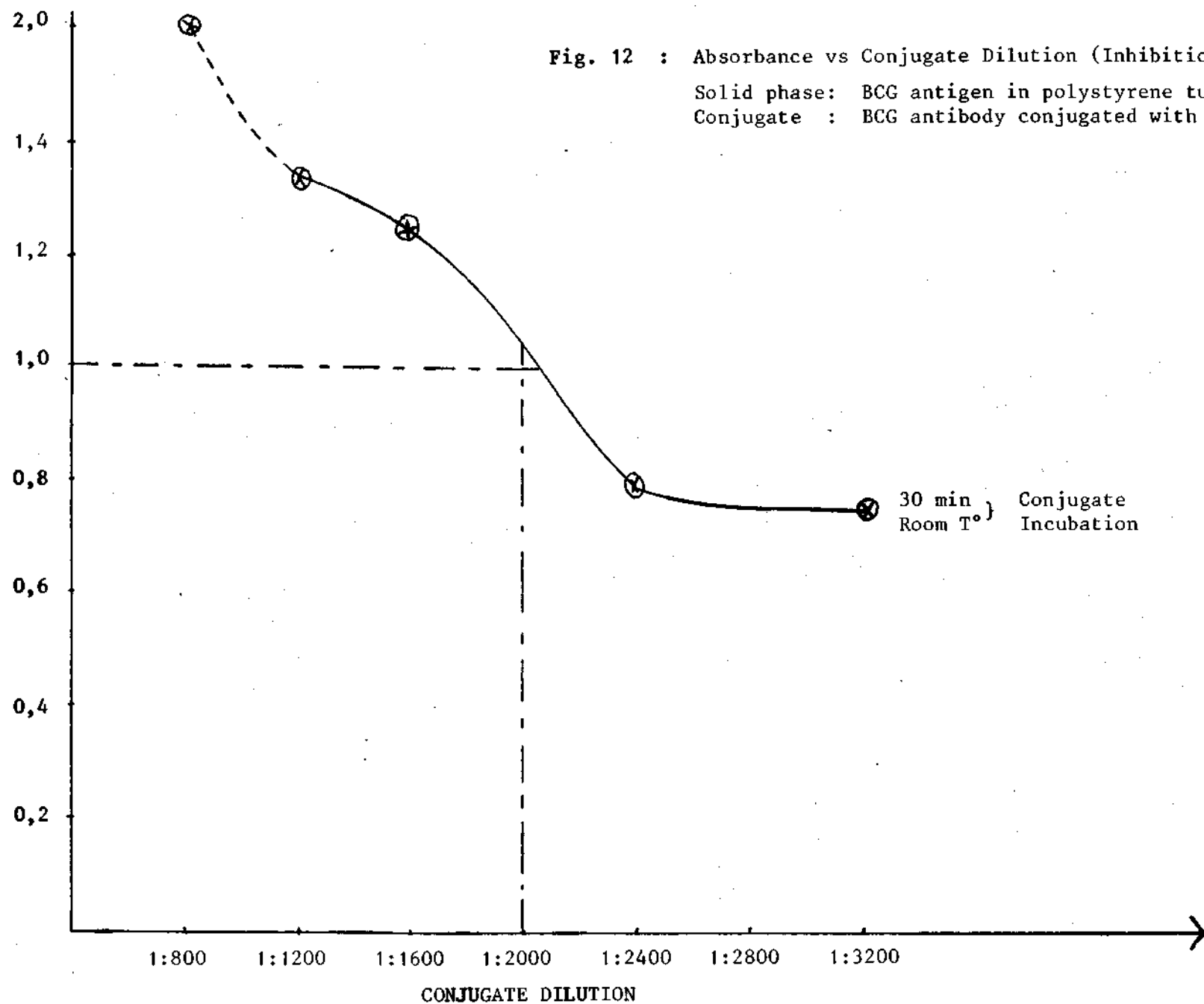


TABLE V : OPTIMAL DILUTION OF CONJUGATE (INHIBITION ELISA)

Conjugate Dilution	Absorbance* (405 nm)
1:100	> 2
1:150	> 2
1:200	> 2
1:300	> 2
1:800	> 2
1:1200	1,325
1:1600	1,248
1:2400	0,790
1:3200	0,748

*After 30 minutes incubation at room temperature.

An enzyme immuno-assay was carried out to determine the optimal working dilution of the conjugate. Polystyrene tubes coated with Human IgG were used to compare absorbance values obtained with various conjugate dilutions. An absorbance value of approximately 1,0 was chosen as the optimum absorbance value.

TABLE VI : VARIATIONS IN ABSORBANCE VALUES
OBTAINED WITH VARIOUS MICROPLATES

TYPE OF MICROPLATE	Mean absorbance + S.D.
NUNC FLAT-BOTTOMED PLATE ✱442404	1,159 \pm 0,047
TITERTEK ACTIVATED PLATE ✱ 77-773-05	1,169 \pm 0,056
DYNATECH REMOVASTRIP SYSTEMS OF IMMULON ®	1,194 \pm 0,049
NUNC FLAT-BOTTOMED STRIPS MODULES F-16 ≠ 469914	1,219 \pm 0,072

An identical enzyme-immunoassay was carried out in all the wells in each of 4 different microtitre plates. The mean absorbance value per plate and the standard deviation for each plate was calculated.

