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Diagnostic Performance of Polymerase Chain Reaction Targeting Insertion Sequence (IS6110) for the Detection of Extra Pulmonary Tuberculosis

A. Chitra¹, B. Usharani², S. Smita¹, C. K. Vidya Raj¹, S. Anbazhagi³, M. Muthuraj^{1,*}

¹State TB Training and Demonstration Centre, Intermediate Reference Laboratory, Government Hospital for Chest Diseases, Puducherry, India

²Department of Biomedical Genetics, Institute of Basic Medical Sciences, University of Madras, Tamil Nadu, India

³Department of Environment Science, Central University, Kerala, India

*Corresponding author: muthuraj1970@gmail.com

Abstract Background: The diagnostic challenges in extra-pulmonary tuberculosis remain to be addressed even though remarkable progress has been made in the diagnostics of pulmonary tuberculosis during the last decade. Methods: This study was conducted to evaluate the use of polymerase chain reaction (PCR) in diagnosis of definitive extra pulmonary tuberculosis patients, and to assess the performance of insertion sequence (IS)6110 based PCR assay as compared to conventional liquid culture by Microbial growth Indicator Tube (MGIT) 960 system. Results: A total of 792 clinical specimens were collected from clinically suspected extra pulmonary tuberculosis patients. The specimens included 22 ascitic fluids, 69 pleural fluids, 240 Cerebrospinal fluids (CSF), 386 endometrial tissues, 47 lymph nodes, 22 pus, one synovial fluid, one fallopian tube, two brain abscess and two ovarian cyst samples. All these clinical samples were subjected to Auromine O staining (FM) for acid fast bacilli (AFB) and culture on MGIT 960 tubes containing Modified Middlebrooks 7H9 broth medium. PCR was performed by targeting 123bp fragment of insertion sequence IS6110 of *Mycobacterium tuberculosis*. Our study of 792 specimens, revealed their 87.5% sensitivity to endometrial samples, 92.31% sensitivity for cerebrospinal fluid, and 66.66% sensitivity in Pleural fluid and 60% sensitivity in Lymph node samples. The combined sensitivity and specificity of the PCR IS6110 was calculated to be 85.71% and 82.91%, respectively. Conclusion: PCR using IS6110 primer was able to pick up more positivity in extra pulmonary samples as compared to conventional culture method for the detection of *M. tuberculosis*.

Keywords: polymerase chain reaction, mycobacterium tuberculosis, extra pulmonary tuberculosis, insertion sequence, cerebrospinal fluid

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1. Introduction

Tuberculosis has been a foremost global public health problem from times immemorial. World Health Organization (WHO) estimates show that globally there are 9.4 million incident cases of TB of which 80% are in 22 countries, with India ranked as the highest burdened country, accounting for one-fifth of the global TB incidence. TB remains the largest infectious killer disease affecting adults in developing countries [1]. TB manifests clinically as pulmonary or extra pulmonary tuberculosis (EPTB), with the former being more common. In India, 10 to 15% of TB cases are estimated to be cases of extra pulmonary tuberculosis (which affects mainly the lymph nodes, meninges, kidney, spine, and growing ends of the bones), with a 25 to 50% case mortality rate within months [2].

The global burden of extra pulmonary tuberculosis constitutes about 15 to 20 % of all cases of TB. The annual global incidence of extra pulmonary tuberculosis has been increasing in the last decade due to the changing TB control practices, spread of HIV (human immunodeficiency virus), the population growth and the cure of infectious cases of TB might have resulted in a relative rise of annual extra pulmonary tuberculosis case detection. HIV pandemic further complicates the situation, as extra pulmonary tuberculosis constitutes more than 50 % of all cases of TB in HIV-positive patients. HIV co-infected TB patients often develop extra pulmonary involvement that may progress rapidly unless the infection is diagnosed and they are treated appropriately [3].

