



Detection of Immune Complexes in CSF of Tuberculous Meningitis cases in Correlation with Antibody Response

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Abstract

A total of 800 CSF samples from patients suspected to TB meningitis were analyzed for anti-TB antibodies and mycobacterial immune complexes. About 24.375% of the cases showed anti-TB immune response by ELISA. Some CSF samples were positive only for anti-TB antibodies whereas some others were positive only for mycobacterial immune complexes. It appears that the immune complexes which are found before the free antibody could be detected. Immune complexes are a mixture of both antigen and antibodies. As the disease progresses the patient would have free antibodies and may become negative for immune complexes. It appears from the present study that the detection of both antibody and immune complexes is significant in the immunodiagnosis of the disease.

Keywords: Tuberculosis, meningitis, ELISA, CSF.

Introduction

Meningitis is inflammation of meninges, the membranes that cover the brain and spinal cord. Meningitis is also defined as inflammation of the Leptomeninges and underlying subarachnoid cerebrospinal fluid (CSF). It is mainly caused by bacteria or viruses. Bacterial meningitis is usually serious and is life-threatening if it is not treated promptly¹.

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB) in humans. Humans are the only reservoir for the bacterium. Tuberculous meningitis (TBM) is meningoencephalitis affecting meninges as well as brain parenchyma and vasculature. The primary pathologic event is formation of thick exudate within subarachnoid space, most prominently the base of brain. Accompanying this exudate is inflammation which affects the adjacent blood vessels. Ischemic cerebral infarction is caused from vascular occlusion found around middle cerebral artery (reflecting presence of exudate within sylvian fissure) and striates arteries as they penetrate the base of the brain^{2,3}. TBM is the clinical manifestation of central nervous system (CNS) involvement in TB⁴. *Mycobacterium tuberculosis* in the meninges is the main feature and the inflammation is concentrated around the base of brain. Infection onsets in lungs and spread to the meninges by various routes; blood-borne spread occurs generally and patients (25 %) with miliary TB have TBM, by crossing the blood-brain barrier; but many patients get TBM from rupture of cortical focus in brain and even from rupture of a bony focus in the spine (Pott's spine)⁵.

The diagnosis of TB mainly depends on clinical suspicion and radiographic findings with bacteriological confirmation by smear examination and culture techniques. The method of

diagnosis by solid media culture usually takes 3 to 8 weeks to reveal the causative organisms. TB serological techniques based on antibody detection for diagnosing and monitoring tubercular infection with low cost and flexibility to adapt to small laboratories makes it a popular diagnostic method⁶.

The main purpose of this study is to reduce the time required for the diagnosis of TB. The average incubation period required for the growth of this organism is estimated to be about 8 weeks. During this time period, the infection might be fatal to the patient and will lead to rise in mortality rate. Even though the gold standard diagnostic tool for the organism is culture, the time duration for incubation masks its specificity. In order to overcome this, a variety of diagnostic tools that have sensitivity rate of culture techniques can be developed.

Material and Methods

Samples: CSF samples (800 samples) used for this study were collected from TB patients diagnosed with active disease, subsided disease and also from patients with no history of TB were obtained from neighboring diagnostic laboratory as well as hospitals.

Staining for AFB (acid-fast bacteria): Preliminary and initial detection of TB was done by Ziehl-Neelsen (ZN) method of staining and graded as per RNTCP (Revised National Tuberculosis Control Programme) guidelines.

Culturing on Lowenstein-Jensen (LJ) medium: Readymade LJ medium slants were inoculated with 0.1 ml of each of the samples and incubated at 37 °C for 8 weeks. The LJ slants were inspected weekly for the growth of the mycobacterial colonies on the LJ slants i.e., rough, tough and buff-colored, were

subjected to ZN staining to confirm the presence of acid-fast bacilli⁷. Samples that show no growth in LJ medium after 8 weeks of incubation at 37 °C were considered as negative.

Culturing in modified Middlebrook 7H9 broth: Middlebrook 7H9 broth with indicator and cocktail of antibiotics (PANTA) (Himedia, Mumbai, India) was inoculated with 0.1 ml of each of the samples. The indicator in the medium makes it possible to visualize growth within 10–14 days. Antibiotic cocktail present in the medium retards the growth of contaminating flora.