Fig. 14 : Inhibition of interaction between the solid phase (BCG) and the antibody-enzyme conjugate by anti-BCG

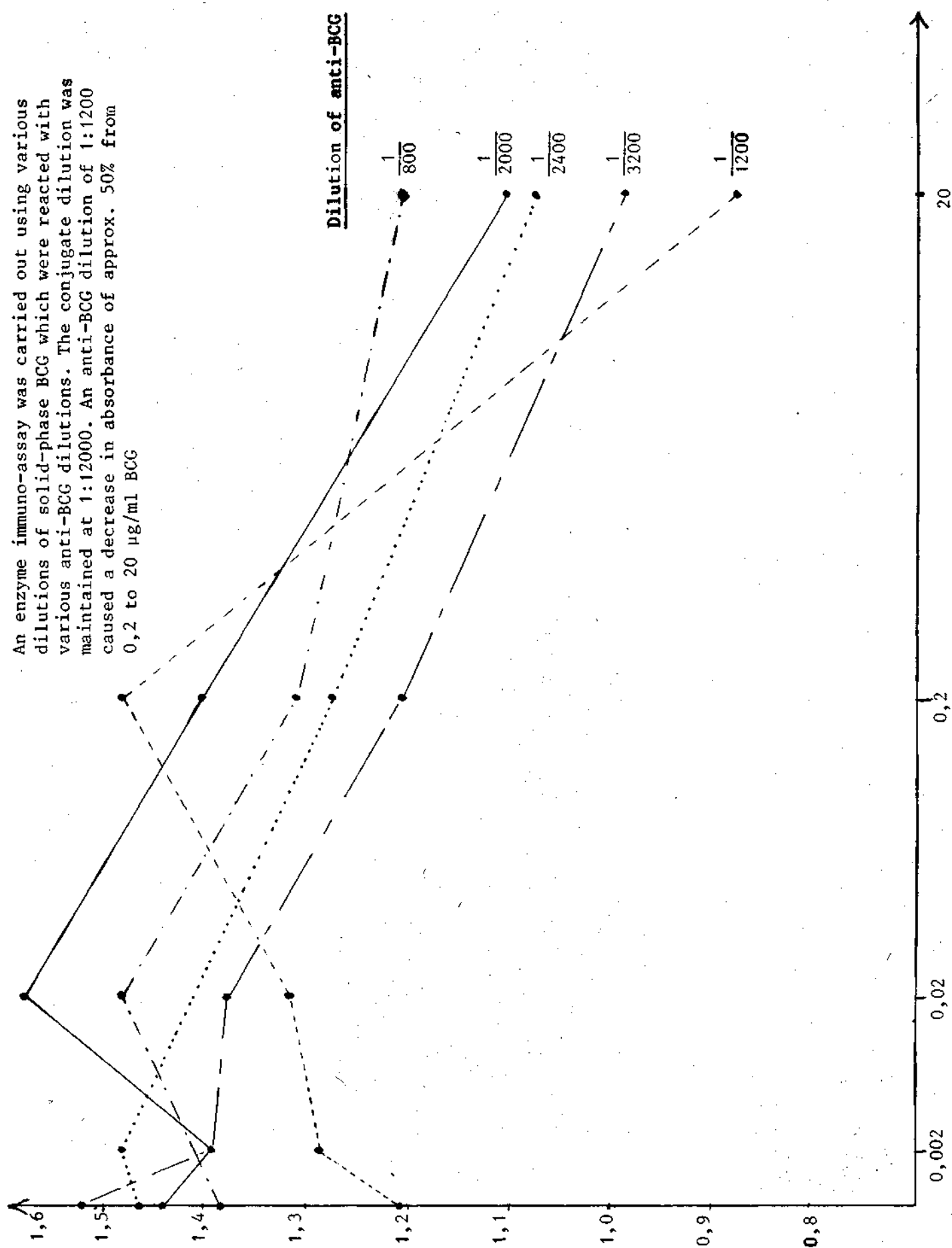


TABLE VIII : RESULTS OF 'INHIBITION ELISA'

(a) Cerebrospinal fluid

Sample	No. tested	Mean \pm S.D.	Range
Normal CSF	25	1,089 \pm 0,224	1,486 - 0,934
Bacterial CSF	10	0,920 \pm 0,029	0,975 - 0,879
TBM CSF	10	0,658 \pm 0,043	0,742 - 0,601
Viral CSF	14	0,903 \pm 0,104	1,070 - 0,597

(b) Pleural and Ascitic fluid

Sample	No. tested	Mean \pm S.D.	Range
TB Pleural	5	0,791 \pm 0,091	0,883 - 0,647
TB Ascitic	10	0,741 \pm 0,065	0,883 - 0,647
Control Ascitic	10	0,966 \pm 0,055	1,052 - 0,903

TABLE IX : ANALYSIS OF VARIANCE TABLES FOR INHIBITION ELISA RESULTS

(a) CSF samples

Source of variation	Sum of squares	Degrees of Freedom	Mean square (variance)	Variance ratio (F)
Between treatments	1,955	2	0,978	49,62
Residual (within group)	0,829	56	0,020	
TOTAL	2,784	58		

(b) Pleural and Ascitic fluid

Source of variation	Sum of squares	Degrees of Freedom	Mean square (variance)	Variance ratio (F)
Between treatments	0,291	2	0,146	36,375
Residual (within group)	0,097	22	0,004	
TOTAL	0,388	24		

Fig. 15 : Distribution of absorbance values of pleural and ascitic fluid samples from patients with tuberculosis and from control groups

