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Research Article

A STUDY ON EARLY AND ACCURATE DETECTION OF MYCOBACTERIUM TUBERCULOSIS AMONG MENINGITIS CASES BY USING CONVENTIONAL STAINING METHODS

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ABSTRACT

Tuberculous meningitis is the most severe manifestation of extrapulomonary TB with a high mortality rate. The aim of the present study was to diagnose Mycobacterium tuberculosis in CSF of TBM cases by different conventional staining methods in its earliest stages among the suspected TBM cases. In our study, the results revealed that staining methods were useful for early detection of Mycobacterium tuberculosis in CSF of TBM patients. In our study we analysed100 suspected cases of Tuberculous meningitis. Among them 12 cases were confirmed in CSF sample of TBM patients. Finally the study concluded that, Fluorescent staining is more sensitive than Ziehl Neelsen staining. TBM is still a diagnostic problem. A high index of suspicion is required in order to make an early diagnosis and treatment.

Keywords :-Meningitis, Ziehl Neelsen staining, Flourescent, Mycobacterium.

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INTRODUCTION

Tuberculosis is a chronic communicable air borne bacterial infection caused by *Mycobacterium tuberculosis*. It continues to haunt the human race as a medical malady with a tragic consequences in the social and economic realms & responsible worldwide for a staggering toll of disease and death [1].

Tuberculous meningitis (TBM) is still one of the common infections of central nervous system and can occur at any age except in the newborn, and poses significant diagnostic and management challenges, because of the inconsistent clinical presentation, low number of bacilli in CSF, lack of rapid sensitive and specific tests, emergence of HIV and drug resistant strains, more so in the developing world. Despite Millennium Development Goals of ``The Stop TB``, despite advances in field of microbiology and though curable, TB remains one of the world's biggest threats. The global incidence of TB is increasing by 0.4% per annum [2]. In 2014, 6 million new cases of TB were reported to WHO, fewer (5.4 million men, 3.2 million women, 1.0 million children) estimated to have fallen sick with the disease. This means that worldwide, 37% of new cases went undiagnosed or were not reported. In 2014, TB killed 1.5 million people (1.1 million HIV-negative and 0.4 million HIV-positive) [2].

India still is a High Burden country for TB with total new and relapse cases of 16, 09,547 out of which

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6% cases aged under 15 years; accounting 26 %(one fifth) of global burden [3]. To reduce this burden, detection and treatment gaps must be addressed.

The rapid and early diagnosis of tuberculosis is crucial first step for tuberculosis control program worldwide especially in the wake of emergence of drug resistant TB and its related implications for HIV infected patients [4].

Diagnosis of tuberculosis is mainly based on clinical presentation, demonstration of Acid fast bacilli in smears, isolation of MTB from culture (conventional and rapid) radioimmunoassay and Enzyme linked immunosorbent assay, which detect the mycobacterial antigen and its antibody in the Cerebrospinal Fluid (CSF) [5,6].

Currently, radiometric assay allows detection of *Mycobacterium tuberculosis* growth and provides antibiotic sensitivity results more rapidly usually within 10 days. However use of the technique is limited because culture medium contains radioactive carbon. Genetic probes are on the other hand quite easy to use and allow identification of bacteria in only a few hours by polymerase chain reaction. *Mycobacterium tuberculosis* strains can be detected directly in the sputum specimen within 2 or 3 hours, but in practice, this method has not become a routine laboratory technique, particularly due to lack of sufficient specificity and sensitivity. Serological tests are currently not reliable enough for the diagnosis of tuberculosis [7]. Microscopic examination and culture are still essential elements of the bacteriological diagnosis of tuberculosis; the diagnosis of tuberculosis is confirmed on the basis of demonstration of tubercle bacilli in the CSF or any other pathological material [8, 7, 9, and 10]. Smear examination is believed to be simple, cheap, quick and practicable and effective case finding method for developing countries. As tuberculosis bacilli are very slow growing organisms, culture results are available after a period of three or six weeks.

The specimen most commonly examined is CSF and mucous secretion coughed up from the lungs [11]. Microscopic examination of Ziehl-Neelsen or auramine stained specimen allows detection of most strains in less than an hour. Ziehl-Neelsen is the most extensively used procedure for the demonstration of *Mycobacterium tuberculosis* in smear [12, 13]. The requisites for the staining procedures are; basic fuchsin, phenol, absolute alcohol, sulphuric acid and methylene blue. Microscopic examination under oil immersion objective reveals *Mycobacterium* as red bacilli. In fluorescent staining, the smear is stained with Auramine. The auramine stain enters the cell wall of *Mycobacterium tuberculosis* and makes them glow against dark background under UV light [9]. Microscopic examination under low power objective will reveal *Mycobacterium* as glowing yellow white, rice like bacteria in the smear. Therefore the present prospective study was under taken to see the efficacy of Ziehl Neelsen method versus fluorescent staining in the detection of *Mycobacterium* in CSF sample.

