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Original Research Article

Ziehl-Neelsen stain: a dated but useful tool for diagnosis of EPTB in resource-constrained settings

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ABSTRACT

Background: Tuberculosis is one of the leading causes of mortality worldwide. The increasing prevalence of extrapulmonary tuberculosis (EPTB) is a cause of concern and its diagnosis still poses a major challenge to health care facilities.
Methods: This was a retrospective study, covering a one-year period, at a tertiary-care hospital in Eastern India. The EPTB smear samples were subjected to Ziehl-Neelsen (Z-N) stain and examined. Also, fine needle aspiration cytology (FNAC) was performed on lesions at suitable sites.
Results: Based on Z-N stain, EPTB was diagnosed in 40 out of 222 suspected cases (18.02%). The highest positivity was observed between 25 to 40 years (45%). The sex ratio was 2:1. Overall, the highest positivity was seen among pus samples (57.14%); followed by bronchoalveolar lavage fluid (40%), and aspirates from FNAC (14.3%, all of which had corroborating cytopathological features). Other samples, constituting of cerebrospinal fluid, synovial fluid, pleural fluid, ascitic fluid, urine and tissue were all negative by Z-N stain.
Conclusion: Technological advances are being developed for faster and specific diagnosis of tuberculosis. However, conventional staining method and microscopic findings still remain useful, especially in resource-limited settings.

Keywords: Acid-fast bacilli, Biosafety cabinet, Extrapulmonary tuberculosis, Pauci-bacillary, Sensitivity, Ziehl-Neelsen stain

INTRODUCTION

Tuberculosis (TB) is a global public health problem with more than 10 million new patients and approximately 1.4 million deaths every year, making it the second commonest cause of death from a single infectious agent, after COVID-19.[1] About a quarter of the global population is estimated to have been infected with TB.[1] A lot of emphasis has been laid on the diagnosis and control of pulmonary tuberculosis (PTB) but gaps exist in the clinical and laboratory diagnosis, prevention and control of extrapulmonary tuberculosis (EPTB). The prevalence of EPTB is rising globally, accounting for 15% to 20% of all cases, 30% of paediatric cases and 50% of cases with HIV-TB coinfection. However, this figure indicates only the tip of the iceberg, as majority remain undiagnosed.[2,3]

Diagnosis of EPTB is difficult, owing to its complex, subclinical presentation, lack of adequate diagnostic resources and paucibacillary nature.[4] Hence in an endemic country like India, there should be a high index of suspicion for tuberculosis. Despite the advent of faster and more sensitive technologies like PCR (polymerase chain reaction), NAAT (Nucleic Acid Amplification Test) and Xpert MTB/RIF assay, unavailability in every healthcare setting and higher costs, limit their use in routine diagnosis. Hence, in many low- and middle-income settings, a presumptive diagnosis is still commonly based on the finding of acid-fast bacilli (AFB)

on microscopic examination of a diagnostic specimen.[14] EPTB diagnosis is majorly lacking in rural health facilities that constitutes about 70% of the population in developing countries.[4] Here, the diagnosis largely depends on conventional smear microscopy with Ziehl-Neelsen (Z-N) stain, which serves as a rapid, practical, cost-effective and accessible tool for detecting acid-fast bacilli (AFB). Literature on the utility of Z-N stain and smear microscopy for diagnosis of EPTB is limited. Hence, the aim of the present study was to evaluate the diagnostic utility of Z-N stain in suspected cases of EPTB.

METHODS

Study design

This was a retrospective, cross-sectional study, at a tertiary-care hospital in Eastern India, covering a one-year period from May 2023 to April 2024.