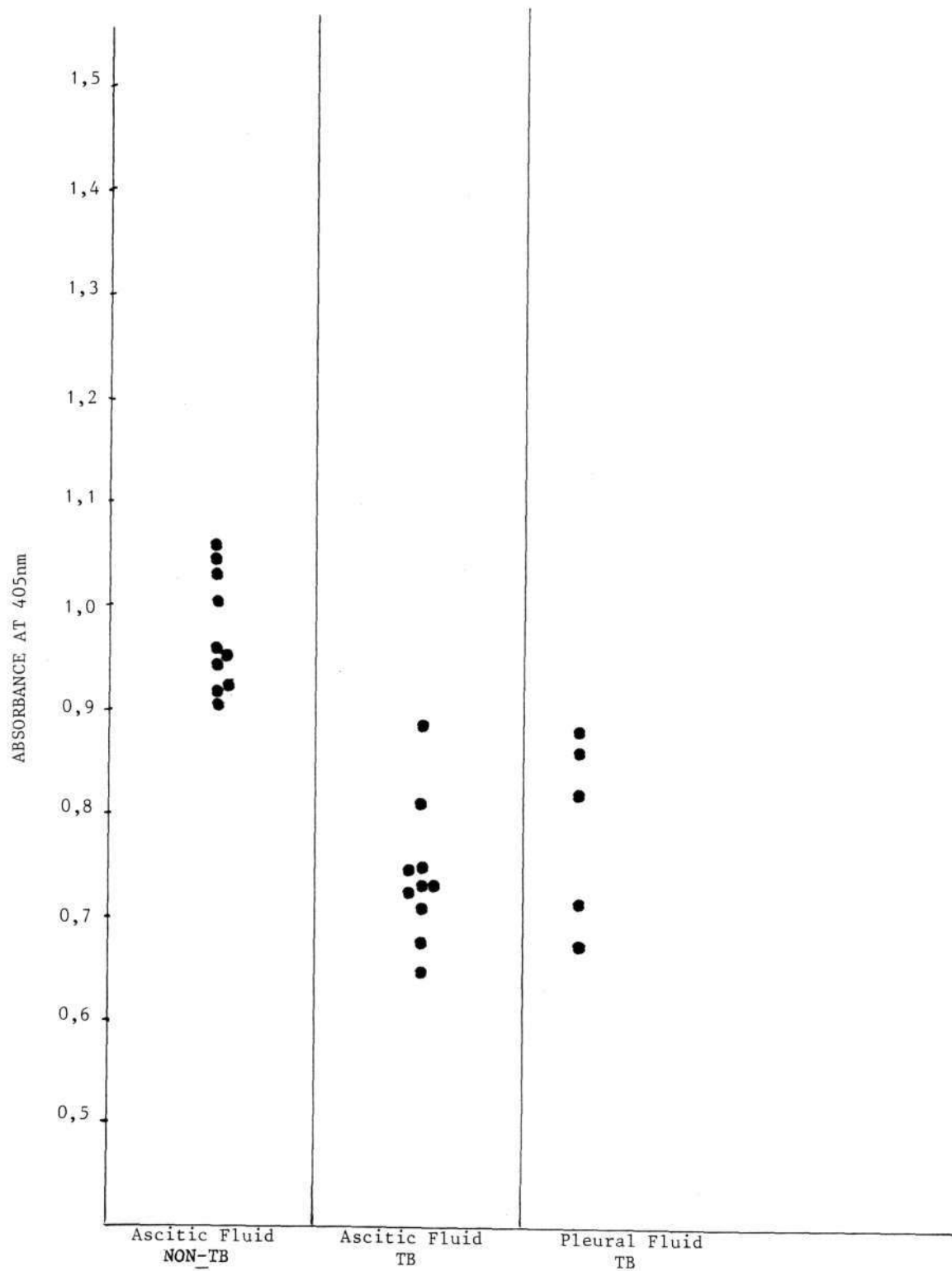
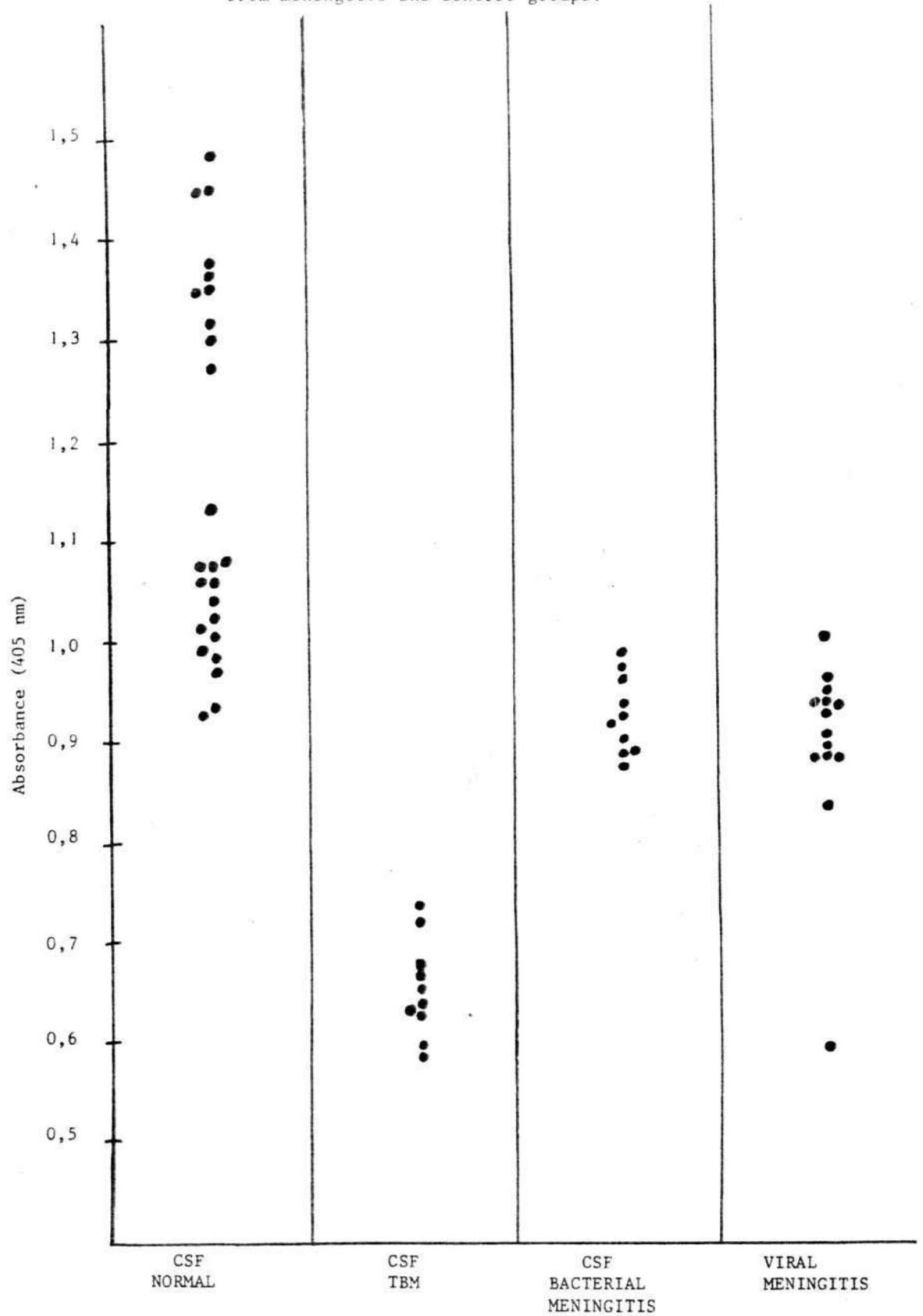