Coating of MTSE antigen and blocking of MTSE plates: 5 ml of 20x PBS solution was taken and volume was made into 1x solution with distilled water. From this 5 ml was taken and 10 µl of MTSE antigen was added into it. 50 µl of this solution was transferred into the wells of ELISA (Microtitre) plates. The plate was kept in the refrigerator for overnight. The next day the MTSE plate was washed with PBST solution for 3 times. 5 ml of 20x PBS solution was taken and its volume was made into 1x solution with distilled water. To this 50 ml of solution 1 g of skimmed milk powder was added. 150 µl of 2 % PBST milk was added into antigen coated ELISA plate. Then the plate was incubated at 37 °C for 2 hrs. The plates were blot dried and were stored in the refrigerator⁸.

Coating of BCG antigen and blocking of immune complexes plates: 5 ml of 20x PBS solution was taken and its volume was made into 1x solution with distilled water. From this 30 ml was taken and 9 µl of anti-BCG antigen was added into it. 50 µl of this solution was transferred into ELISA (Microtitre) plates which were marked as IcM and IcG. These plates were kept in the refrigerator for overnight. The next day the BCG coated plates were washed with PBST solution for 3 times. 5 ml of 20x PBS solution was taken and its volume was made into 1x solution with distilled water. To this 50 ml of solution 1 g of skimmed milk powder was added. 150 µl of 2% PBS milk was added into Ag coated microtitre ELISA plates. Then the plates were incubated at 37 °C for 2 hrs. The plates were blot dried and were stored in the refrigerator⁹.

Procedure for ELISA of MTSE: 1% PBST Milk was prepared (0.2 g of skimmed milk powder was added to 20 ml of PBST solution. This is the sample diluent). 400 µl of PBST milk was added into each dilution tube. 100 µl of sample was added to it. These 1:5 dilutions were mixed well. The microtitre plate was marked as MTSE. The first well is the blank and to the second well MTSE Positive control was added. From the dilution tubes 50 µl was transferred to the ELISA plate wells in duplicates and plate was incubated at 37°C for 1 hr and 30 min. After the incubation was over the plate was washed for six times with PBST solution and it was blot dried. The conjugate IgG HRP and IgM HRP of 5 µl were added to 15 ml and 5 ml of PBST milk (diluent). The IgG conjugate was added to the plate (1:3000 dilution). The plate was then incubated at 37°C for 1 hr. After the incubation was over the plate was washed with the

wash buffer (PBST) for 8 times and it was blot dried. 75µl of the substrate was added in the ELISA plate into each well. The plate was then kept in a dark place for 10 min for colour development. The colour change was noted after stopping the reaction by using the stop solution 1N sulphuric acid (50 µl) into each well of ELISA microtitre plate. The plate was read using the ELISA reader (492 nm)⁸.

Procedure for ELISA of immune complexes: 1% PBST Milk was prepared (0.2 g of skimmed milk powder was added to 20 ml of PBST solution (sample diluent). 400 µl of PBST milk was added into each dilution tube. 100 µl of sample was added to it. These 1:5 dilutions were mixed well. The microtitre plate was marked as IcM, IcG. The first well is the blank and to the second well IgM Positive control was added to the second well of IgM plate. The first well is the blank and to the second well IgG Positive control was added to the second well of IgG plate. From the dilution tubes 50 µl of the diluted samples was transferred to the ELISA plate in duplicate and it was incubated at 37 °C for 1 hr and 30 min. After the incubation was over the plate was washed for six times with PBST solution and it was blot dried. 5 µl of the conjugate IgG HRP and IgM HRP was added to 15 ml and 5 ml of PBST milk (diluent). The IgG conjugate was added to the IcG plate (1:3000 dilution). The IgM conjugate was added to the IcM plate (1:1000 dilution). The plates were then incubated at 37 °C for 1 hr. After the incubation was over the plates were washed with the wash buffer (PBST) for 8 times and it was blot dried. 75 µl of the substrate was added in the ELISA plate into each well. The plates were then kept in a dark place for 10 min for colour development. The colour change was noted after stopping the reaction by using the stop solution 1N sulphuric acid (50 µl) into each well of ELISA microtitre plate. The plates were read using the ELISA reader (492 nm)⁹.