General setting

Globally, the reported number of newly diagnosed TB cases was 7.5 million in 2022, being the highest since WHO began global TB monitoring. Among the eight high TB burden countries (constituting for two-thirds of the global total TB cases), India accounted for 27% of the world's TB cases.[1] The year 2022 showed a record high notification of 24.2 lakh cases of TB in the country, an increase of 13% as compared to 2021.[2]

As per the National prevalence survey, the TB incidence among men is comparatively more than women. In 2022, out of the total TB cases notified, 61% were male, 39% were female and less than 1% belonged to LQBTQIA++.[2] Data showed that, there had been a gradual increase in the proportion of notified TB cases who were in older age groups (>54 years) along with a simultaneous decrease in the younger age group population (<25 years). 5.6% belonged to paediatric age group (<=14 years) and 23.6% were aged 55 years or above.[2] TB is greatly influenced by socio-economic determinants and health-related risk factors. Undernourishment, alcohol use disorder, smoking, diabetes and HIV infection attributed to the major number of TB cases in India.[2]

According to India TB report 2023, approximately 24% had EPTB, 88% had newly diagnosed TB, and 3.7% had relapsing TB.[2] The common sites of EPTB were lymph node (26.3%), pleural (23.3%), abdomen (17.4%), spine (4.8%), meninges (2.8%), bone (excluding spine, 2.7%) and genitourinary tract (1.6%).

Specific setting

The study was conducted in Department of Microbiology in a tertiary-care medical college and hospital in West Bengal.

During the study period, about 696 sputum samples and 222 EPTB specimens were received (combining both inpatient and out-patient departments) in our laboratory. Out of them, 96 (14%) sputum smears and 40 (18%) EPTB smear samples were positive by Z-N staining method. As per our hospital follow-up reports, approximately 70% of the patients adhered to the treatment guideline and about 2% mortality was noted.

Keeping in mind the aim of the study, we subjected all the PTB and EPTB samples to Z-N staining method for diagnostic purpose. All the patients who were found to be positive by Z-N stain smear microscopy, were referred to our local sub-divisional hospital for proper treatment and follow-up, as per government protocol. Those who were negative by Z-N stain smear microscopy but had strong clinical or radiological suspicion of TB were referred to higher centre for CBNAAT or Gene-Xpert assay.

Sample population

The specimens of all clinico-radiologically suspected cases of EPTB, which were sent to the laboratory, were included in the study. The specimens in which any other bacteria or fungi were observed on Gram stain and/or had isolated growth on culture were excluded from the study.

The independent variables were age, gender, type of TB and site of TB.

Data sources and collection

The demographic characteristics of the patients were collected from hospital information system. Frequency of different EPTB samples, distribution of positive cases and prior history of anti-tubercular therapy were obtained from microbiology and pathology laboratory registers. None of the patients had any prior history of treatment with anti-tubercular therapy.

Laboratory procedures

Smears were prepared from the respected specimens on sterile, unscratched slides in a Class II A2 biological safety cabinet, and subjected to Z-N staining as per National Tuberculosis Elimination Programme (NTEP) guidelines.[5,6] Thereafter, at least 300 oil-immersion fields were examined for AFB. In addition, lesions at suitable sites were subjected to fine needle aspiration cytology (FNAC) and evaluated for cytomorphology and smear microscopy. All slides were examined by two independent observers before being declared negative.

Analysis strategy

Relevant data obtained were entered in Microsoft excel spreadsheet (Office 2021) and analyzed by SPSS (statistical package for social sciences) software (version16). Demographic data were expressed in ratio and percentages. Distribution of EPTB samples were

presented as frequencies and percentages. Since this was a record-based study, all samples suspected of EPTB, which were sent to microbiology and pathology laboratories were included in this study. The total sample size was 222.

RESULTS

Demographic findings

The highest positivity was observed between 25 to 40 years (45%) (Table 1). The sex ratio was found to be 2:1 (Figure 1).

Laboratory findings

AFB appeared approximately 0.3 to 3 um long, slender, beaded and pink-colored rods against blue background (Fig. 2). EPTB was diagnosed in 40 out of 222 suspected cases (18.02%) on the basis of positive Z-N-stained smears. Overall, the highest positivity was seen in pus samples (57.14%); followed by bronchoalveolar lavage fluid (40%), and aspirates for FNAC (14.3%, all of which also had corresponding cytopathological features) (Figure 3). Other samples included cerebrospinal fluid, synovial fluid, pleural fluid, ascitic fluid, urine and tissue, which were all negative by Z-N stain (Table 2).

Table 1: Distribution of positive cases according to age group.

