Comparative Study of Different Diagnostic Methods for Detection of Mycobacteria in Clinically Suspected Patients of Extrapulmonary Tuberculosis at IGIMS, Patna

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Abstract---Background: Tuberculosis is a global problem and incidence of extra pulmonary tuberculosis is increasing day by day. Diagnosis of extra pulmonary tuberculosis is a little bit difficult than pulmonary tuberculosis. Aim and Objective: The aim of this study is to compare different diagnostic methods like ZN staining, LED-FM and GeneXpert assay for detection of Mycobacterium tuberculosis in extrapulmonary tuberculosis (EPTB) cases in respect to their sensitivity, specificity, PPV and NPV respectively. Methodology: This prospective study was carried out in the Department of Microbiology,

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IGIMS, Patna, Bihar. Samples from 210 suspected extrapulmonary cases were collected and processed for ZN-stain, LED-FM, GeneXpert assay and MGIT culture. Taking the MGIT culture result as Gold standard, result of ZN-stain, LED-FM and GeneXpert were compared. Result: Out of 210 samples, detection rate of ZN staining, LED-FM, GeneXpert assay and MGIT culture were 10.47%, 14.28 %, 30.47% and 36.66% respectively. The sensitivity, specificity, PPV and NPV of ZN staining and LED-FM were 28.27 %, 100%, 100%, 70.74% and 39.96%, 100%, 100%, 73.88% respectively. The sensitivity, specificity, PPV and NPV of GeneXpert were 59.74%, 86.46%, 71.87% and 78.76% respectively.

Introduction

Tuberculosis (TB) is a communicable disease that is a major cause of ill health, one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent (ranking above HIV/AIDS). In 2019, about 10 million people developed TB and 1.4 million died. TB can affect anyone anywhere, but most people who develop the disease (about 90%) are adults; there are more cases among men than women; and of those who fell sick with TB in 2019, 87% were in 30 high TB burden countries.[1] India is the highest TB burden country in the world having an estimated incidence of 26.9 lakh cases in 2019 (WHO). 2019 marks another milestone year for TB surveillance effort in India, with a record high notification of 24 Lakh cases; an increase of over 12% as compared to 2018. Of the 24 lakh TB cases 90% (N=21.6lakhs) were incidental TB cases (New /Relapse/Recurrent). With a focus on eliminating Tuberculosis (TB) from the country by 2025, in January 2020 the Government of India renamed the 'National Tuberculosis Control Program' to the 'National Tuberculosis Elimination Program (NTEP) (India TB Report 2020).

Mycobacterium tuberculosis (MTB), the causative agent of the disease tuberculosis (TB), is of great global epidemic importance. The bacterium affects not only lungs, but also the other parts of the body system which is generally termed as extra-pulmonary tuberculosis (Fanning, 1999; Sharma & Mohan, 2004). The following forms of EPTB are classified as severe: meningeal, pericardial, peritoneal, bilateral or extensive pleural effusive, spinal, intestinal, genitourinary. Lymph node, pleural effusion (unilateral), bone (excluding spine), peripheral joint and skin tuberculosis are classified as less severe (Maher et al., 1997). Extra-pulmonary infection are paucibacillary in nature, hence more often the smear is found to be negative as compared to those of pulmonary case, which make the diagnosis difficult (Pfyffer et al., 1997). About 15 to 20 percent of all cases of tuberculosis in immunocompetent patients comprises of extra pulmonary tuberculosis and accounts for more than 50 percent of cases in HIV-positive individuals (Sekar et al., 2008). In India and other developing countries, lymph node(LN) TB constitute to be the most common form of EPTB followed by pleural effusion, bone and joint TB, genitourinary TB, TB meningitis, and others