The diagnosis of extra pulmonary tuberculosis poses a particular challenge for clinician because of the protean ways in which the disease presents. Diagnosis of this condition requires high clinical suspicion and special

diagnostic procedures. The diagnosis of extra pulmonary tuberculosis may not be difficult in a few cases with concomitant pulmonary involvement. However, patients with the extra pulmonary tuberculosis are more likely to have negative sputum smear results and many extra pulmonary tuberculosis cases do not have direct lung involvement. The extra pulmonary tuberculosis diagnosis is lacking more in rural health facilities which are being used by about 70% of the population in developing countries. Here, the diagnosis largely depends on unreliable methods such as ZN microscopy, histology, and tuberculin test [4]. Direct AFB (acid fast bacilli) smear and L-J (Lowenstein-Jensen) culture lack sensitivity for diagnosis of extra pulmonary tuberculosis and culture takes at least two weeks. Smear for AFB is reported to be positive in 10 to 37 percent of patients and L-J culture is reported positive in variable proportion (12 to 80%) in different body fluids from suspected extra pulmonary tuberculosis cases [5]. The existing methodologies remain ineffective due to less number of mycobacteria and/or because of time consuming procedures. Accurate and early diagnosis of TB is crucial for its effective management and timely treatment. At present, nucleic acid amplification based assays are the most suitable choices for the identification of *Mycobacterium tuberculosis* in smear negative samples with high degree of sensitivity and specificity [6,7]. Several studies have been performed to detect *M. tuberculosis* in pulmonary and extra pulmonary clinical samples using PCR targeting different DNA sequences of *M. tuberculosis* [8]. The purpose of this study was to evaluate the effectiveness of PCR in diagnosis of definitive and probable extra pulmonary tuberculosis patients, and to assess the performance of insertion sequence (IS) 6110 based PCR assay as compared to conventional culture by MGIT method for the diagnosis of extra pulmonary tuberculosis.

2. Materials and Methods

All samples were received from eight Medical Colleges in Puducherry state, Government Hospitals and Primary Health centres and were assigned Laboratory number to process the samples at Intermediate Reference Laboratory of Government Hospital for Chest Diseases. The study was done prospectively in a blinded manner in which the clinical diagnosis was not available to the Laboratory personnel. Seven hundred ninety two samples from 792 patients were included in the study and included pleural fluids (n-53), endometrial tissues (n-383), Cerebrospinal fluids (n-237), Pus (n-25), lymph node biopsies (n-73), Ascetic fluids (n-29), ovarian cyst (n-2), Fallopien tubes (n-2), Synovial fluid (n-1), and Brain abscesses (n-1) (Table 1). The inclusion criteria adopted in the selection of patients were untreated patients with features of fever, cough, pleuritic pain, malaise, anorexia, and extensive exudative pleural effusion on diagnostic pleural tap, a with clinical working diagnosis of tuberculous pleural effusion. Patients with histories of tuberculosis in the preceding 2 years, those already receiving antituberculosis therapy, and those with other coexisting medical and surgical illness were excluded from the study.

3. Processing of Extra Pulmonary Samples for MGIT 960 System

3.1. Pus and Other Muco-purulent Specimens

Pus and mucopurulent specimens were decontaminated by N-Acetyl L-Cysteine (NALC) or sodium hydroxide (NaOH) procedure for 15-20 min and centrifuged at 4000 rpm for 15 min. After the falcon tubes were removed from the centrifuge, the supernatant was carefully poured off into a suitable container containing a mycobactericidal disinfectant. The falcon tubes were filled with 20 ml of sterile phosphate buffer (pH 6.8), shaken by hand to mix the deposit and were centrifuged for 15 minutes at 4000 rpm. The supernatant was poured off and using a sterile transfer pipette added 0.5 ml of the processed sediment to a MGIT 960 tube containing Modified Middlebrooks 7H9 broth previously inoculated with 0.8 ml of the PANTA-growth supplement mixture. Tubes were loaded into instrument on the same day as processing/inoculation [9].

3.2. Tissue

The endometrial tissue was homogenized in a pre-sterilised tissue grinder with a small quantity of sterile saline (2-4 ml) and the homogenized specimen was decontaminated using NALC-NaOH procedure. Following centrifugation, the pellet in each tube was suspended in 2.5 ml of phosphate buffer pH 6.8. After resuspension of the sediment with phosphate buffer, inoculated 0.5 ml into MGIT 960 tube. Any processed specimen remaining after initial cultures was stored at -20°C for the duration of the study to allow for re-testing of specimens in case of invalid results [9].

3.3. Other Body Fluids (Cerebrospinal Fluid, Ascitic fluid, Pericardial Fluid, Synovial Fluid and Pleural Fluid)

Sterile body fluid samples (ascitic fluid, pleural fluid, Cerebrospinal fluid, synovial fluid, pericardial fluid and pancreatic cyst fluid) were decontaminated by NALC/NaOH procedure for 20 min and centrifuged at 4000 rpm for 15 min. After the falcon tubes were removed from the centrifuge, the supernatant was carefully poured off into the disinfectant bath. The falcon tubes were filled with 20 ml of sterile distilled water, shaken by hand to mix the deposit and were centrifuged for 15 minutes at 4000 rpm. The supernatant was poured off and using a sterile transfer pipette added 0.5 ml of the processed sediment to a MGIT 960 tube previously inoculated with 0.8 ml of the PANTA-growth supplement mixture. Tubes were loaded into instrument on the same day as processing/inoculation [10].

