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Microbiology

SPECTRUM OF EXTRAPULMONARY TUBERCULOSIS IN A TERTIARY CARE HOSPITAL IN WESTERN UTTAR PRADESH

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ABSTRACT

AIMS & OBJECTIVES- i)To determine the frequency of extra-pulmonary tuberculosis in clinically suspected cases ii)To evaluate the efficacy of Polymerase Chain Reaction (PCR)as a rapid and sensitive diagnostic tool in comparison to gold standard (i.e. culture).

METHOD-A total of ninety seven samples, collected from clinically suspected cases of extra-pulmonary tuberculosis, were processed for detection of *Mycobacterium tuberculosis* by direct microscopy (ZN staining), automated liquid culture method (BacT/ALERT 3D system) and PCR assay.

RESULTS- The frequency of EPTB cases in the present study was 11.3% (11/97). The most common site was endometrial tissue (5/11, 45.4%), followed by pleural fluid (4/11, 36.4%), and lymph node aspirate and fallopian tube (each 9.1%). Amongst the positive cases, predominant age group was 21-30 years, and females outnumbered males with a ratio of 4.5:1. The positivity rate of PCR was 11.3% (11/97) as compared to that of culture and smear that is 7.22% each respectively (7/97). The sensitivity, specificity, PPV and NPV of PCR was 100%, 95.6%, 63.6% and 100% respectively as compared to culture.

CONCLUSION: PCR assay targeting 123 bp fragment of IS6110 is a rapid, sensitive and specific method for the early diagnosis of extra-pulmonary tuberculosis, especially in samples which are pauci-bacillary in nature.

KEYWORDS : Extra-pulmonary tuberculosis, ZN stain, culture, PCR, IS6110

INTRODUCTION

Tuberculosis is a chronic infectious disease, which remains a major public health problem globally, India being one of the high burden countries.[1] The clinical manifestations of tuberculosis are of two types: pulmonary tuberculosis (PTB) & extra-pulmonary tuberculosis (EPTB), the former being commonest. In India, EPTB forms 10 to 15% of all types of tuberculosis.[2] EPTB is an important clinical problem, which is due to dissemination of tubercle bacilli from an initial focus in the lungs, primarily by way of the lympho-haematogenous route in almost all of the organs & tissues of the body. The disease usually occurs some years after the initial infection when the patient's immune system breaks down for some reason other than the presence of tuberculosis bacilli in the lung.[1,2] In EPTB highly vascular areas such as lymph nodes, meninges, kidney, spine & growing ends of the bones are affected.[2] The WHO's "End TB" strategy aims to reduce TB deaths by 95%, reduce new cases by 90% between 2015 and 2035 and ensure that no family is burdened with catastrophic expenses due to TB.[3]

MATERIALS AND METHOD:

The prospective study was carried out in the Department of Microbiology, Subharti Medical College, and associated Chhatrapati Shivaji Subharti Hospital, Meerut, from January 2017-December 2017 after clearance by institutional ethical committe. **Inclusion criteria:** i) Patients clinically suspected of EPTB, irrespective of age and gender. ii) Patients symptomatic for at least six weeks. **Exclusion criteria:** i) Patients with active pulmonary tuberculosis ii) Patient on ATT for more than six weeks.

SAMPLE PROCESSING:[4,5,6] All the non-pulmonary samples received in the laboratory were processed in Biosafety cabinet class II A_2 using standard precautions. Samples were first subjected to decontamination process (using N-acetyl-L-cysteine and NaOH)and then divided into three parts for carrying out the following tests; i) Direct smear microscopy ii)culture iii) PCR.