Adults with TBM often present with the classic meningitis symptoms of fever, headache and meningismus (stiff neck) along with focal neurological deficits, behavioural changes, and alterations in consciousness. A history of or current positive tuberculin skin test, history of exposure to tuberculosis, or the identification of particular risk factors for tuberculosis raises the concern of TBM, although a history of tuberculosis is elicited in only approximately 10% of patients. The presence of active pulmonary tuberculosis on chest X ray ranges from 30 to 50% in recent series. Patients co-infected with HIV do not appear to have an altered presentation of TBM [14].

The aim our study is to analyse and diagnose by microscopic examination using conventional staining methods and identify the features of patients with TBM in Gandhi Medical college, Secunderabad, Telangana, India.

MATERIALS AND METHODS

This study consisted of a retrospective analysis of all TBM cases which were admitted to the Gandhi general Hospital. This study was carried out in the Department of Microbiology, Gandhi Medical College, Secunderabad, and Telangana during the period March 2014 –August 2015. Ethical clearance was obtained from institutional ethical committee. Informed or written consent was taken from the patients or relatives and clinical and demographic data was obtained by structured proforma. A case was considered confirmed if *Mycobacterium tuberculosis* was isolated in the CSF or if nucleic acid of *M. tuberculosis* was detected in the CSF. A case was considered as probable if the clinical profile of TBM, is consistent with CSF laboratory findings compatible with TBM, and a clinical response to anti-tuberculous treatment.

Study Period

The present study was conducted over a period of one and a half year from March 2014 – August 2015. Sample siz.e for the study was 100 clinically suspected cases of tuberculous meningitis.

Sample collection

CSF samples drawn aseptically by the treating physician were collected. One part was processed immediately, and another part was stored in -20^0c for molecular methods.

Gram staining, ZN staining, fluorescent staining, CSF analysis, and culture was done by inoculation of a centrifuged CSF sample on chocolate agar and blood agar incubated at 37°C to rule out pyogenic meningitis.

Microscopical smears

For each sample, three smears were made and

deposit on new glass slides. Smears were air dried and heat fixed. Gram staining, Ziehl Neelsen staining (ZN) & Fluorescent staining was done for each sample. The staining was done as per the standard protocol.

Data analysis

All of the smear slides stained and observed under oil immersion objective. A total of 300 visual fields on each slide were observed, among which AFB-positive fields were counted by three experienced observers independently. All of the data were displayed as means ± standard errors of the means (SEM) analyzed Differences were considered statistically significant when $P < 0.05$.

RESULTS

In the present study among 100 clinically suspected Tuberculous meningitis (TBM) cases, 12 cases were confirmed as TBM cases by PCR (Table 1).

Table 1. TBM Positives among clinically suspected cases(n=100)

	TBM positive	TBM negative	Total
cases	12 (12%)	88 (88%)	100

Among 100 clinically suspected TBM cases, positivity for AFB by Ziehl-Neelsen staining was 1% (Table 4, Figure: 4)

Table 2. Demonstration of AFB By Ziehl-Neelsen staining

Sample	No. of cases	No. of Positives
CSF	10	1 (1%)
Controls	20	NIL

Among 100 clinically suspected TBM cases, detection of AFB by Fluorescence Microscopy was 2% (Table 2, Figure:1).

Table 3. Demonstration of AFB by Auramine phenol staining

Sample	No. of cases	No. of Positives
CSF	100	2 (2%)

Sensitivity and specificity of Ziehl Neelsen staining was 8.3% and 100% respectively when compared to PCR.

Figure 1. Acid fast bacilli on ZN staining

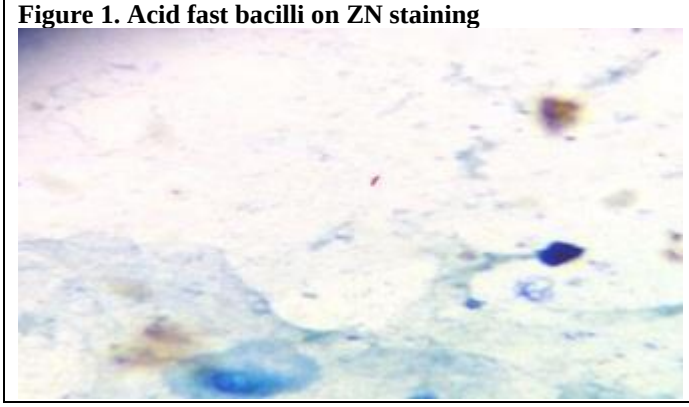
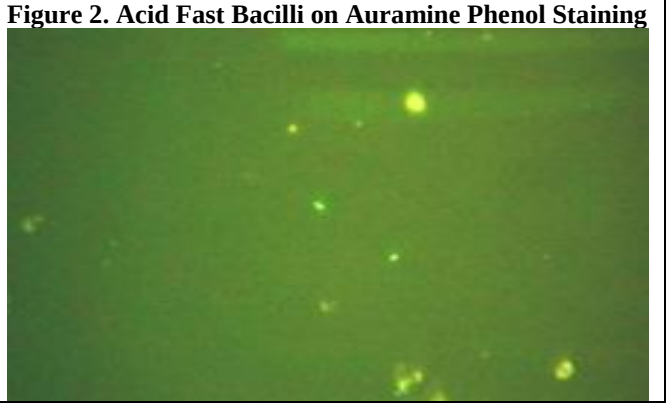


