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Molecular Epidemiology of Female Genital Tuberculosis leading to infertility

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A B S T R A C T

Diagnosis of Female Genital tuberculosis (FGTB) is difficult using conventional diagnostic methods. This study was conducted to evaluate the use of polymerase chain reaction (PCR) in diagnosis of definitive and probable Female Genital tuberculosis and to assess the performance of insertion sequence (IS) 6110 based PCR assay as compared to conventional culture by Lowenstein-Jensen (L.J), Liquid Culture (MGI-960) methods for the diagnosis of FGTB. This study was conducted with one hundred and twenty six endometrial samples with the definite PCR and microbiological diagnosis of genital tract tuberculosis. Diagnosis of extra pulmonary tuberculosis is often difficult, because of the lower sensitivity and specificity of the methods as well as the invasive procedure of acquiring biopsy specimens. Of the 126 endometrial biopsy tissues specimens from highly-probable FGTB patients (based on clinical features), 27, 32 and 41 were positive by L.J (21.43%), MGIT (25.40%) and PCR (32.54%) respectively, whereas microscopy results were negative. PCR offered more sensitive in determining tuberculosis etiology in female infertility than any other conventional method in the diagnosis of clinically suspected Female Genital Tuberculosis

Introduction

Tuberculosis (TB) is an increasing public health concern worldwide. Genital TB is one form of extra pulmonary TB and is not uncommon. The global prevalence of genital TB is estimated to be 8-10 millions cases, with a rising incidence in the industrialized and developing counties partly as a result of its association with HIV virus infection and emergence of multidrug resistance. It is estimated that 5-13 percent of females presenting in infertility clinics in Indian have genital TB and majority are in age

group of 20-40 years (Agarwal *et al.,* 1993). The actual incidence may be under reported due to asymptomatic presentation of genital TB and paucity of investigations. Genital TB frequently presents without symptoms and diagnosis requires a high index of suspicion (Arora *et al.,* 2003). It is estimated that at least 11% of patients lack symptoms (Bhanu *et al.,* 2005). Diagnosis of early TB is very difficult. Early diagnosis may be associated with a more favorable result before extensive genital damage occurs (Gatongi *et al.,* 2005).

Microscopic examination of acid-fast bacilli (AFB) requires presence of at least 10,000 organisms/ml in the sample.Mycobacterial culture is more sensitive compared to AFB microscopy, requiring as little as 10-100 organisms/ml. BACTEC (MGIT) Mycobacterium Growth Indicator Tube culture had decreased the time required for bacteriologic confirmation to 2 to 3 weeks and also rate of contamination is lower as it is a closed system. BACTEC MGIT has a sensitivity of 80-90 percent versus L.J (Lowenstein-Jensen) medium, which has sensitivity of 30-40 percent (Shetty *et al.,* 2006). Whether cultured by L.J medium or BACTEC, the detection of a positive culture depends on various factors (Agarwal *et al.,* 1993) Number of organisms in the specimen – heavy smear positive specimens may turn positive as early as 48 hours, but if the bacterial load is low, it takes longer to grow the bacilli (Arora *et al.,* 2003). Treatment status of the patient – if the patient is already on treatment; the bacilli are debilitated and may require a longer time to grow. With new diagnostic molecular tests like PCR it is now possible to pick up latent endometrial TB (Riffat *et al.,* 2006). Rapid nucleic acid amplification techniques such as polymerase chain reaction (PCR) allow direct identification of M.tuberculosis on clinical specimens. It can detect less than 10 bacilli per ml of the specimen and the results are available within 1-2 days (Shetty *et al.,* 2006). False positive cases reported in TB PCR are basically because of contamination from air inside the laboratory. Good Laboratory Practice (GLP) in molecular laboratory markedly decreased the incidence of false positive cases. It has sensitivity of 90-94% and specificity of 70-78%. Therefore it can be applied to specimens, where culture is difficult due to bacterial load (Shetty *et al.,* 2006; Suman *et al.,* 2009).

Therefore, the present study was designed to assess the diagnostic value of molecular technique (PCR) to diagnosis of genital tuberculosis in infertile women and compare various bacteriological diagnosis methods.

Materials and Methods

Endometrial Samples processing

A total of 126 endometrial biopsy tissues specimens were received from Rajiv Gandhi Government Women and Child Hospital, Puducherry and all the specimens were processed at Department of Microbiology, State TB Training and Demonstration Centre, Intermediate Reference Laboratory, Government Hospital for Chest Diseases, Puducherry, South India, during the period from January 2006 to November 2010.