Results and Discussion

The control of TB depends mainly on early detection of infection and effective treatment. Various attempts have been made in improvement of diagnosis of TB by implementing clinical and laboratory methods^{10, 11}. Laboratory diagnosis have limitations: microscopic examination gives a low sensitivity, whereas culture techniques require more time and sometimes fail to detect the disease early¹². The development of the ELISA technique¹³ and dot-immunobinding assay¹⁴ using mycobacterial antigens has revolutionized serological tests for the diagnosis of mycobacterial infections.

A total of 800 CSF samples from patients suspected to TBM were analyzed for anti-TB antibodies and mycobacterial immune complexes. It is observed that 195 samples were found to be positive for either anti-TB antibodies or mycobacterial immune complexes. The percentage of positivity is 24.375%. The aim of this present study is to detect the presence of immune complexes IcG and IcM and MTSE antibody in CSF samples from TBM cases by ELISA.

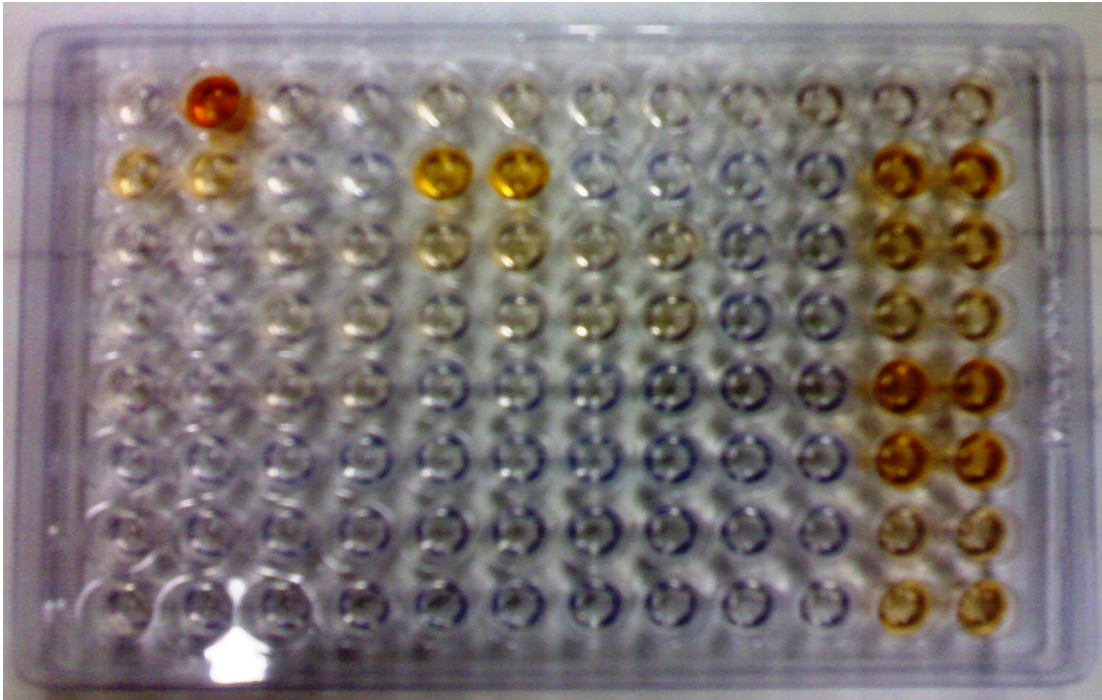


Figure-1
A microtitre plate module with positive results

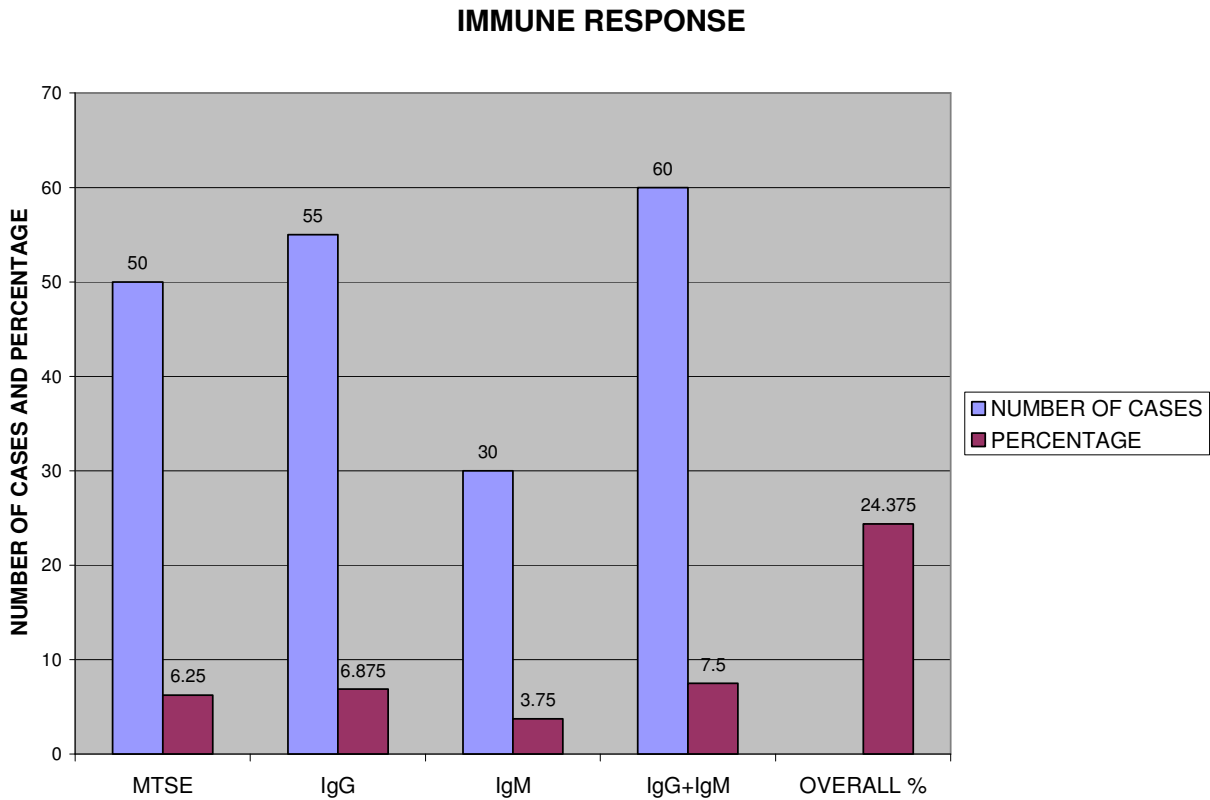


Figure-2
Bar graph representation of total immune response positive cases out of 800 samples tested for tuberculous meningitis immune complexes by ELISA