1. **Direct Smear Microscopy:** Body fluids and urine samples were centrifuged at 3000 rpm for 15minutes and smears were made from deposit. Tissue samples were minced in pestle and mortar. Smears were stained by Ziehl Neelsen(ZN) staining to look for beaded acid-fast bacilli.
2. **Automated liquid Culture (BacT/ALERT 3D, BioMerieux):** Reconstitution of MB/BacT antibiotic supplement was done as per manufacture's protocol. For culture of non-sterile samples, 0.5ml of the reconstituted MB/BacT antibiotic supplement was added to each process bottle. For culture of sterile samples, 0.5 ml of the reconstitution fluid was added to each process bottle. Each MB/BacT bottle was inoculated with 0.5ml of the processed sample and incubated as per manufacturer's protocol. If it flagged positive, culture smear was made and stained by ZN stain to look for acid fast bacilli.
3) **Conventional PCR:** Sample preparation and DNA extraction were carried out according to manufacturer's protocol. DNA amplification was done in thermocycler **(Applied Biosystem)**.

M.tuberculosis specific IS 6110 loci were used to design the primers.
Primer 1 - 5' CCT GCG AGC GTA GGC GTC GG 3'
Primer 2- 5' CTC GTC CAG CGC CGC TTG GG 3'

First Amplification Master Mix: i) first amplification pre mix (8.2µl) ii) Genei hot start Taq DNA Polymerase (0.33 µl) iii) Uracil DNA glycosylase (0.50 µl)

Amplification Master Mix (9 µl)was aliquoted into each of the labeled vials. 3 µl of each extracted DNA was added to the vials. Amplified product was stored at 2-8^{0}C.

Second Amplification Master Mix :II amplification pre mix (14.7 µl)and Genei Hotstart Taq DNA Polymerase (0.33 µl)

15 µl of the amplification master mix II was added to each tube after PCR is completed.

Second PCR program was performed as per instructions manual.

Post Amplification - Analysis of amplified product was done by gel electrophoresis (GeNei, Bangalore) as per manufacturer's protocol.

Interpretation: Positive Result- band at 123bp & (Internal control band at 340 bp) Negative Result – band at 340 bp (Internal Control)

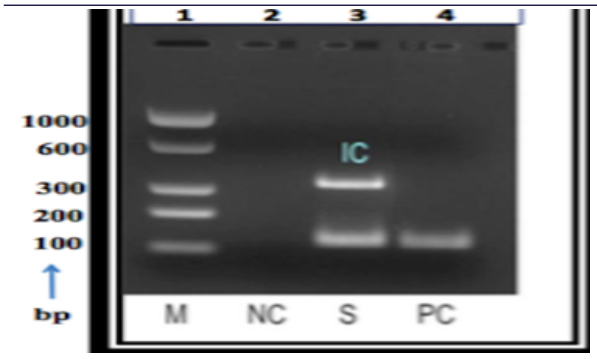

Figure 1: Gel documentation- Lane 1(M): Molecular markers, Lane 2 (NC):Negative Control, Lane 3 (IC):Internal Control for *Mycobacterium* species as per commercial kit manufacturers protocol,Lane 4 (PC): Positive Control,bp: base pairs

Statistical analysis: Stata 14.0 statistical software was used for data analysis.

Results:

Table 1: Sample-wise distribution of clinically suspected cases (n=97)

Types of Sample	No. of cases
CSF	23
Endometrial tissue	23
Pleural fluid	20
Ascitic fluid	16
Lymph node aspirate	8
Fallopian tube	3
Menstrual blood	2
Pericardial fluid	1
Bone tissue	1

Table 2: Sample wise distribution of positive cases (n=11)

Type of Sample	Positive cases (n=11)	Percentage(%)
Endometrial tissue	5	45.4
Pleural Fluid	4	36.4
Lymph node aspirate	1	9.1
Fallopian Tube	1	9.1

TABLE 3: Age and gender wise distribution of positive samples (n= 11)

Age (in years)	Total no. of cases	Male	Female	No. of positive cases
0-10	13	7	6	0
11-20	11	5	6	1
21-30	29	10	19	4
31-40	15	5	10	2
41-50	9	5	4	2
51-60	7	3	4	0
≥ 61	13	8	5	2
Total	97	43	54	11

Figure 2: Comparison of PCR positives with other modalities (n=11)