Fig. 16 : Distribution of absorbance values of CSF samples from meningitis and control groups.



(v)

The enzyme-linked immunosorbent assay (ELISA) for detection of mycobacterial antigens appeared to be reasonably sensitive. Sensitivity is defined as the number of cases the test calls positive, specificity as the fraction on non-cases the test calls negative. In order to evaluate the specificity of the assay, seven different types of patients were selected. ELISA tests on proven cases of TBM were carried out to get an estimate of false negative results. Incidence of false positivity was evaluated by tests on pleural, ascitic and CSF samples, normal and abnormal, but of non-tubercular aetiology.

The specificity of the assay (98,3%) requires further investigation. The total number of 10 cases of TBM was too small to draw any conclusions about the validity of the assay in the clinical situation. However, the results obtained are favourable towards INHIBITION ELISA as a possible diagnostic procedure for TBM. Forty-eight samples of CSF definitely not from patients of TBM were non-reactive (25 samples were normal, 14 samples were from viral meningitis patients, and 10 were from bacterial meningitis patients). Only one sample (from a patient with viral meningitis) reacted falsely. It is significant that the bacterial antigens present in the bacterial meningitis group of patients did not cross-react with mycobacterial antigens ($p < 0.01$).

The INHIBITION ELISA has potential for improvement. The use of monoclonal antibodies raised against *M. tuberculosis* or antibodies against antigenic components exclusive to *M. tuberculosis* such as antigen 5 or 6 may serve to increase the specificity of the assay.

(vi) Conclusion

The inhibition assay was reactive for mycobacterial antigens in all ten patients who were clinically diagnosed as having TBM. Of the 84 patients studied, 49 proved to be of non-tuberculous aetiology and all but one were non-reactive for mycobacterial antigens in the ELISA test. The INHIBITION assay hence appears to be a promising approach for a definitive diagnosis of TBM.

The assay may have potential in simplifying the diagnosis of abdominal and pleural tuberculosis. Fifteen pleural and ascitic fluid samples of tubercular aetiology were distinguishable from ten similar samples of non-tubercular aetiology. Further evaluation of the assay in the clinical situation is required before it may be adopted as a diagnostic aid for the detection of mycobacterial infections.

3. Double Antibody Sandwich ELISA

(i) Objectives

- (a) To determine whether the detection of mycobacterial antigens in the CSF of patients with TBM by double antibody sandwich ELISA is rapid and reliable.
- (b) To confirm the findings of Sada et al,⁽⁴⁷⁾ who found the double antibody sandwich ELISA to be useful for the detection of soluble antigens of mycobacteria in CSF of patients with TBM.

ELISA has been used in antigen detection of several infectious diseases and is a simple and rapid method. On the basis of a report by

Sada et al,⁽⁴⁷⁾ in 1983 that it was possible to detect mycobacterial antigens in CSF of patients with TBM it was decided that the reproducibility of Sada's assay would be investigated in the South African context.

Before the ELISA could be performed on CSF samples, various preliminary investigations and techniques had to be performed. The enzyme-antibody conjugate was not available commercially and had to be prepared and tested before use. In addition, the acceptability of various microtitre plates was investigated; the selection of optimum amount of the solid-phase antibody and the suitability of various conjugate and sample incubation conditions was also investigated.

(ii) Method

The method used was essentially that of Engvall and Perlmann⁽²²⁾ which had been adapted by Sada et al,⁽⁴⁷⁾ for the detection of mycobacterial antigens. The assay had to be standardized prior to the testing of CSF samples.

(iii) Results

Working dilution of conjugate - Absorbance of approximately 1,0 given by a 1:150 dilution of the conjugate after 90 minutes incubation at 45°C was considered optimum and was selected for the next step.

Selection of optimum amount of the solid-phase antibody - The absorbance recorded with 0,1 ml of the anti-BCG diluted 1:800 as the solid-phase antibody was found to be optimum and was selected for the next step.