3.4. Extraction Target DNA from Clinical Samples

DNA was extracted according to the CTAB-phenol chloroform extraction method. Briefly, the pellet was

suspended in 100 µl of TE buffer (Tris EDTA, pH7.4). The entire pellets were treated with 50 µl of lysozyme (10mg/ml) at 37°C for overnight incubation. Added 70 µl of 14% SDS and 6 µl Proteinase K (10mg/ml), mixed and incubated at 65°C for 15 minutes. After incubation, 10 µl of 5M NaCl and 80 µl of high salt CTAB buffer (containing 5M NaCl, 1.8%CTAB) was added and mixed followed by incubation at 65°C for 10 minutes. An approximate equal volume (0.7-0.8 ml) of Phenol: Chloroform: Iso amyl alcohol(25:24:1) was added, mixed thoroughly and centrifuged at 4°C for 10 minutes in a micro centrifuge at 12000rpm. The supernatant was separated and then mixed with 0.6 ml volume of ice cold isopropanol to get precipitates. The precipitated nucleic acids were washed with 70% ethanol, dried and re-suspended in 25 µl of 1X TE buffer [11].

3.5. PCR Amplification

The extracted DNA template was used for the amplification using IS6110 insertion sequence primer specific to Mycobacterium tuberculosis complex. The sequences of the two primers used were: Forward primer F 5'GTGAGGGCATCGAGGTGG 3': R 5'CGTAGGC GTCGGTCACAAA 3' [12]. The amplification was performed on the total reaction volume of 50 µl with 2 µl of extracted DNA, 25 µl of Master mix (Eppendorf 2X), 2 µl of each primer (10pmol/µl) using Master cyclor

gradient (Eppendorf, USA). Initial denaturation at 95°C for 5 minutes was proceeded by 35 cycles each of denaturation (95°C for 1 min), annealing (57°C for 1 min) and extension (72°C for 1 min) followed by a final extension at 72°C for 10 minutes. The amplified product was electrophoresed into 1% agarose gel. The gels were stained with ethidium bromide and visualized in a UV-transilluminator (Spectroline, France).

4. Results

Among the seven hundred and ninety two extra pulmonary specimens received (Table 1), 169 (21.34%) were definitive extra pulmonary tuberculosis. Out of the 169 confirmed tuberculosis, 58 were Cerebrospinal fluid, 52 were endometrial tissue, 20 were Pleural fluid, 20 were Lymph node and 9 were ascitic fluid (Table 3) 159(66.25) Cerebrospinal fluid samples were received from paediatric and positivity was 40(25.16%). 51(15.32%) definite positive tuberculosis were among the 333 (86.27%) samples received from age group between 15 to 45 years (Table 2). Among 47 Lymph node cases, 20(42.55%) were definitive extra pulmonary tuberculosis. Among the 331 body fluid samples, the definite extra pulmonary tuberculosis were 9 (40.91%), 20 (28.99%) and 58 (24.17%) in ascitic fluid, pleural fluids and Cerebrospinal fluid samples respectively (Table 3).

Table 1. Diagnostic categories of specimens

Specimens Extra pulmonary of specimens tuberculosis	Total no n(t)	Age groups (years)			
		<15yrs.	>15 to < 45yrs.	>45 to <60 yrs.	>60 yrs.
Cerebrospinal fluid	240	159	60	15	6
Pleural fluid	69	42	16	6	5
Ascetic fluid	22	9	8	5	-
Endometrial tissues	386	-	384	2	-
Pus	22	3	16	1	2
Brain abscesses	2	1	1	-	-
Lymph node	47	20	26	-	1
Synovial fluid	1	-	1	-	-
Fallopian tube	1	-	1	-	-
Ovarian cyst	2	-	2	-	-
	792	234	515	29	14