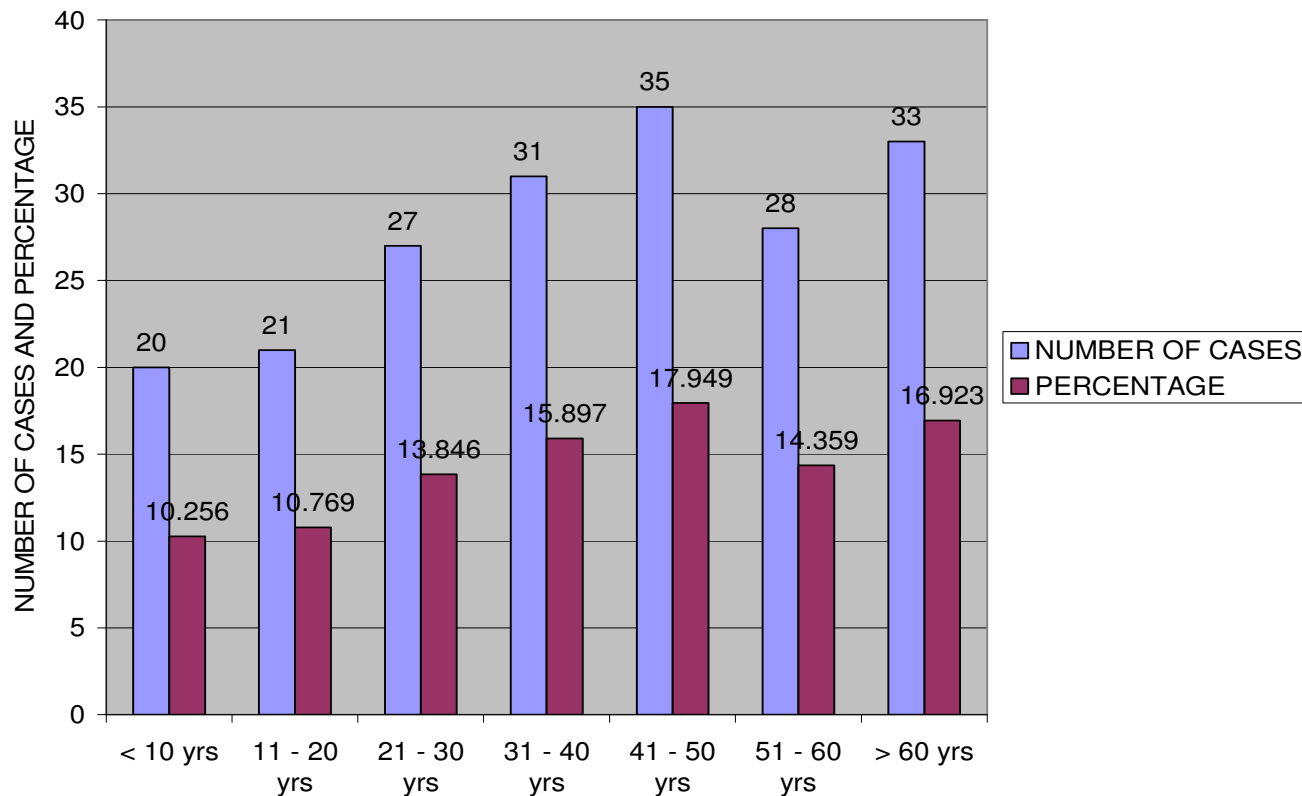


Figure-3
Bar graph representation of analysis of anti tuberculosis immune response in relation to age

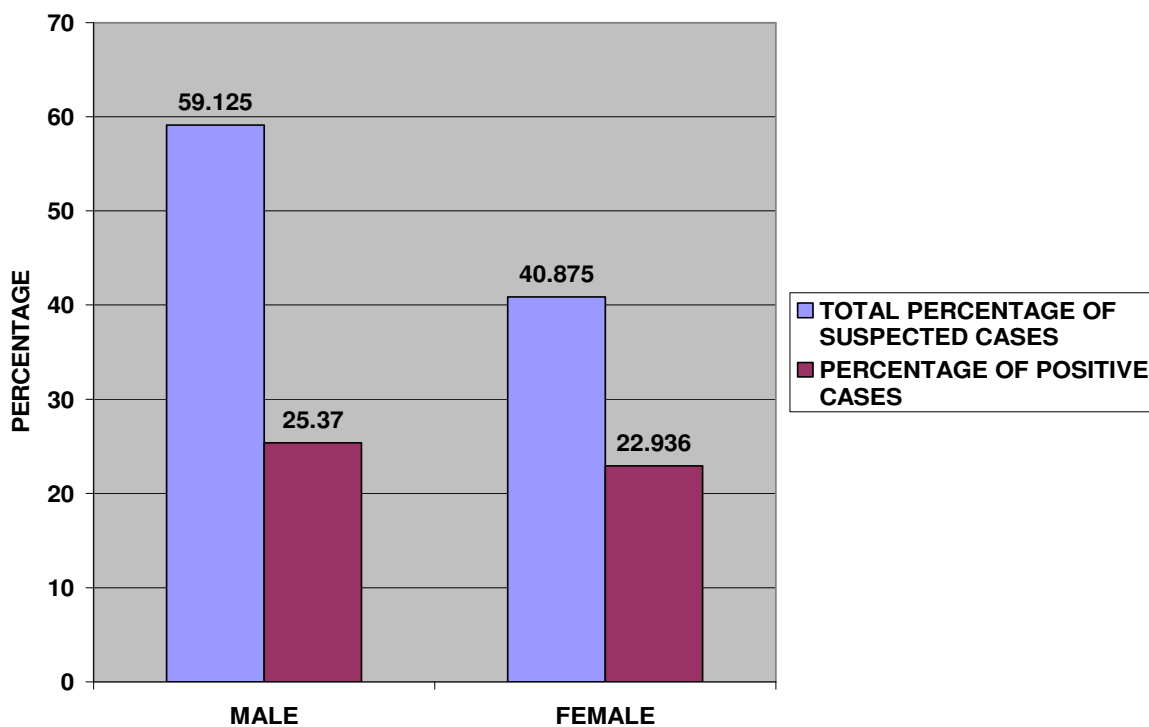


Figure-4
Bar graph representation of anti tuberculosis immune response analysis gender wise