ELISA technique - 100 μ l amounts of CSF samples from 20 normal and abnormal patients of non-tubercular aetiology were tested in order to evaluate the incidence of false-positivity. In addition, a row of positive standards consisting of commercial BCG solutions was included. A bright yellow colour (indicating a positive test) was found to have developed in all the wells, including those containing normal CSF. The entire assay was repeated (including the conjugation procedure and the standardization of the assay). Similar results (intense colour development) were recorded on repetition of the assay.

(iv) Discussion

The DOUBLE ANTIBODY SANDWICH ELISA for the detection of soluble antigens of mycobacteria in CSF did not appear to be either specific or sensitive; contrary to reports by Sada et al.⁽⁴⁷⁾

The optimal dilution of antibody selected to coat the solid-phase was identical to that determined by Sada et al, i.e., 1:800. The conjugate (prepared in a similar manner to that by Sada et al) was found to be active (activity was assayed by EIA), however, a more concentrated solution was used (1:150 as compared to 1:1000).

The incidence of false-positivity recorded was thought to be caused by non-specific binding of the antibody-enzyme conjugate to the solid-phase antibody. Various antigenic components present in CSF may have bound to the solid-phase antibody, and therefore also to the conjugate, hence causing intense colour development. These antigenic components, if identical or nearly identical to the correct mycobacterial antigens at the binding point of the anti-BCG, can be recognized by the anti-BCG. This reactivity towards incorrect antigen is therefore not a correct reactivity and is called cross-reactivity.

The assay is based on the fact that *M.bovis* BCG and *M.tuberculosis* have the same surface antigens (as tested by reference antisera), thus anti-BCG is used in the assay as the solid-phase antibody.

One way to improve the sensitivity and specificity of the test would be to use monoclonal antibodies raised against *M.tuberculosis*. Coates et al,⁽⁷⁾ have reported the production of murine monoclonal antibodies, using the hybridoma technique. These monoclonal antibodies are able to distinguish between strains of *M.bovis* and *M.tuberculosis* and even between certain strains of *M.tuberculosis*.

Alternatively, antibodies raised against antigens 5 and 6 of *M.tuberculosis* by Daniel and Janicki⁽¹³⁾ which are specific for *M.tuberculosis*, if used as the solid-phase antibody, could possibly improve the specificity of the reaction.

(v) Conclusion

The detection of soluble *M. tuberculosis* antigens in CSF of patients with TBM was not possible using the DOUBLE ANTIBODY SANDWICH ELISA of Sada et al. The false positivity recorded with 20 normal CSF samples made these indistinguishable from positive control BCG standards. The results obtained by Sada et al, were hence not reproducible even on duplication of the entire procedure. The specificity and sensitivity of the assay may be improved by using monoclonal antibodies or antibodies raised against antigens 5 and 6 which are specific for the *M. tuberculosis* bacillus as the solid-phase antigen.

TABLE X : OPTIMAL DILUTION OF CONJUGATE
(DOUBLE ANTIBODY SANDWICH ELISA)

Conjugate Dilution	Absorbance ^{*1} (405 nm)
1:125	1,245
1:150	0,795
1:200	0,743
1:400	0,420

Conjugate Dilution	Absorbance ^{*2} (405 nm)
1:50	1,163
1:100	0,509
1:150	0,467
1:200	0,353
1:300	0,182
1:400	0,130
1:600	0,117
1:800	0,104
1:1200	0,104
1:1600	0,096

*1 After 90 minute incubation at 45°C

*2 After 30 minute incubation at room temperature.

Fig. 17 : Absorbance vs Conjugate dilution
(DOUBLE ANTIBODY SANDWICH ELISA)