(Dandapat et al., 1990). Direct staining for acid-fast bacilli (AFB) is the most rapid method, and takes less than 1 hour (Kent, 1985). However, a minimum concentration of at least 105 organisms per milliliter of specimen is required for visualization by light microscopy, whereas by fluorescent microscopy that number decreases to 104 per milliliter (Forbes et al., 2007). Moreover, it can not distinguish Mycobacterium tuberculosis from other mycobacteria and considered useful only as a screening test (Moore & Curry, 1995). LEDs provide a cheap and reliable light source with a more robust and long lifespan (>50,000 hours); additionally, no darkroom is required for their operation; LED microscopy has been shown to have equivalent specificity and improved sensitivity over conventional ZN microscopy (Bonnet et al., 2011). More recently, the WHO endorsed the GeneXpert (Xpert MTB/Rif assay) for the diagnosis of TB (lnternational standard for tuberculosis care, 3rd edition, 2O14). The GeneXpert utilizes a DNA-PCR technique for simultaneous detection of Mycobacterium tuberculosis and Rifampicin resistance related mutations. It is the first fully automated bench top cartridge based nucleic acid amplification (CB NAAT) assay for TB detection that includes all necessary steps of DNA PCR. It gives results within 2 hours. Diagnostic accuracy of GeneXpert for pulmonary TB has been reported high (Shah & Gupta, 2015; World Health Organization, 2013). Extra pulmonary cases in whom AFB smear examination is usually negative, are the most likely to be benefited from GeneXpert. Mycobacterium Growth indicator Tube (MGlT) is a liquid broth medium that is known to yield better recovery and faster growth of Mycobacteria. ln addition to Middlebrook 7H9 liquid media, the MGlT tube contains an oxygen-quenched fluorochrome. It detects oxygen consumption induced by growing micro-organisms (Tortoli et al., 1999). In many of the studies conducted worldwide, MGIT culture technique has been considered as a gold standard (Kent, 1985; Forbes et al., 2007).

Aims and objectives

This study was done with an aim to determine the prevalence of extra-pulmonary tuberculosis in the cases attending at IGIMS, Patna and to compare the different diagnostic methods like ZN staining, LED fluorescent microscopy and GeneXpert assay considering MGIT culture as gold standard, with respect to their sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPP).

Materials and Method

This Prospective, laboratory based comparative study was conducted at microbiology department, IGIMS Patna, Bihar. In a duration of 18 months, from September 2018 to February 2020, 210 Patients with complaints of suspected extra pulmonary tuberculosis were included in the study.

Inclusion criteria

Outpatients and inpatients with clinical symptoms suggestive of extrapulmonary tuberculosis such as:

- Painless lymphadenopathy with or without abscess formation.
- Pleural aspirates from cases suspected of Tuberculous pleural effusion

- Discharge pus and draining fluid from cases suspected of bone and skeletal tuberculosis.
- Endometrial tissue
- CSF in cases suspected of tuberculous meningitis.
- Gastric aspirate
- Other body fluids like ascitic, pericardial with suspected extra pulmonary tuberculosis involvement.
- Both children, paediatric and adult patients irrespective of age and sex

Exclusion criteria

- Pulmonary tuberculosis cases
- Cases infected with Atypical mycobacteria
- Patient not giving consent
- Sample of urine, stool and blood were not included in the study

Ethical consideration

Ethical and research clearance was obtained from the Ethical committee Indira Gandhi Institute of Medical Sciences, patna-14 Permission to conduct the study was sought from the respective hospital authorities. Informed consent was obtained from the patients before enrolment in to the study.

Laboratory methods

All the patients were clearly explained about the procedure and the samples were collected after following universal precautions. Samples received in department of microbiology included Lymph node aspirates, Pus, Pleural fluid, cerebrospinal fluids (CSF), Ascitic fluid, gastric aspirates, Endometrial tissue and pericardial fluids, from clinically suspected cases of extra pulmonary tuberculosis. All the samples were collected in duplicate that is in 2 falcon tubes one for ZN smear microscopy, & Auramine Rhodamine stain and another for GeneXpert and MGIT BACTEC 960 liquid culture. After taking the samples for GeneXpert, the leftover samples from the second tube (not less than 2ml) was used for MGIT culture. GeneXpert testing was performed according to the manufacturer's instructions (Xpert MTB RIF kit 301-1404). Sample reagent was added to untreated samples at a ratio of 2:1. The sample was vortexed and kept at room temperature for 10 minutes. It was again vortexed and kept at room temperature for 5 minutes.

Furthermore, 2 ml of the inactivated material was transferred to the test cartridge and inserted into the test platform. Only electronic results were used for comparison. Direct Smear microscopy was performed to investigate presence of acid-fast bacilli with the samples in first falcon tube, using conventional ZN staining method and Auramine-rhodamine staining method. In ZN smear microscopy, slides showing red coloured acid- fast bacilli were taken as positive and the ones without any acid-fast bacilli were taken as negative.[20] In Fluorescent microscopy, the slides were examined microscopically under the same light source as used for fluorescent microscopy. Slides were screened under high power (400X) objective lenses. If Fluorescent AFB were seen, the smear was reported

as AFB positive and plus signs (+ to +++) were given to indicate the number of bacilli present. If no fluorescent rods were seen, the smear was reported as NO AFB seen.