Processing of endometrial samples for L.J and L.J-P medium

The endometrial specimens are collected and transported in SK medium. Carefully placed the tissue inside a sterile petriplate inside the BSC. Using sterile scissors and forceps, cut the tissue in to tiny pieces and transferred to a sterile tissue grinding tube – added a little water to the petriplate to facilitate transferring. Sterile distilled water

was added to the tube (not more than 5 ml) and Homogenized using a sterile Teflon grinding rod using a foot operated tissue grinder. Prepared a direct smear from the homogenate and the homogenate was Centrifuged at 3000 x g for 15 minutes. The supernatant was decanted carefully in to the disinfectant bath and 1 ml of sterile distilled water was added to the deposit. One drop was added to the direct smear, air dry, fix and stain and 1ml of 5% H_2SO_4 was added to the remaining pellet, add 1ml of 5% H_2SO_4 Two slopes each of L.J and L.J-P were inoculated with one loopful of deposit for each slope and transferred the remaining deposit in to one bottle of Selective Kirchner's (SK) medium .The slopes and Selective Kirchner's medium were inoculated at 37°C, along with the Selective Kirchner's medium used for transporting

Processing of endometrial samples for MGIT 960

The endometrial tissue was homogenized in a 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. Processed sediments from the same patient were pooled and mixed thoroughly. Samples were cultured using the BACTEC MGIT 960 system (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA).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 (Telenti *et al.*,1997).

Staining

Ziehl-Neelsen Method: Take large yellow purulent portion of the sputum samples and spread evenly onto central portion of the microscopic slide and fix the smear to the slide. Cover the slides with freshly filtered 1 % carbol fuchsin and heat underneath until steam rises from the stain and allow the hot carbol fuchsin to react for at least 5 minutes. Add 25% sulphuric acid to react for 2-4 minutes after water washing. Counter stain with 0.1% Methylene blue for 30 seconds and wash as before with water and slope the slides to air dry. Examine the slides under the Microscope to observe for the presence of tubercle bacilli (Muthuraj *et al.,* 2010)

Fluorescence Method: Smear the specimen onto the centre portion of the slide and allow smears to air-dry for 15 minutes and fix the smear to the slide. Flood the slides with freshly filtered auramine-phenol. Let stand for 7-10 minutes. Decolorize by covering completely with acid-alcohol for 2 minutes, twice. Wash well with running water, as before to wash away the acid alcohol. Counter stain with 0.1% potassium permanganate for 30 seconds. Wash as before with water and slope the slides to air dry. Positive smears were graded into four degrees of positivity using the 20x, 25 x objectives along with 10 x eyepieces (Kathirvel *et al.,* 2013).

Mycobacterium DNA Extraction

DNA was extracted according to the CTAB-phenol chloroform extraction method. Briefly, 0.2 ml of homogenized tissues material was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet suspended in 100 µl of TE buffer (Tris EDTA, pH 7.4). The entire pellets were treated with 50 µL of lysozyme (10 mg/mL) at 37 °C for overnight incubation. Add 70 µl 10% SDS and 6 µl proteinase K (10 mg/ml), mixed and incubated at 60°C for 10 min. After incubation, 10 µl of 5 M NaCl and 80 µl of high-salt CTAB buffer

(containing 4 M NaCl, 1.8% CTAB (cetyl-trimethyl-ammonium bromide) was added and mixed followed by incubation at 65°C for 10 min. An approximate equal volume (0.7–0.8 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed thoroughly and centrifuged at 4°C for 10 min in a micro centrifuge at 12,000 rpm.The supernatant was separated and then mixed with 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 75% ethanol, dried and re-suspended in 50 µl of 1x TE buffer (Muthuraj *et al.,* 2010; Maruthai *et al.,* 2013).

PCR Amplification for Species Identification

The target for the PCR assay was IS6110 insertion element specific to *Mycobacterium tuberculosis* complex. The sequences of the two primers used were: Forward primer F 5'GTGAGGGCATCGAGGTGG 3' (10 pmol/µL) R 5'CGTAGGCGTCG GTCACAAA 3' (10 pmol/µL)(Fletcher *et al.*,2003).DNA amplification by PCR was performed in the total reaction volume of 50 µl with 2 µl of extracted DNA, 25 µl of eppendorf Master mix (2X), 2 µl (10 pmol/µL) of each primer .Amplification was carried out on a programmable Mastercycler gradient (Eppendorf, USA).