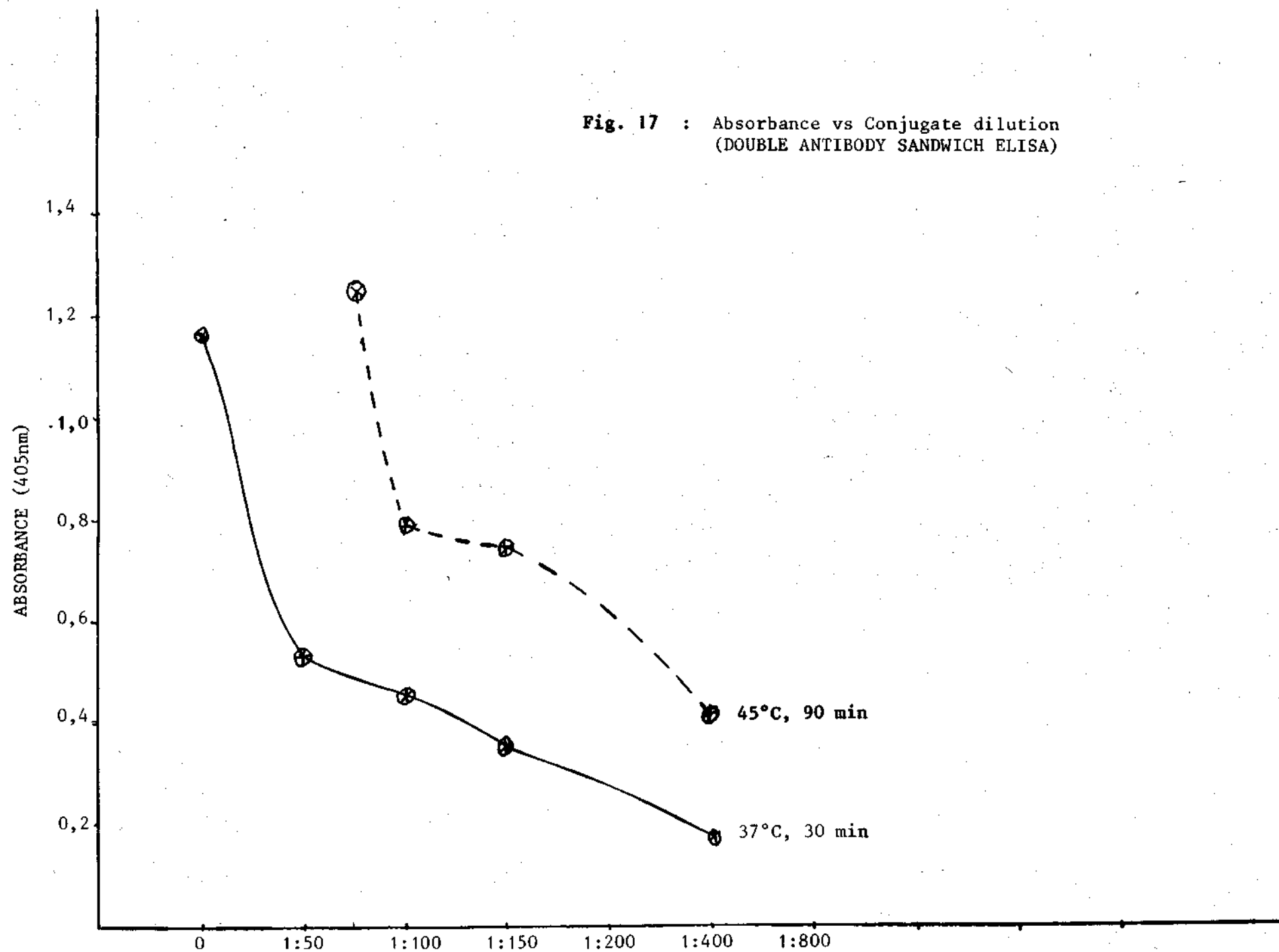


TABLE XI : RESULTS OF CHEQUERBOARD TITRATION USING RABBIT IgG ANTI-BCG AND REFERENCE ANTIGENS

Positive Antigen Diluted	Antibody Dilution						Antibody Dilution						Negative Antigen Dilution
	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	$\frac{1}{50}$	$\frac{1}{100}$	$\frac{1}{200}$	$\frac{1}{400}$	$\frac{1}{800}$	$\frac{1}{1600}$	
2 µg/ml	0,758	0,954	0,667	0,859	1,395	0,816	0,125	0,120	0,119	0,121	0,122	0,113	2 µg/ml
0,5 µg/ml	0,844	0,731	0,534	0,876	1,107	0,830	0,091	0,089	0,090	0,093	0,091	0,085	0,5 µg/ml
0,2 µg/ml	0,823	0,786	0,811	0,816	0,876	1,068	0,108	0,111	0,109	0,108	0,122	0,106	0,2 µg/ml

The values represent spectrophotometer readings (E405)