Figure 2. Acid Fast Bacilli on Auramine Phenol Staining



DISCUSSION

India has a long history of research and demonstration projects on TB. The detection of Acid fast bacilli is often considered as the evidence of the infected stage. Thus, the laboratory plays a critical role in the diagnosis of TB [15]. In developing countries, microscopy of the specimen is by far the fastest, cheapest, and most reliable method for the detection of AFB [11, 10]. However fluorescent staining has been added in Revised National Tuberculosis Control Program (RNCP) because of more sensitivity and rapid results and thus can be used in field areas.

Mycobacteria are distinguished from other microorganisms by thick lipid-containing cell-walls that retain biochemical stains despite decolourisation by acid-containing reagents (so-called 'acid-fastness'). Smear sensitivity is further reduced in patients with extra-pulmonary TB and those with HIV-co-infection [16]. Conventional fluorescence microscopy is on average 10% more sensitive than ZN microscopy. Conventional fluorescent microscopy has therefore been recommended by WHO at intermediate laboratory level where more than 100 smears are examined per day [17].

In the present study, Ziehl Neelson staining could detect 1% of cases among 100 clinically suspected TBM cases. These findings were similar or closer to earlier studies (1%) [18]. In previous studies reported variation in sensitivity and positivity, in different staining methods [19, 20, 21].

The detection limit of microscopy is 10^4*Mycobacterium* per milliliter whereas most patients with TBM have fewer *Mycobacterium* in their CSF samples and are thus missed as tuberculous meningitis cases.

Detection of AFB by Auramine phenol staining in the present study was almost similar to other findings [22, 23, and 24]. Even microscopic detection of tubercle bacilli in CSF by Auramine phenol staining requires at least 10^4 organisms, but due to more intensive binding of mycolic acids of the bacilli to phenol Auramine, it was having more positivity than ZN staining.

Detection of AFB by Auramine phenol staining requires 10^4organisms/ml like ZN staining, but Fluorescent microscopy is 10% more sensitive than ZN staining and more slides can be screened in lesser time.

The use of Fluorescent Microscopy greatly improves the diagnostic value of CSF smear especially in patients with low density of bacilli that are likely to be missed on Zeihl Neelsen stained smears. The method is economical in both time and expense and recommended for laboratories handling large number of CSF specimens [12]. Fluorescent staining is superior to that of ZN staining in the presence of a low bacterial load as seen in smears.

Using fluorescent microscopy, the tubercle bacilli when examined under ultra violet illumination, appears as bright rods against a dark back ground. Since there was a contrast, the bacilli were readily seen and therefore in very less time large area could be examined. Images were then captured with the digital camera and enhanced through imaging processing techniques [25]. While in ZN staining acid fast bacilli appeared bright red rods in blue background and the images were captured. The potential benefits of automated screening for tubercle bacilli are: rapid, acute, inexpensive diagnosis; the ability to screen large number of people; increased resources to monitor patients; and reduction in health risk to staff.

Thus the study reveals that CSF smears stained by the florescent method is useful and reliable for. Since the fluorescent microscopy is costly some laboratories cannot afford to buy florescent microscopy, so in these laboratories Ziehl-Neelsen staining is most employed [25,26].

CONCLUSION

CSF examination for the tubercle bacilli is usually performed for patients clinically and/ radiologically suspected of TBM cases. However, the standard method of CSF examination, that is, ZN staining is not sensitive enough and a large number of the suspected cases miss diagnosis. Moreover, many cases remain unsuspected and don't seek treatment. Fluorescent staining is a more efficient over ZN Staining in detecting Tubercle bacilli in sputum. Since screening is done under low power of magnification (40X), fluorescence has been found to be less time consuming compared to ZN method (100X) in the diagnosis of tuberculosis. Hence, it has been advocated to be method of choice where large numbers of CSF smears are to be examined. The fluorescent bacilli are easily identifiable and cause less eye-strain.

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