For liquid culture, samples were processed using the N-acetyl-L cysteine- Sodium hydroxide method (NALC-NaOH) as per the manufacturer's instructions, inoculated in MGIT media and incubated in MGIT BACTEC 960 liquid culture system (Siddiqi & Rüsch-Gerdes, 2006). Sodium hydroxide (NaOH) is a decontaminating agent and also acts as emulsifier and NALC acts as a mucolytic agent and also reduces the concentration of NaOH required (BA, 2007). When the tubes were flagged positive by the system, ZN staining and subculture on BHI agar/ 5% sheep blood agar were performed from the MGIT tube directly to see the presence of any AFB or any contamination respectively as per the manufacturer's instructions. All tubes were checked for 42 days before declaring them negative. Differentiation between Mycobacteria other than tubercle bacilli (MOTT) and *Mycobacterium tuberculosis* from positive culture tubes were done by rapid immunochromatography test kit using MPT 64 antigen according to the manufacturer's instructions.

Method of statistical analysis

The data was tabulated in Microsoft excel spreadsheet in a master chart and studied for correlation. Statistical analysis of the data was conducted with statistical package for the social science system version SPSS27.0. The sensitivity, specificity, PPV, NPV and p value was calculated for AFB smear microscopy, LED Fluorescent microscopy and the GeneXpert, using MGIT culture of *Mycobacterium tuberculosis* from extrapulmonary specimens as gold standard. By taking culture method as reference, samples that were positive and negative in culture were considered true positive and true negative. Culture negative and GeneXpert positive samples were taken as false positive samples. Culture positive and GeneXpert negative samples were considered false negative.

Result

Table 1
Extrapulmonary samples

Samples	Male	Female	Total no.	ZN Staining positive	FM positive	GeneXpert positive	MGIT culture positive
FNAC Lymph node	14(10.76%)	15(18.75%)	29(13.8%)	7(31.81%)	10(33.33%)	18(28.12%)	14(18.18%)
Pus	43(33%)	20(25%)	63(30%)	15(68.18%)	20(66.66%)	29(45.31%)	28(36.36%)
Cerebrospinal fluid	13(10%)	06(7.5%)	19(9%)	00	00	06(9.3%)	08(10.38%)
Pleural fluid	38(29.23%)	18(22.5%)	56(26.66%)	00	00	09(14%)	13(16.88%)
Ascitic fluid	14(10.76%)	06(7.5%)	20(9.5%)	00	00	00	06(7.79%)
Gastric aspirate	06(4.6%)	02(2.5(%)	08(3.8%)	00	00	00	02(2.59%)
Endometrial tissue	00	10(12.5%)	10(4.76%)	00	00	01(1.5%)	04(5.19%)
Pericardial fluid	2(1.5%)	3(3.7%)	05(2.38%)	00	00	01(1.5%)	02(2.59%)
Total	130(100%)	80(100%)	210(100%)	22(100%)	30(100%)	64(100%)	77(100%)

Out of 210 Extrapulmonary samples, more samples were from male patients (62%) as compared to female patients (38%). The maximum number of samples

40.95% (86/210) were from patients belonging to age group 19-39Yrs. The case detection rate of ZN staining and LED-FM were 22/210 (10.47%) and 30/210 (14.28%) respectively. GeneXpert /RIF assay detected 64/210 (30.47) % cases. The case detection rate of MGIT culture for Extrapulmonary specimens was 77/210 (36.66%).

Table 2
Comparison of various diagnostic methods for diagnosing Extrapulmonary tuberculosis

Diagnostic methods	Sensitivity	Specificity	PPV	NPV	P value
ZN	28.27%	100%	100%	70.74%	0.001
FM	39.96%	100%	100%	73.88%	0.001
GeneXpert	59.74%	86.46%	71.87%	78.76%	0.001

Prevalence of extra pulmonary tuberculosis among suspected patients

Out of 210 EPTB suspected patients, 77 (36.66%) were AFB positive by MGIT culture method. The prevalence of extra pulmonary tuberculosis among male and female cases were 48/130 (36.92%) and 29/80 (36.25%) respectively. From the positive cases the highest proportion 48/77 (62.33%) was males. The uppermost prevalence of extra pulmonary TB cases was observed in the age group of 19–39, 39/86 (45.34%) .