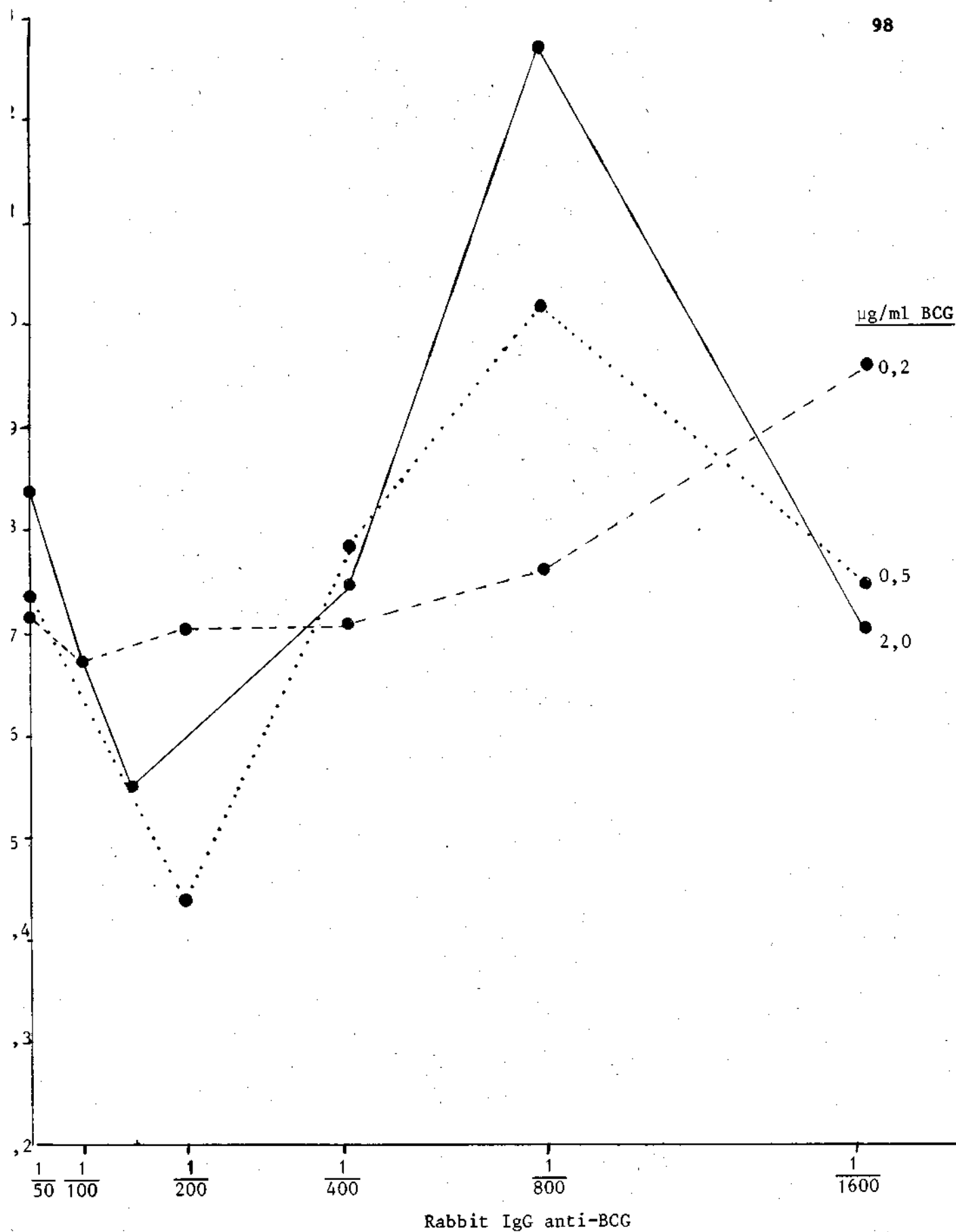


Fig. 18 : Graph of absorbance versus serum dilution for chequerboard titration using reference antigens

V. DISCUSSION

DISCUSSION

TBM always causes an illness grave enough to require hospital admission. It is virtually impossible for a patient to recover spontaneously without treatment. The average duration of the disease from onset to death is only about 17 days if it goes undetected. Diagnosis of TBM is difficult in the early stages of the disease. The clinical picture presented is non-specific and onset is typically insidious. It has been established that the severity of the outcome of TBM (either death or neurological and intellectual impairment in the majority of cases) is linked to the stage of the disease at which diagnosis and chemotherapy is introduced. The earlier the diagnosis, the less severe the sequelae. Fig. 19 shows the outcome of TBM according to stage at diagnosis.

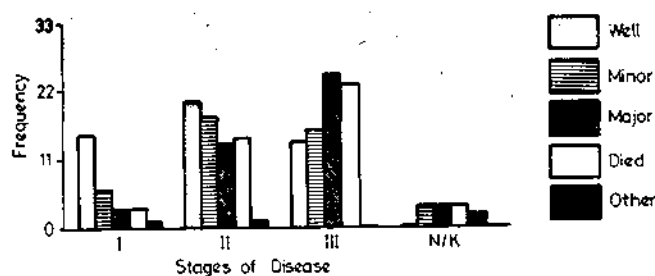


Fig. 19 : Outcome of TBM according to stage at diagnosis.

(after Deeny, J.E., et al, 1985)

Presently the diagnosis of TBM is made on the basis of past exposure to tuberculosis, clinical picture, positive Tuberculin skin test and CSF laboratory profile. The only definitive investigation is the identification of *M.tuberculosis* in CSF. However, the bacillus is only identified in a minority of cases and positive cultures have

been obtained from CSF samples with no apparent cellular content. Culture of the bacillus takes six to eight weeks and is only positive in a few patients (<10%). Other methods have been used in the diagnosis of TBM (radioactive bromide partition test; CSF ADA levels etc.) but though helpful, none have proven confirmatory for the disease. In addition, expensive and complicated equipment is required which is not always available in underdeveloped regions where the disease is most common.

The use of ELISA as a rapid and simple method to detect antigens and antibodies raised against several infectious diseases has been established. Sada et al, reported the use of ELISA to detect mycobacterial antigens. This technique as well as the INHIBITION ELISA were evaluated in the detection of soluble mycobacterial antigens.

The INHIBITION ELISA appeared to be reasonably sensitive. The sensitivity and specificity was almost 100% - no false negative and only one false positive result occurred among the CSF samples tested. It is realized that a total number of ten cases of TBM is too small to draw any definite conclusions on the specificity of the assay in the clinical situation. Many more samples need to be tested before the INHIBITION ELISA can be adopted as a diagnostic aid in TBM.

The use of the INHIBITION ELISA to detect mycobacterial antigens has potential clinical application as an accurate test in the diagnosis of tuberculosis. The ELISA was shown to detect mycobacterial antigens in body fluids (pleural and ascitic) in human tuberculosis. On further evaluation, the INHIBITION ELISA may prove useful in

simplifying the diagnosis of human TB. Possibilities also exist for the detection of mycobacterial antigens in veterinary science. Many animals and much income is lost annually in South Africa as a result of mycobacterial infection of cattle. The INHIBITION ELISA may prove useful in diagnosing these infections.

It is significant that the bacterial antigens in CSF samples did not cross-react with the mycobacterial antigens in the INHIBITION ELISA. Cross-reactions between polysaccharides of mycobacteria and other bacterial genera have been reported by several groups of researchers. It is doubtful whether sputum or urine samples from tuberculous patients could be used in the ELISA (INHIBITION). This is so because the antigens of mycobacteria are known to cross-react extensively with those of saprophytic mycobacteria, *Nocardia*, *Corynebacteria* and many other genera. Sputum and urine are known to have a large commensal population of bacteria which would undoubtedly cross-react in the INHIBITION ELISA, thus causing a loss in specificity of the assay.

The INHIBITION ELISA for the diagnosis of TBM and human and bovine tuberculosis may be improved and made more specific if antibodies which are more specific than anti-BCG are used. Monoclonal antibodies raised against *M. tuberculosis* or antibodies directed against antigens 5 and 6 which are specific for the bacillus may be used to improve the assay. This is also true for the DOUBLE ANTIBODY SANDWICH ASSAY. Alternatively, these specific antigens may be used to detect mycobacterial Ag's in CSF by simplifying the ELISA if latex is used as the solid-phase. If successful, this technique would prove useful to doctors in rural areas as a minimum of equipment would be required.