Table 2. Performance of PCR Positivity among age groups

Specimens of Extra pulmonary of specimens tuberculosis	Total no n (t)	Age groups (years)							
		<15yrs.		>15 to < 45 yrs.		>45 to <60 yrs.		>60 yrs.	
		n (p)	n(n)	n (p)	n(n)	n (p)	n(n)	n (p)	n(n)
Cerebrospinal fluid	240	40	119	13	47	4	11	1	5
Pleural fluid	69	18	24	2	14	0	6	0	5
Ascitic fluid	22	5	4	4	4	0	5	0	0
ndometrial tissues	386	0	0	51	333	1	1	0	0
Pus	22	1	2	3	13	0	1	1	1
Brain abscesses	2	1	0	1	0	0	0	0	0
Lymph node	47	11	9	9	17	0	0	0	1
Synovial fluid	1	0	0	1	0	0	0	0	0
Fallopian tube	1	0	0	1	0	0	0	0	0
Ovarian cyst	2	0	0	1	1	0	0	0	0
	792	76	158	86	429	5	24	2	12

n (n-623); n(p-169).

Table 3. MGIT culture and PCR IS6110 positivity rate in various specimens of suspected extra pulmonary tuberculosis patients (N=792)

Specimens of Extra pulmonary of specimens tuberculosis	Total no	MGIT Results		PCR results	
		Positive (%)	Negative	Positive (%)	Negative
Cerebrospinal fluid	240	13(5.42)	228	58(24.17)	182
Pleural fluid	69	9(13.04)	63	20(28.99)	49
Ascitic fluid	22	4(18.18)	18	9(40.91)	13
Endometrial tissues	386	16(4.15)	372	52(13.47)	334
Pus	22	2(9.09)	20	5(22.73)	17
Brain abscesses	2	0	2	2(100.00)	0
Lymph node	47	5(10.64)	44	20(42.55)	27
Synovial fluid	1	0	1	1(100.00)	0
Fallopian tube	1	0	1	1(100.00)	0
Ovarian cyst	2	0	2	1(50.00)	1
	792	49(6.19)	743	169(21.34)	623

Table 4. Comparison of PCR IS6110 results with conventional MGIT Culture results

N=792	MGIT Culture Positive for <i>M.tuberculosis</i> n (49)	MGIT Culture negative for <i>M.tuberculosis</i> n (743)
PCR IS6110 positive n (169)	42	127
PCR IS6110 negative n (623)	7	616
Sensitivity (%)	85.71	
Specificity (%)	82.91	
PPV (%)	24.85	
NPV (%)	98.88	
Efficiency (%)	6.19	

PPV-positive predictive value; NPV-negative predictive value.

Table 5. Performance of PCR in diagnosis of extra pulmonary tuberculosis

Parameters	Endometrium n (386)		CSF n (240)		Pleural fluid n (69)		Lymph node n (47)	
	MGIT (+); MGIT (-)	MGIT (+); MGIT (-)	MGIT (+); MGIT (-)	MGIT (+); MGIT (-)	MGIT (+); MGIT (-)	MGIT (+); MGIT (-)	MGIT (+); MGIT (-)	
PCR (+)	14	38	12	46	6	14	3	17
PCR (-)	2	332	1	181	3	46	2	25
Sensitivity (%)	87.5		92.31		66.66		60.00	
Specificity (%)	89.73		79.74		76.67		59.52	
PPV (%)	26.92		20.69		23.08		15.00	
NPV (%)	99.40		99.45		93.88		92.59	
Efficiency (%)	4.15		5.42		13.04		10.64	

PPV, positive predictive value; NPV, negative predictive value.

IS6110 PCR has shown 85.71 percent sensitivity (with 95% confidential interval) and 82.91 percent specificity (with 95% Confidential Interval). Overall positive and negative predictive value of IS6110 PCR was observed as 24.85 per cent (with 95% CI) and 98.88 per cent (with 95% CI) (Table 4). The IS6110 PCR has shown 92.31%, 87.5%, 66.66% and 60.00% sensitivity for Cerebrospinal fluid, endometrial tissue, Pleural fluid and Lymph node samples respectively. The specificity of the PCR IS6110 was calculated for the endometrial tissues, Cerebrospinal fluid, Pleural fluid and Lymph node and was 89.73%, 79.74%, 76.67% and 59.52 % respectively (Table 5).

