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PERFORMANCE OF LED FLUORESCENT MICROSCOPY AND XPERT MTB/RIF ASSAY IN DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS.

Microbiology

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ABSTRACT

Microbiological diagnosis of extrapulmonary tuberculosis [EPTB] is based on microscopy, culture and molecular tests. A prospective study was done to determine sensitivity and specificity of Xpert MTB/RIF assay and LED fluorescent microscopy in clinically suspected cases of EPTB in comparison with solid culture. Consecutive 275 extrapulmonary specimens were mainly pleural fluid (8.14%) followed by CSF (36.2%), pus (37.5%) and lymph node biopsy (40.90%). ZN and LED microscopy, Xpert assay and culture detected 14, 19, 59 and 70 cases respectively. LED microscopy and Xpert assay had sensitivity and specificity of 27.14%; 100% and 80%;98% respectively. Xpert assay detected MTB in 59 (21.45%) specimens with 22.03% rifampicin resistance. 17 specimens showed discordant results. Xpert MTB/RIF assay is a rapid and time-saving diagnostic test and should be used as the initial diagnostic test for detection of tuberculosis as well as rifampicin resistance in all EPTB. LED microscopy should replace ZN microscopy.

KEYWORDS

Extrapulmonary tuberculosis, LED microscopy, Xpert assay.

INTRODUCTION

Pulmonary tuberculosis (PTB) caused by *Mycobacterium tuberculosis* (MTB) is a leading cause of death [1]. The prevalence of extrapulmonary tuberculosis (EPTB) is on the rise in India [2]. Globally, EPTB accounts for 10% to 42% of TB cases [3]. In India, 15 to 20% of TB cases are estimated to be EPTB [3]. The common sites involved are lymph node, pleura, meninges, spine, skin, gastrointestinal tract or central nervous system through blood or lymphatic spread [4]. Diagnosis of EPTB is difficult due to its paucibacillary nature and limited quantity of sample retrieved from deep seated lesions.

EPTB is diagnosed usually by composite criteria which include clinical, radiological, histopathological and microbiological findings. Amongst this, microbiology provides specific diagnosis. Microbiological diagnosis of tuberculosis is based on microscopy, culture and molecular assays. Microscopy technique is simple, inexpensive and rapid. Microscopy by Ziehl Neelsen [ZN] is highly specific but with low sensitivity (20%-80%) in pulmonary tuberculosis and still lower (0-40%) in a paucibacillary disease like EPTB [5,6]. Fluorescent LED microscopy has 10% more sensitivity than ZN stained light microscopy mainly in PTB [6].

Culture is considered as a gold standard investigation with a prolonged turnaround time of 4-8 weeks. Identification of drug resistant TB takes another 4-6 weeks. Liquid culture has reduced the turnaround time by half [7]. Molecular assays have revolutionised the diagnosis of tuberculosis especially PTB with improved sensitivity compared to microscopy [8].Xpert ® MTB/RIF assay, Cepheid, (Xpert assay) is a cartridge-based nucleic acid amplification test based on hemi-nested PCR technique that can be done directly on untreated EPTB specimens. It uses molecular beacons and simultaneously detects the presence of molecular beacons and resistance to rifampicin within 2 hours [8]. It requires minimum handling of specimen and technique is very easy. With better sensitivity in all forms of TB disease, this assay was endorsed by World Health Organization (WHO) in 2013 as the initial diagnostic test in EPTB [3].

The present study was undertaken to determine the performance of LED microscopy and Xpert assay in diagnosis of EPTB in a tertiary care teaching hospital in comparison with culture and to determine the additional yield with these tests in comparison to the conventional methods.

MATERIALS AND METHODS

A cross sectional study was carried out over a period of one year (April 2016 to March 2017) in a tertiary care teaching hospital after obtaining institutional ethics committee permission [ethics no. EC/181/2015]. Patients of any age and gender, referred by clinicians for laboratory confirmation of clinically suspected EPTB, submitting extrapulmonary specimens (minimum 5 ml) other than stool and blood and willing to give written informed consent were included in the study. Patients already on anti-tuberculosis treatment were excluded from the study.