Unfortunately the normal CSF samples tested with the DOUBLE ANTIBODY SANDWICH ELISA were indistinguishable from positive control BCG solutions on the basis of colour. A uniform intense yellow-coloured product was formed in all wells tested, even on repetition of the entire assay procedure, including the conjugation process. This may be attributed to non-specific binding of the antibody-enzyme conjugate to the solid-phase antibody. Alternatively, antigenic components in the normal CSF may have become partially bound to the solid-phase antigen, and thus captured the conjugate, hence the coloured product. The solid-phase antibody may not be suitable for use in the assay but this is doubtful since Sada et al (1983) reported a measure of success with the same method and similar reagents.

Unlike the Serum/CSF Bromide ratio, the BLOOD/CSF chloride ratio did not tend towards unity as a result of increased permeability of the blood-brain barrier in patients with TBM. The BLOOD/CSF chloride ratio was found to be raised by a similar quantity in all types of meningitis (bacterial, viral, and TBM). Hence the BLOOD/CSF chloride ratio is of negligible use in the differential diagnosis of meningitis.

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VII. APPENDIX

APPENDIX A

LOWRY METHOD OF PROTEIN DETERMINATION

Materials and Equipment

- (i) Protein to be measured
- (ii) Buffer in which protein is dissolved
- (iii) Standard protein (Albumin, bovine serum-BSA)
- (iv) 2% (w/v) copper sulphate, hydrated ($5H_2O$)
- (v) 4% (w/v) sodium potassium tartrate
- (vi) 3% (w/v) sodium carbonate in 0,2 N sodium hydroxide
- (vii) Folin and Ciocalteu's phenol reagent
- (viii) Visible light spectrophotometer.

Procedure

1. Make a 1 mg/ml solution of the standard protein and calculate the exact concentration from its absorbance at 280 nm.

For BSA, the extinction coefficient for $E_{280}^{1\%}$ (the absorbance of a 10 mg/ml solution at 280 nm) is 6,7.
2. Pipette an aliquot of the unknown solution containing 5-50 μ g of protein, the same volume of the buffer blank, and 0, 2, 5, 10, 20, 35 and 50 μ l of the standard solution (the zero tube is the water blank) into separate tubes.
3. Add water to bring the contents of each tube to the same volume (preferably < 200 μ l).
4. Mix 1 ml of the copper sulphate solution and 1 ml of the tartrate solution with 48 ml of freshly prepared carbonate solution. Add 1 ml of this to each tube, mix and incubate for 10 minutes at room temperature.
5. Add 50 μ l of phenol reagent to each tube, mix again and incubate for 25 minutes.
6. Mix again and 5 minutes later, read the absorbance of each tube at 640 nm, using water to blank the spectrophotometer.

APPENDIX B

MATERIALS USED IN THIS STUDYBlocking Buffer

pH 7,4

NaCl	8,0g
KH ₂ PO ₄	0,2g
Na ₃ HPO ₄ · 12H ₂ O	2,9g
KCl	0,2g
BSA	0,5g
NaN ₃	0,2g
Distilled water q.s. ad	1000 ml

Coating Buffer

pH 9,5

Na ₂ CO ₃	1,59
NaHCO ₃	2,93g
NaN ₃	0,2g
Distilled water	1000 ml

Washing Buffer

pH 7,4

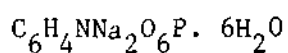
NaCl	8,0g
KH ₂ PO ₄	0,2g
Na ₃ HPO ₄ · 12H ₂ O	2,9g
KCl	0,2g
Tween 20	0,5 ml
NaN ₃	0,2g
BSA	10g
Distilled water q.s. ad	1000 ml

TRIS BUFFER (pH 8,0)

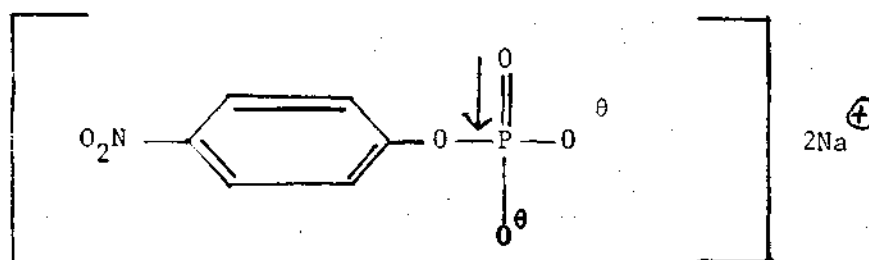
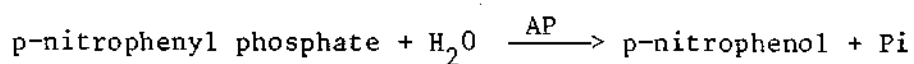
TRIS BUFFER (SIGMA X T-1378)	6,05 g
BSA	10 g
NaN ₃	0,02 g
Distilled water	1000 ml

Substrate

p-Nitrophenyl phosphate disodium salt



MW 371,15

Linkage which can be split enzymaticallyReaction:

The p-nitrophenol produced is measured spectrophotometrically at 405 nm.

Substrate Buffer (pH 9,8)

10% diethanolamine buffer

diethanolamine	97 ml
H ₂ O	800 ml
NaN ₃	0,2 g
Add a 1 mol/l solution of HCl to give pH 9,8	
Distilled water q.s.	ad 1000 ml

PBS (pH 7,4)

NaCl	8,0 g
KCl	0,2 g
KH ₂ PO ₄	0,2 g
NaN ₃	0,02 g
Distilled water	1000 ml