Age group (Years)	No. of patients	%
0-12	3	7.5
13-25	4	10
25-40	18	45
40-60	13	32.5
>60	2	5

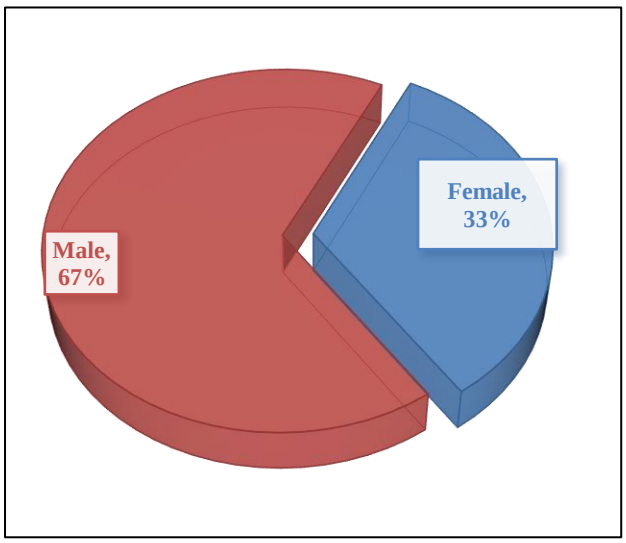

Figure 1: Sex distribution.

Table 2: Frequency distribution of different samples of EPTB.

EPTB Samples	Frequency of cases	%
Broncho-alveolar lavage fluid	65	29.28
Pus	14	6.3
Cerebrospinal fluid	36	16.21
Pleural fluid	27	12.16
Ascitic fluid	12	5.4
Synovial fluid	9	4
Tissue	8	3.6
Urine	9	4
Aspirates for FNAC	42	19

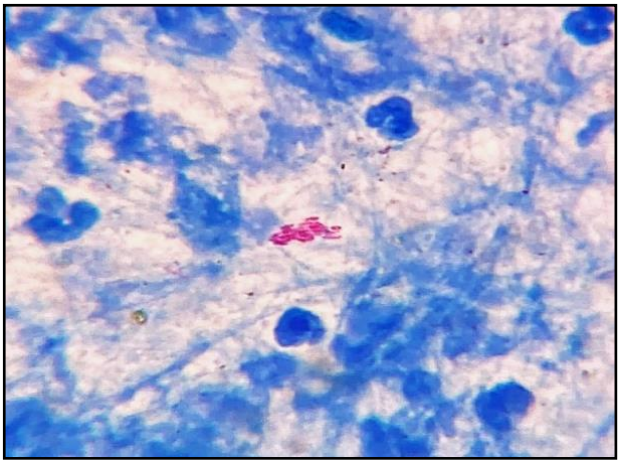

Figure 2: Z-N stain of AFB positive EPTB sample.

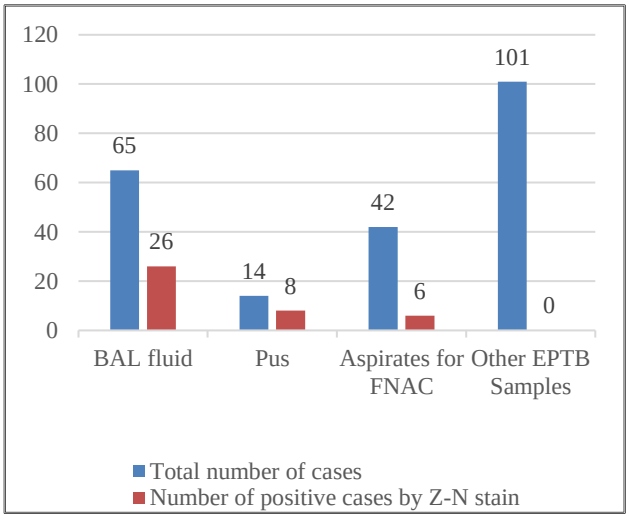

Figure 3: Distribution of EPTB samples.