STUDY PROCEDURE

All processing was carried out in Bio Safety Cabinet (BSC) class 2 and level 2 bio safety practices were followed. All body fluids were centrifuged at 3000 rpm for 15 minutes. Highly viscous specimens were digested by NALC – NaOH method before centrifugation. For each specimen, two direct smears were prepared and labelled as D1 and D2. Two smears prepared after concentration and labelled as C1 and C2. D1 and C1 were stained by ZN stain and screened for the presence of acid fast bacilli D2 and C2 were stained by fluorescent stain and screened for the presence of fluorescent bacilli. Specimen was considered positive if any of the direct or concentrated smear showed presence of acid fast or fluorescent bacilli suggestive of Mycobacteria as per Revised National Tuberculosis Control Programme guidelines [9].

Pellet obtained after centrifugation was inoculated on Lowenstein Jensen (LJ) medium and incubated aerobically at 37°C. Any growth observed on LJ medium was identified as MTB or mycobacterium other than tuberculosis using phenotypic characteristics and MPT 64 immunochromatography assay. Any acid fast isolate which was a slow grower, formed buff coloured colony on LJ slant and was MPT64 positive was characterized as MTB.

Xpert assay was carried out as per manufacturer's instruction preferably on concentrated pellet [8]. Negative results were generated as "MTB not detected". Positive results were generated as MTB detected with rifampicin sensitive / resistant / indeterminate.

RESULTS

290 extrapulmonary specimens from an equal number of consecutive patients, referred during study period to the laboratory were tested by microscopy, culture and Xpert assay. Of these, 10 specimens showing error in Xpert assay and 5 specimens showing contamination in culture could not be retested due to unavailability of additional specimen. So 275 specimens were included for final analysis. Of these 275 patients, 117 (42.54 %) were in the age group of 15-30 years and 158 (14.90 %) were in pediatric age group (≤ 14 years). Male to female ratio was 1:0.87.

Specimens obtained were mainly pleural fluid (86, 8.14 %) followed by CSF (58, 36.2%), pus (48, 37.5%) and lymph node (LN) biopsy (44, 40.90%). [Table 1]

ZN and LED fluorescent microscopy, Xpert assay and culture detected 14 (4.41%), 19 (6.5%), 59 (21.45%) and 70 (25.45%) cases respectively. Culture had the maximum diagnostic yield followed by Xpert and LED microscopy. Sensitivity/specificity of ZN and LED fluorescent microscopy was 20%; 100% and 27.14%;100% respectively. LED fluorescent microscopy detected five additional cases in comparison to acid fast stained microscopy. All specimens positive by microscopy were also positive by culture. Both the microscopy techniques did not have any additional yield over culture. ZN stained microscopy detected TB in 9 (3.27%) direct smears and 14 (5.09%) concentrated smears. LED fluorescent microscopy detected TB in 13 (4.73%) direct smears and 19 (6.91%) concentrated smears. This difference was not statistically significant.

Xpert assay detected MTB in 59 (21.45 %) specimens. [Table 2] 14 specimens were positive by culture but negative by Xpert assay. 3 specimens were positive by Xpert assay but were negative on culture. Sensitivity and specificity of Xpert assay in comparison with culture was 80% and 98% respectively using single specimen. Bacillary load observed in the specimens was medium (10, 16.94%), low (30, 50.84%) and very low (17, 28.81%) on Xpert assay. Only two specimens (both pus from psoas abscess) had high bacillary load.

In the present study, rifampicin resistant was detected in 13/59 (22.03%) MTB positive specimens by Xpert assay.

DISCUSSION

Present study on performance of Xpert assay, a cartridge based nucleic acid amplification test and LED fluorescent microscopy in extrapulmonary TB, has provided expected findings. Xpert assay detected greater number of cases than any microscopy technique and LED fluorescent microscopy performed better than acid fast stained light microscopy. The need for a more accurate test in settings of EPTB to provide confirmatory evidence of clinical and/or radiological suspicion has long been felt. Xpert assay also provided rapid information on rifampicin sensitivity, thereby playing a dual and significant role.