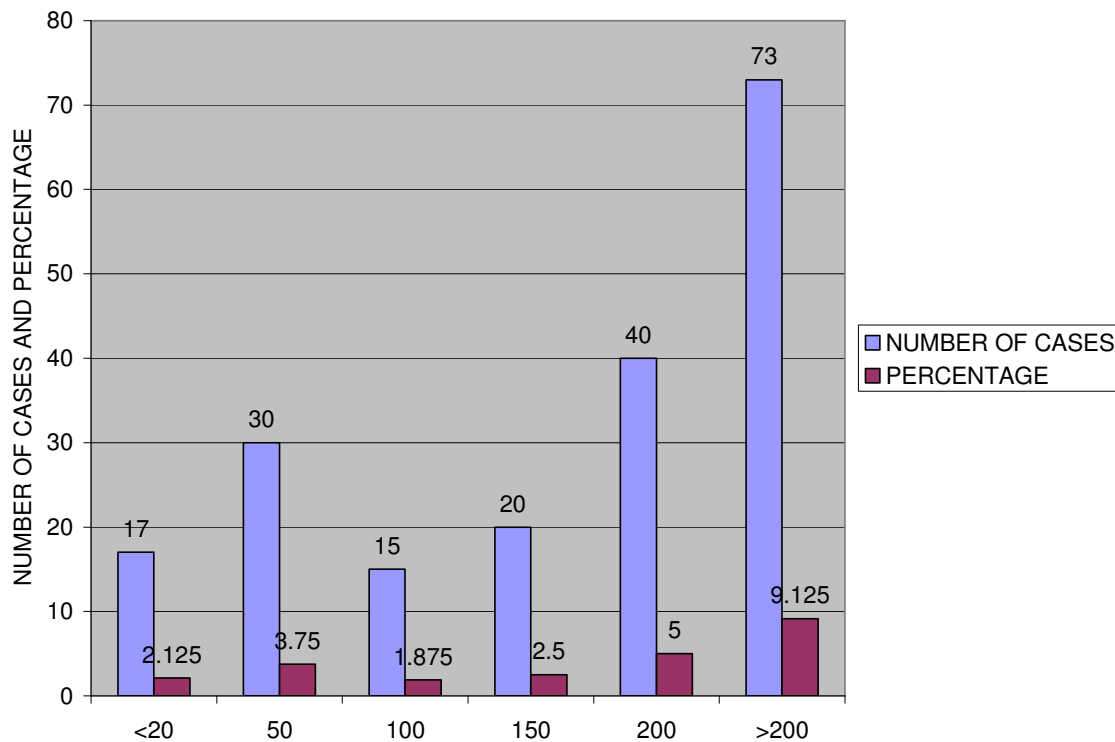


Figure-5
Bar graph representation of antibody response in relation to cell count

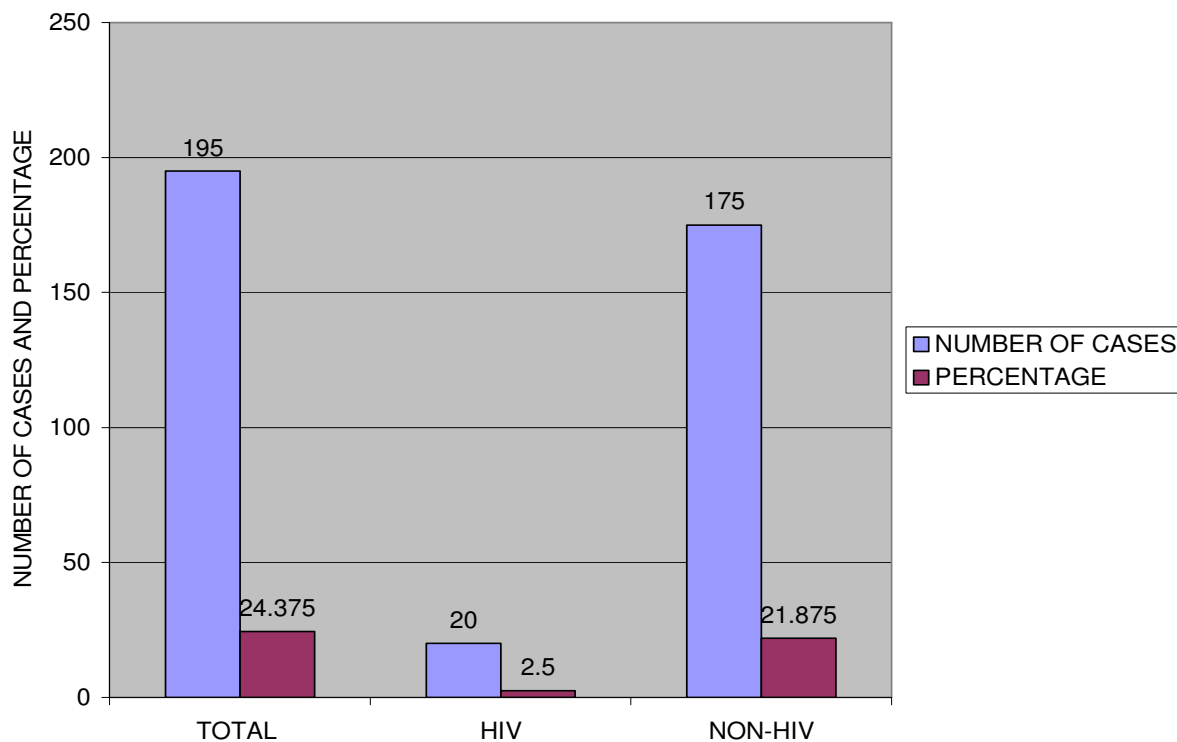


Figure-6
Bar graph representation of immune response and immune status of positive cases

It was observed that the total immune complexes are more when compared with total MTSE antibody. Out of 195 positive cases 60 (30.769 %) were total immune complexes. Then the IgG positive cases were 55 (28.205 %), IgM positive cases were 30 (15.385 %) and MTSE antibody positive cases were 50 (25.641 %). The study shows increased amount of antibodies and immune complexes in the age group between 41 and 50 (17.949 %). In other words the age group between 41 and 50 are mostly affected by TB when compared with other age groups according to the results obtained from this table. When compared to gender's males (25.37 %) showed higher positivity to both antibody and immune complexes than females (22.936 %). Thus this table suggests that when compared to gender's males showed higher positivity to both antibody and immune complexes than females. Whenever the cell count is high the positivity is also high and whenever the cell count is low the positivity is also low. Hence this means that when the cell count is low the severity of the disease might be less. Thus immune response and cell count might be interlinked with each other.

Earlier studies demonstrated that the detectable anti-TB antibody was found in cerebrospinal fluid (CSF)¹⁵⁻¹⁷. The detection of antibodies correlated well with the disease and was positive in 68 to 80% of the cases. Scientists have shown that antigen or antibody detection is useful in the diagnosis of pulmonary, meningeal, pleural and abdominal TB by radioimmunoassay^{15,18,19}. In a limited study of 10 CSF samples from patients with TBM, the potential usefulness of ELISA was demonstrated in the detection of antigen²⁰. Studies reported that in a cross sectional study on changes in IgG and IgA and IgM levels along with other serum proteins in pulmonary TB during