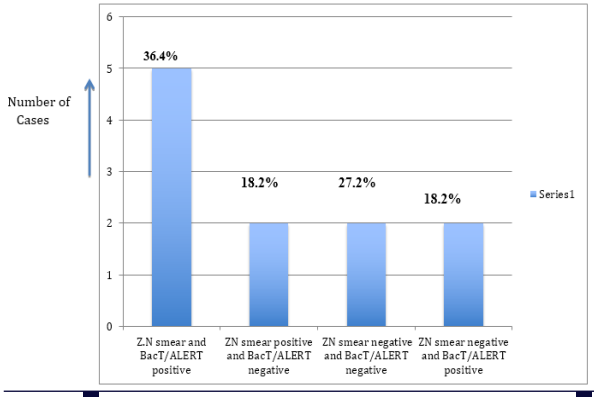

Table 4- Statistical analysis: PCR versus Culture

	Culture		
PCR	**Positive cases**	**Negative cases**	**Total**
Positive cases	7	4	11
Negative cases	0	86	86
Total	7	90	97
Sensitivity (95% C.I)	100% (64.6, 100)		
Specificity (95% C.I)	95.6% (89.1,98.3)		
PPV (95% C.I)	63.6% (35.4, 84.8)		
NPV (95% C.I)	100% (95.7, 100)		
Diagnostic Accuracy (95% C.I)	95.9 (89.9, 98.4)		
Likelihood Ratio test (95% C.I)	22.5 (13.8, 36.7)		

TABLE 5: Distribution of PCR positive samples with other modalities

Type of Sample	Smear and Culture positive	Smear positive and Culture negative	Smear negative and Culture negative	Smear negative and culture positive
Pleural fluid (n=4)	1	1	2	-
Lymph node aspirate (n=8)	1	-	-	-
Endometrial tissue (n=5)	2	1	-	2
Fallopian tube (n=1)	1	-	-	-
Total (n=11)	5	2	2	2

DISCUSSION

Extra-pulmonary tuberculosis occurs due to dissemination of tubercle bacilli from an initial focus in the lungs after primary infection. In our set up the frequency of extra-pulmonary tuberculosis in clinically suspected cases was 11.3%. Our data was comparable with the study done by Ajantha *et al.*[7] in Dharwad, which had reported the positivity rate of EPTB as 12.1%. However, a study done by Mavila *et al.*[8] reported EPTB as high as in 52.08% of cases. In 2013, Sandgren *et al.* reported that during surveillance from 2002 to 2011, EPTB accounted for 19.3% of all notified cases in 30 member states, ranging from 5.8% to 44.4%.[9]

In the present study, most common site of EPTB was endometrial tissue (45.4%), followed by pleural fluid (36.4%), lymph node aspirate and fallopian tube (9.1% each). The overall frequency of genital tuberculosis (endometrial and tubal) was 54.5%. Female genital TB is a form of EPTB affecting female genital organs with fallopian tubes being affected most commonly, followed by the endometrium and the ovaries.[10,11]. As per study done by Singh et al in 2011 the urogenital system is a common site of EPTB in adults, but the true incidence of UGTB is less clear, and reports have varied from 4% to 73%. In 2016, a study done in North East India [13] reported that among EPTB cases, pleural effusion (30.04%) was commonest followed by lymphadentitis (24.58%), abdominal tuberculosis (13.91%) and central nervous system tuberculosis (12.35%). Similarly, in 2015, Prakasha *et al.*[2]also reported that pleural TB was the commonest type of EPTB (28.03%), followed by lymph node (24.81%).Various other studies have also reported that mostly pleura and regional lymph nodes are commonly involved in EPTB cases.[14-17]However, Bisht *et al.*[18]observed that EPTB was maximum in skin (24.27%), followed by lymph nodes,(19.42%), and musculoskeletal system (16.50%).

The positivity rate of endometrial tissue in our study was 21.73% (5/23) followed by 20% (4/20) in pleural fluid, 12.5% (1/8) in lymph node aspirate and 33.33% (1/3) in fallopian tube. In 2014, Shrivastava e*t al.* [19] reported that amongst the endometrial tissue samples obtained from suspected cases of genital tuberculosis, positive cases were 50.6%.