In the present study, majority of the specimens were obtained from pleura (86, 31.27 %). Similar findings have been reported in other studies. [10,11]. Pleural tuberculosis is thought to be more common than other forms of TB. It is believed to be due to delayed hypersensitivity reaction to mycobacterial antigens in the pleural space [12]. Rupture of sub-pleural foci of mycobacteria result in entry of bacilli into the pleural space [12] accounting for its higher frequency in EPTB. Lymph node TB has also been reported as the most frequent form of EPTB in many studies [13]. It is caused due to the spread of infection from primary site to the draining lymph-node through lymph-haematogenous route [14]. In the present study, LN TB accounted for 16% (44/275) of total cases.

Overall smear positivity of 6.91 % in present study was concordant with smear positivity reported in other studies [15,16]. Of the 19 cases detected by any microscopy, five were detected only by LED microscopy. The grading of these five additional cases was scanty. The marginal higher yield by LED was statistically significant**.** Higher positivity by fluorescent staining as compared to acid fast staining has also been reported in literature [16,17].

Low smear positivity in EPTB can be attributed to the paucibacillary nature of the disease [3]. Positivity by direct smear and concentrated smear was 3.27%/ 5.09% and 4.73%/ 6.91% by ZN and LED fluorescent staining method respectively. The marginally higher yield with concentrated smear in both the staining technique was not statistically significant. Similar findings have been reported by Jedthani et al (1999) [18]. Every additional case detected by any method even though statistically not significant is important in terms of patient outcome (morbidity and mortality) [19,20] and disease transmission especially in the setting of concomitant PTB.

Presently under RNTCP, LED fluorescent microscopy has been phased into most of the designated microscopy centres and replaced acid fast stained microscopy. At present, it is used mainly for microscopy of sputum specimens. LED fluorescent microscopy has lots of advantages. As fluorescent bacilli are easily visible against dark background, even scanty bacilli are easily picked up resulting in more sensitivity. The smear is screened under low or high power lens as compared to oil immersion lens. ZN staining require examination of every smear for at least 5 minutes and 100 oil immersion fields should be observed before recording it as negative. As more time needs to be spent on reading slides, there is a possibility of missing scanty bacilli especially in laboratories with very high load, resulting in false negativity. LED fluorescent staining technique is comparatively simple as heating is not required. The error reported due to either over heating or under heating can be avoided. Any smear with doubtful reading can be restained easily with ZN staining for confirmation.

xo

The drawback with LED fluorescent microscopy is that staff should be properly trained to differentiate between acid fast bacilli and artefact. For better reporting the smears should be stored in dark and examined within 24 hours after staining. Training, competency and confirmed quality assurance should be done regularly for better results.

In the present study, Xpert assay detected less number of cases as compared to culture with a sensitivity and specificity of 80% and 98% respectively using a single specimen. The sensitivity and specificity of Xpert assay reported in literature varies from 70.6% to 80% and 97.3% to 100% respectively [21,22]. Maximum positivity was observed in pus (33.33%) and lymph node (29.54%) followed by pleura (19.76%). The yield of positivity also depends on the nature of sample. Presently Index TB guidelines on use of Xpert assay, recommends that this test should be used as an adjunctive test for CSF sample. In the present study, Xpert assay detected MTB in 29.31% (17/58) of CSF specimens.

Three specimens (one FNAC and two CSF) were positive by Xpert assay but negative by culture. This may be due to the paucibacillary nature of extrapulmonary specimens with uneven distribution of the bacilli and formation of clumps in CSF [23]. The sample processing done with Xpert assay is reported to better homogenize and liquefy than traditional NALC-NaOH method [23]. False negative results by culture may also be because Xpert assay is not affected by contaminating bacteria in the sample.