therapy, serum IgG and IgA levels have mostly reported to be increased while most of the authors have reported unchanged IgM levels²¹. Measurements of different classes of immunoglobulins using antigenic preparations have shown that IgM antibody levels have been found to be so low that their reliable measurement has been difficult. In the present study, IgM is found to be positive in less number when compared with IgG and MTSE antibody. Overall the presence of immune complexes is marginally more predominant than mycobacterial antibody (MTSE). It appears that the immune complexes which are found before the free-antibody could be detected, as immune complex is a mixture of both antigen and antibodies.

Conclusion

TBM is meningoencephalitis affecting meninges as well as brain parenchyma and vasculature. The primary pathologic event is formation of thick exudate within subarachnoid space, most prominently the base of brain. A total of 800 samples suspected from TBM were analyzed for anti-TB antibodies and mycobacterial immune complexes by using ELISA. It is observed that 195 samples were found to be positive for either anti-TB antibodies or mycobacterial immune complexes. The percentage of positivity is 24.375%.

The positive results were further analyzed for total immune response, immune response in relation to cell count, age wise analysis of immune response, gender wise analysis of immune response and immune status of the patients.

Some CSF samples were positive only for anti-TB antibodies whereas some others were positive only for mycobacterial immune complexes. It appears that the immune complexes which are found before the free antibody could be detected. Immune complexes are a mixture of both antigen and antibodies. As the disease progresses the patient would have free antibodies and may become negative for immune complexes. It appears from the present study that the detection of both antibody and immune complexes is significant in the immunodiagnosis of the disease.

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