Discussion

In our study the most commonly processed sample was Pus samples 63 (30%) followed by Pleural fluid 56 (26.66%), Lymph node aspirate 29 (13.8%), Ascitic fluid 20 (9.5%), CSF 19 (9.04%), Endometrial tissue 10 (4.7%), Gastric aspirate 8(3.8%) and Pericardial fluid 5(2.38%) samples. This finding correlates to study conducted by Chander et al. (2012), where pleural fluid samples were found to be the most common i.e. 53 cases (61.6%), followed by lymph node aspirates i.e. 20 cases (23.2%) and ascitic fluid samples i.e. 8 cases (9.3%). In our study, out of 210 EPTB cases there were 130/210 (62%) males and 80(38%) females. Male to Female ratio was 1.6:1. Out of 130 males, MTB was detected in 48 (36.92%) and out of 80 females, MTB was detected in 29(36.25%). These findings correlate with study conducted by Sanjay et al. (2017), in which out of 65.16% male, 14.9% were found to be MTB positive and out of 34.83% female, 22.5% were found positive. Extra pulmonary tuberculosis is one of the highly prevalent diseases in developing countries including India. In this study the prevalence of culture-positive extra pulmonary tuberculosis infection was 36.66% which is higher than the prevalence reported in Turkey (25.9%), Southern region of Ethiopia (28%) , Addis Ababa (15.9%) Gunal et al. (2011); Yassin et al. (2006); Deribew et al. (2011), and Gondar 28.3% (Tessema et al., 2009).

Age distribution of sample with positivity rate

In our study we found majority of suspected EPTB cases 40.95% (86/210) were in age group 19 to 39 years and this age group also showed maximum positivity by

ZN microscopy 19.76% (15/86), FM microscopy 22% (19/86), GeneXpert 39.53% (34/86) and MGIT culture 45.34% (39/86). The reasons that make this age group (19 to 39 years) more vulnerable to TB may be that they are socially more active and are more exposed to open cases of TB than others. Arora & Gupta (2006), conducted a study on trends of EPTB and found higher detection of EPTB cases in younger age group.

Positivity by ZN, and LED-FM

In this study, out of 210 specimens, ZN detected 22/210 (10.47%) and Fluorescent Microscopy detected 30/210 (14.28%). The smear positivity for AFB by conventional ZN method in the study done by Bagdia et al. (2018), was 9.28% while the positivity increased to 17.52% by modified fluorescent method. In a similar study done by Vamseedhar Annam et al. (2009), the positivity rates were 44.11% by ZN method and 81.37% by modified fluorescent method. Out of 30 samples which were detected positive by LED-FM, 20 samples were pus and 10 were lymph node aspirates. In a study done by Mohi Siddiqui et al. (2013), lymph node aspirates showed higher percentage of positivity (33%).

Positivity by ZN and MGIT culture

In this study ZN smear positivity was 10.47% and culture positivity was 36.66% which correlates with the study done by Munir et al. (2009), where culture positivity of extra-pulmonary specimens was 18.46% and was considerably high as compared to the ZN smear positivity of 3.85%, thus proving that culture is a sensitive tool in the diagnosis of extra-pulmonary TB.

Table 3
Comparison of culture positivity by MGIT in extra pulmonary specimens with other studies

Study	MGIT Culture positivity
In this study	36.66% (77/210)
Rishi et al	34.10% (88/258)
M. Kashif Munir et al	18.46%
Mohammed Abdul Mohi Siddiqui et al	15%

Among 77 cases of Mycobacteria which were detected by MGIT, 70 (90.9%) belonged to Mycobacterium tuberculosis complex, whereas 07 (9.09%) comprised of MOTT (Atypical mycobacteria). As far as the sample yield is concerned, MGIT could detect Mycobacteria more from pus samples and Lymph node samples, which were 36.66% and 18.18% respectively. In this study, 6.2% (13/210) of MGIT cultures were contaminated with gram positive and gram-negative organisms, which is similar with study done, where contamination rate was 6.1%. The mean time to detection of MTBC from smear-positive specimens and smear-negative specimens by MGIT was 10.2 days and 21.4 days respectively, which is in accordance to the study conducted, where the mean time to detection of MTBC from smear-positive specimens and smear-negative specimens was 9.9 days and 20.3 days respectively.