DISCUSSION

EPTB involves a variety of presentations related to different organ systems. The rise in number of EPTB cases may be attributed to improper diagnosis or poor adherence to treatment.[4] The International standards for

TB care (ISTC) recommends that appropriate specimens must be collected from suspected sites for microbiological and histological examination.[7]

Although EPTB cases were found in all age groups in our study, majority were between 25 to 40 years, corroborating with other studies.[8,9] This group constitutes young, reproductive and economically productive population; thus, highlighting the socio-economic burden of EPTB on Indian economy. Globally, women are found to be more at risk of EPTB, whereas, in our study, male preponderance was observed, which correlates with few other studies.[8,11] Low detection of EPTB in female sex in our study could be due social stigma associated with TB, which hinders female population, especially those belonging to rural areas, to seek medical assistance.

The percentage positivity in our study, based on Z-N stain, correlated with a study by Kant et al. (21.7%), while in some other studies the positivity was 10-14%.[12] Even though smear microscopy using Z-N stain has limited sensitivity, it is a simple, useful and cheap diagnostic method accessible to peripheral laboratories.[11,13]

In our study, the highest positivity was seen among pus samples; but in other studies, lymph node and pleura were most often positive.[8,10] The bacterial load of a clinical specimen has direct association with the sensitivity of Z-N stain. Smear positivity is generally achieved at a concentration of ≥10 organism/ml of the specimen.[4,13] Due to pauci-bacillary nature of EPTB samples, only a minority of these are positive, and it requires patience to detect the few bacilli present in a smear.

Culture methods are more sensitive because fewer bacilli (10-100 bacilli/ml of concentrated material) can be detected but they are time-consuming; besides, they need skilled technicians and appropriate bio-safety measures.[4]

NAAT have high sensitivity (1-10 organisms in specimen) and high positive predictive value (98–99%) from various EPTB specimens.[4] The sensitivity of nested or real time PCR is more compared to Z-N staining or culture but its higher cost limits its use in routine diagnosis. The sensitivity of the Xpert MTB/RIF assay for EPTB ranges from 25% to 96.6%. In settings where the above facility cannot be implemented, TB-LAMP assay (loop-mediated isothermal amplification) may be a possible alternative as it is simple to use, cheap and requires minimal infrastructure.[14] In a study by Singh P et al., sensitivity of LAMP assay was 85.71% when compared with culture and 88% when compared to Gene-Xpert assay.[15]

The sensitivity of FNAC smears is between 34.6 to 66%.[16] In our study, the percentage positivity based on Z-N stain was 14.3%, whereas in a study by Gulati HK et al., it was 39.4%.[17] Thus, cytopathological and microbiological examinations of the FNAC aspirates can be useful to diagnose tuberculous lymphadenitis.

CONCLUSION

This study provides a valuable insight about the demographic characteristic, site predilection and overall trend of EPTB in a tertiary healthcare setting, which caters to a varied type of population. There were also certain limitations of this study. The study being retrospective, it did not include information about important risk factors such as HIV status, nutritional status, smoking, alcohol intake, drug addiction, etc. We also could not comment on the disease status of the AFB negative specimens as they were subjected to Z-N staining only which has a relatively lower sensitivity.[13]

Further studies with larger sample sizes employing Z-N stain, and comparing it with more sensitive tests as the gold standard, may be necessary to validate the efficacy of Z-N stain in the diagnosis of EPTB samples.

Recommendation

Based on the above findings, the following recommendations are forwarded (a) more sensitive and specific diagnostic tests like NAAT or GeneXpert should be made available for better diagnosis of EPTB, in every healthcare setting, (b) large scale, community-based studies and awareness programme should be conducted for decreasing the burden of EPTB.

EPTB remains a significant health problem in developing countries. Without treatment, the death rate from TB disease about 50% whereas, with treatments currently recommended by WHO, about 85% of people with TB can be cured.[1] Thus, it is very important to diagnose the disease as early as possible. Although new technologies are constantly being developed for faster and specific diagnosis of tuberculosis, the screening of EPTB samples by Z-N stain remains an inexpensive yet useful option, especially in resource-constrained settings. It requires sincere examination of smears from extra pulmonary sites. In laboratories, where culture and molecular facilities are unavailable, commitment and thorough search for AFB from a Z-N-stained smear may be rewarding.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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