14 samples were culture positive and Xpert assay negative. Xpert assay result was valid as sample processing control (SPC) was amplified and probe check control (PCC) also worked. Xpert assay result may be false negative due presence of inhibitors in specimens which inhibited the amplification of the *M. tuberculosis* genome without any effect on the internal control. Xpert assay result depends on capture of intact bacilli from the specimen within the cartridge. With reported limit of detection of Xpert assay (131 CFU/ml), not enough bacilli may be captured and lysed. In literature similar findings have been reported within the range of 3.73% to 59.34% for Xpert assay and range 4.98% to 66% for culture [24,25].

Ten Specimens showed "Error" by Xpert assay. These were FNAC from lymph node (7) and bone tissue (3) specimens. Collection of extrapulmonary specimens is an invasive procedure. Traces of blood are likely to be present in extrapulmonary specimens. But if the specimen contains significant amount of blood, presence of RBCs reportedly can produce error result by Xpert assay. Proper homogenization of bone pieces may not be achieved by specimen reagent resulting in error.

Low and very low bacterial load was seen in 79.66% of the specimens tested positive on Xpert assay. Similar findings have been reported in literature [22] where 78.4 % of specimens showed low and very low load. This result correlates with the fact that EPTB is a paucibacillary disease.

Rifampicin resistance was detected in 13/59 (22.03%) positive specimens. Of these 5(8.47%) were new cases and 8 (13.56%) has history of taking anti-TB treatment in past. This correlates with the resistance rates reported from Mumbai MDR TB accounts for 24%–30% of new cases and 11%–67% of treated cases in Mumbai. The corresponding figures from other parts of the country are 1%–13% and 12%–40% respectively [26]. A study done in Mumbai in 2016 reported rifampicin resistance in 19.2% cases [27]. Three specimens showed rifampicin indeterminate result by Xpert assay. All three specimens had very low load by Xpert assay and could not be retested by Xpert assay as second specimen was not available.

WHO has endorsed use of Xpert assay for EPTB specimens in 2013 as the initial diagnostic test in cerebrospinal fluid (CSF) and as an add-on test in lymph nodes and tissues [28]. International Standard for TB Care and Standards for TB Care in India, updated in 2014, recommend Xpert assay

for EPTB [29]. As per policy document on Index TB guidelines for extrapulmonary tuberculosis published in 2017, Xpert assay may be used as an adjunctive test for TB meningitis [30]. Considering significant positivity in pleural fluid and lymph node obtained in the present study, it is advisable to perform the Xpert assay on these specimens due to its rapid turnaround time and information about rifampicin susceptibility status.

Use of liquid culture instead of solid culture could have detected more cases. Also, correlation of laboratory result with the treatment outcome would have given a clearer picture of the true incidence.

CONCLUSIONS

Xpert MTB/RIF assay is a rapid and time-saving diagnostic test when compared to solid culture. Xpert assay should be used as the initial diagnostic test for detection of tuberculosis as well as rifampicin resistance in all EPTB cases along with culture. LED fluorescent microscopy should replace ZN microscopy considering its better sensitivity.

TABLE 1: Specimen-wise results of various tests.

Specimen	Total tested	Total positive	% positivity	Test Positivity					
				Culture	Xpert assay	ZN microscopy		LED microscopy	
						Direct smear	Concentrated smear	Direct smear	Concentrated smear
Lymph Node	44	18	40.90%	18	13	2	2	1	2
Pus	48	18	37.5%	18	16	4	6	5	7
Pleural	86	7	8.14%	7	5	0	2	4	5
CSF	58	21	36.21%	19	17	0	1	0	1
Abdomen	17	1	5.88%	1	1	1	1	1	1
Bone	11	5	45.45%	5	5	1	1	1	2
Pericardial fluid	5	0	0	0	0	0	0	0	0
Other	4	2	50%	1	2	1	1	1	1
Joint	2	1	50%	1	0	0	0	0	0
Total	**275**	**73**	**26.54%**	**70**	**59**	**9**	**14**	**13**	**19**

TABLE 2:Comparison of results of Xpert assay and LED microscopy with culture.

	Culture		Total
	Positive	Negative	
Xpert assay			
Positive	56	3	59
Negative	14	202	216
Total	70	205	275
LED Microscopy			
Positive	19	0	19
Negative	51	205	256
Total	70	205	275

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