Specimen wise GeneXpert result and Rifampicin sensitivity

GeneXpert /RIF assay detected 64/210 (30.47) % and the overall diagnostic yield of GeneXpert MTB/RIF in various extra pulmonary samples in order of frequency were as follows: Lymph node 18 out of 29 samples (62%), Pus 29 out of 63 samples (46%), CSF 6 out of 19 samples (31.57%), pericardial fluid 1 out of 5 samples (20%), pleural fluid 6 out of 56 samples (10.7%), and lastly Endometrial tissue 1out of 10 samples (10%). Thus Lymph node has the maximum diagnostic yield. Out of 64 cases diagnosed as EPTB, 11/64(17.18%) were Rifampicin resistant and 53/64(82.81%) were Rifampicin sensitive as detected by GeneXpert. Among these 11 Rifampicin Resistant Mycobacteria, 5/11(45.45%) were from pus, 2/11(18.18%) from pleural fluid, 2/11(18.18%) from lymph node and 2/11(18.18%) from CSF. Among 53 Rifampicin sensitive Mycobacteria, 24 were from pus, 16 from lymph node, 7 from pleural fluid, 4 from CSF, 1 from Endometrial tissue and 1 from pericardial fluid samples.

These findings of our study were similar to the study conducted by Srwar A et al[35] in which positivity of GeneXpert was 37/100(37%), out of which 31/60(51.7%) was from pus sample, 3/19(15.8%) from pleural fluid, 1/16(6.3%) from ascitic fluid and 2/5(40%) from CSF samples. Similar study was done by Monteiro et al. (2016), at Goa medical college in which Out of 212 samples 51 were positive for Mycobacteria Tuberculosis (MTB) by GeneXpert i.e.average of 24%. Out of 51 cases which were positive by GeneXpert, 38 were of lymph node TB i.e 64%, 6 of TB pleural effusion i.e. 7.3%, 2 of urogenital TB i.e. 16.6%, 2 of abdominal TB i.e.16.6%, 1 of CNS TB i.e. 3.4%, 2 of TB involving the skeletal system i.e. 25%. Out of 51 cases diagnosed as EPTB, 5 were Rifampicin resistant as detected by GeneXpert. Among these 5 Rifampicin resistant cases, 3 were of lymph node TB, 1 Was of scrotal TB and 1 was of abdominal TB. The finding of this study was in concordance with our study. Moure et al. (2012), in 2012 conducted a study in which 58.3% samples were positive with the GeneXpert assay for Mycobacterium tuberculosis.

In our study the positivity was very less in pleural fluid where only 9 out of 64 positive cases (14%) were found. This is higher than the study done by Hillemann et al. (2011); Causse et al. (2011), where pleural fluid sample was found to be positive for EPTB in 3% and 11.8% cases respectively. The main disadvantage of GeneXpert is that, it is designed to detect only MTBC, and not mycobacteria other than tuberculosis (MOTT), because this does not have the primer for MOTT. In our study sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of ZN staining and GeneXpert was 28.27%, 100%, 100%, 70.74% and 59.74%, 86.46%, 71.87%, 78.76% respectively. Study conducted by Bajrami et. al[40]. showed Sensitivity, specificity, PPV and NPV for GeneXpert and ZN staining as 82.3%, 97.6%, 93.3%, 93% and 94.1%, 85.7%, 53.3%,98.8% respectively. Study of Agrawal et al. (2016), showed the result of Sensitivity, specificity, PPV and NPV for GeneXpert and ZN staining as 72.7%, 100%, 100%, 76.9% and 100%, 90%, 91.6%, 100% respectively.

Conclusion

Male patients were predominant than female patients as far as testing samples for suspected EPTB cases are concerned. Also, the maximum suspected cases for EPTB lie in age group 19-39 years. Among all the testing methods used for detecting MTBC in extrapulmonary samples, MGIT is most superior, followed by GeneXpert, LED-FM and ZN staining. Among GeneXpert, LED-FM, and ZN staining, GeneXpert has maximum sensitivity, whereas LED-FM and ZN staining has almost 100% specificity.

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Conflicts of interest

There are no conflicts of interest.

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