5. Discussion

Extra pulmonary tuberculosis is a significant health dilemma in both developed and developing countries. A

high degree of suspicion abetted by intensive investigations is important in the diagnosis of the extra pulmonary tuberculosis. The laboratory diagnosis of extra pulmonary tuberculosis is challenging due to the lack of adequate sample amounts or volumes; the paucibacillary nature of the specimens; the distributing of the sample for various diagnostic tests (histology/cytology, biochemical analysis, microbiology, and PCR), resulting in nonuniform distribution of microorganisms; the presence of inhibitors that undermine the performance of nucleic acid amplification-based techniques; and the lack of an efficient sample processing technique universally applicable on all types of extra pulmonary samples. The poor performance of conventional microbiological techniques in extra pulmonary specimens has encouraged the increased use of PCR tests in the laboratory diagnosis of tuberculosis. The exact diagnostic role of PCR assay for *M. tuberculosis* in high prevalence areas for tuberculosis has been assessed in appropriate

specimens and control groups, particularly in the case of extra pulmonary tuberculosis. In modern practice, clinicians neither start nor stop treatment for this condition based exclusively on PCR results. Taking this into account, the present blind study was conducted to prospectively evaluate the role of the multipurpose. We show here that PCR would be mainly useful for the diagnosis of tuberculous pleural effusion, lymphadenopathy in patients and diagnosis of tuberculosis in Cerebrospinal fluid and endometrial samples where conventional diagnosis fails and where the provisional diagnosis of tuberculosis is made on the basis of clinical presentation and histology/cytology examination without evidence of AFB.

Several genotypic methods have been tried so far for diagnosis of extra pulmonary tuberculosis [13], which include gene coding for the 38 kDa, 65kDa heat shock proteins, 85B antigen, IS6110 insertion sequences and 16SrRNA. There is wide variation in specificity and sensitivity for detection of mycobacterial DNA from extra pulmonary samples using IS6110 as target sequence [14]. IS6110 is a long 1191bp repetitive insertion sequence that is usually present 6-20 times in the *M. tuberculosis* complex genome than other repetitive sequence [15]. Makesh Kumar *et al* reported the amplification of the same 123bp fragment targeted IS6110 in their study. Among 178 samples, 87.2 percent were PCR positive. Negi *et al* reported 83 percent PCR positivity and Ogusku *et al* showed 92.1 percent of their samples to be IS6110 positive when compared with 65 kDa, 38 kDa and MPB 64 specific primers [16,17].

Makesh Kumar *et al* reported 42.85 percent positivity rate in pus, 36 percent in Cerebrospinal fluid and 50 percent in urine samples. In comparison, our study revealed 13.47% percent positivity in endometrium, 24.17 percent in Cerebrospinal fluid, 28.99 percent in Pleural fluid and 42.55 percent in Lymph node samples [16]. An earlier Indian study [18] has also reported 63 per cent positivity by PCR using IS6110 element in specimens of extra pulmonary. Most of the studies which used IS6110 based PCR, had reported 90 per cent sensitivity in Cerebrospinal fluid, pleural fluid, ascitic fluid and other extra pulmonary specimens. In comparison, our study revealed 87.5 percent sensitivity in endometrium, 92.31 percent sensitivity in Cerebrospinal fluid, 66.66 percent sensitivity in Pleural fluid and 60 percent sensitivity in Lymph node samples. Tiwari *et al* had shown 62 percent total positivity rate among extra pulmonary samples and detection of *M. tuberculosis* DNA in 57 percent of AFB smear negative extra pulmonary samples. Our study has shown a low number of PCR positives among the 331 body fluid samples, *i.e.* 40.91 per cent in ascitic fluid, 28.99 per cent pleural fluids and 24.17 percent Cerebrospinal fluid samples. IS6110 is specific for *M. tuberculosis* complex and generally occurs in 1-20 copies per cell, which are dispersed in the *M. tuberculosis* genome and it is an ideal target for amplification, one locus, the direct repeat region, has on high frequency of carriage of IS6110 and has been proposed as a "hot spot" for integration of this element, although most of the copies are located at a single site [19]. These insertion elements are present in multiple copies of the genome of *M. tuberculosis*, with 16 copies of IS6110, 6 copies of IS1081 and 2 copies each of IS1547 [20]. The variable copy number of IS6110 among different strains of

the tubercle bacilli has led to its extensive use as a genetic marker to investigate the epidemiology of tuberculosis [21].

The advantages of IS6110 PCR are that they are very rapid, easy to perform and result can be delivered for early treatment and can prevent further transmission of tuberculosis infection and the disadvantage with PCR assay is that it is not able to differentiate live bacilli from dead organisms. Further, IS6110 PCR test is more sensitive even when both smear examination and culture results are considered in conjunction. In conclusion, the present study revealed that IS6110 PCR could detect more positives in extra pulmonary tuberculosis when compared with conventional methods. Proper positive and negative control checks and good laboratory practice can eliminate the chances of false positive results.

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