In Pune, Mani *et al.*[20] reported tubercular endometritis in 13.6% of cases clinically presenting with primary/secondary infertility.Our data suggests that amongst the positive cases, females outnumbered males with a ratio of 4.5:1. The maximum numbers of positive cases were seen in age group 21-30 years followed by 31-40 years.

Similar observation was made by Bisht *et al.*[18]in 2016 that maximum number of EPTB cases belonged to the age group of 21-30 years. They also reported that there were almost equal cases in both the genders with a slight preponderance of females.

A study done in Shimla by Kaushik *et al.* [21] reported that the incidence of EPTB was more in females (1%) as compared to males (0.63 %).Various other authors have also reported that burden of EPTB was more in the reproductive age group.[22,23]

In our study, PCR was positive in 11.3% of cases clinically suspected of EPTB. Amongst the PCR positive cases, 36.4% were also smear and culture positive, 18.2% were smear positive but culture negative, 27.2% were both smear and culture negative and 18.2% were smear negative but culture positive (Figure 2). However, all the PCR negative cases (88.65%) were also smear and culture negative. Sensitivity and specificity of PCR was found to be 100% and 95.6% respectively.

A study done in Dharwad [7]showed that cases which were PCR positive but smear and culture negative were 45.5%, PCR and culture positive cases were 27.3%, only ZN smear positives were 9.1%, PCR and ZN smear positives were 4.5%. PCR, culture and ZN smear positive cases were 13.6%. However, the cases were subjected to conventional LJ medium. In another study the positivity rate of PCR in suspected cases of EPTB was 45.3% and 54.7% were negative by PCR.[24] Out of PCR positive samples, only smear positives were 14.9%, only culture was positive in 19.4%, both smear and culture positives were 26.9%. Both smear and culture negatives were 38.8%. The sensitivity and specificity of PCR was 91.9% and 88.4% respectively. However, the samples were subjected to real time PCR and direct fluorescent microscopy. A study done in Ahemdabad by Patel *et al*. observed that 2.26 % suspected samples were positive for acid fast bacilli by ZN staining.[23]

Our data suggests that amongst the endometrial cases, 40% cases were positive by smear, culture and PCR, 20% were positive by smear and PCR but culture negative and the remaining 40% were smear negative but positive for culture and PCR. In our hospital the positivity rate of PCR was 11.3% (11/97) and culture and smear was 7.22% each (7/97) showing that PCR could detect 4 more cases (n=11) than that by automated culture (n=7) thus more sensitive to detect the cases with low bacterial yield. A previous study carried out in AIIMS, Delhi by Singh*et al.* in 2000[25] observed that cases of EPTB are more often culture negative. A study done by Lakshmi *et al.*[26] in Vishakhapatnam found that 9% of the cases were positive for AFB by ZN staining, whereas Siddiqui *et al*.,[27] reported 5% positivity which are comparable to our study. Desai *et al.* observed smear positivity in 14.28% cases of EPTB in Bhavnagar, Gujarat.[28]

The low smear positivity rate in our setup could be due to the reason that ZN stain requires 10^5 bacilli /ml.[1] In the present study, positivity by PCR targeting the IS6110 element (11.34%) was found to be higher as compared to other techniques, which was observed in studies conducted by various workers.[29-35] Extra-pulmonary tuberculosis remains a challenging diagnosis for both clinicians and microbiologists due to the paucity of acid fast bacilli in extra-pulmonary samples.[36] The limitation of the present study being the use of one target in the PCR. Thus, by using more than one target in the PCR, the positivity rate would have been increased and aided in the diagnosis of EPTB.

CONCLUSIONS:

Our study highlights that PCR was found to be a sensitive and specific method for low grade bacteraemia. Thus, utilization of PCR may give vital evidence in cases of extra-pulmonary TB with low bacterial load as compared to other diagnostic modalities.

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