Initial denaturation at 95ºC for 5 min. was proceeded by 35 cycles each of denaturation (95ºC for 1 min.), annealing (57ºC for 1 min) and extension (72ºC for 1 min) followed by a final extension at 72ºC for 10min.The amplified product was electrophoresed into 1% agarose gels. The gels were stained with ethidium bromide and visualized in a UV-transilluminator (Spectroline, France). The presence of a 123 bp fragment indicated a positive test (Kathirvel *et al.,* 2013).

Electropherogram Analysis of PCR Amplified Products

DNA dye concentration and DNA gel matrix were allowed to equilibrate at room temperature.25 µL of dye concentration was added to DNA gel matrix, vortexed and transferred to spin filter and centrifuged at 2240 g for 15 minutes. The gel dye was allowed to settle at room temperature for 30 minutes. A new DNA chip was placed on the chip priming station. 9 µL of gel dye mix was pipetted into the well marked as G and the chip priming station was closed. The plunger was pressed down until it is held by the chip for 60 seconds. After 5 seconds the plunger was pulled back slowly to 1 mL position. The chip priming station was opened and 9 µL of gel dye was pipetted into the well marked G and 1 µL of ladder was added to the well labeled ladder. 5 µL of marker was pipetted into all 12 sample wells and in ladder well.1µL of sample was added into the well. The chip was placed in the Laser Induced Fluorescent instrument (Bioanalyzer-Agilent, 2100) and the results were interpreted (Hathaway *et al.,* 2010).

Results and Discussion

In this study, overall 126 patients were analyzed by an IS6110 insertion elements targeted PCR to evaluate its diagnostic efficacy for rapid and specific diagnosis of Female Genital tuberculosis. Based on the clinical criteria, 126 patients with Female genital tuberculosis and a control group of 6 patients having non-tubercular endometrial tissues included in the study were analyzed for Female Genital tuberculosis (Table 1). The positive results of Genital tuberculosis among the 126 suspected patients are tabulated in Table 2 and 3 for the conventional and molecular methods. Turnaround time for each positive sample in liquid culture (MGIT) was studied. (Figure

1). A clear PCR product band of 123 base pairs (bp) was observed on a 2% agarose gel confirming the *M. tuberculosis* (Figure 2). The PCR products were analyzed on a Bioanalyzer (Agilent 2100) to check the purity and specificity of the products. Electropherogram analysis confirmed the molecular size (123 bp) of the products (Figure 3). Microscopic examination by ZN-staining indicated absence of AFB in all endometrial samples. The detection limit of microscopy is 10^4 mycobacteria per milliliter whereas in view of the fact that delayed hypersensitivity is the underlying immune response in FGTB, the paucibacillary state could be accounted for these negative results. Thus in practical terms, it is a presumptive test with low sensitivity. In this study none of the patients had positive ZN staining (Butt *et al.*, 2003). Bacterial culture has 100% specificity but a major problem is that, in addition to high cost, weeks and months are required for precise identification of the species. Lowenstein-Jensen (LJ) medium is used for this purpose; however, mycobacterium tuberculosis is very difficult to grow. In this study no positive culture was obtained.

Female genital tuberculosis is an important cause of infertility. Early diagnosis and treatment in young patients with genital TB may improve the prospects of care, before the tubes are damaged beyond recovery. Culture of Mycobacterium TB remains the gold standard of diagnosis of genital TB, but early TB being a paucibacillary disease, can be missed in culture which can only be detected in PCR. PCR can detect less than 10 bacilli per ml of specimen. If the patients are adequately treated before their tubes are irreversibly damaged, the chance of successful pregnancy is reasonably good with a 20% pregnancy rate reported in one study(Sin SY 1995).In this study, PCR detected 32.54%(41/126) of the suspected cases. The Conventional (L.J) solid culture showed 21.43% (27/126) positive cases and Liquid Culture (MGIT) showed 25.40% (32/126) positive cases. Our results depicted that PCR is best method of diagnosing genital TB.

An earlier Indian study also reported 63 per cent positivity by PCR using IS 6110 element in specimens of EPTB. Most of the studies which used IS6110 based PCR, reported 90 per cent sensitivity in CSF, pleural fluid, ascetic fluid and other extra pulmonary specimens (Narayanan *et al.*, 2001). Tiwari *et al*., showed 62 per cent total positivity rate among EPTB samples and detection of *M. tuberculosis* DNA in 57 per cent of AFB smear negative EPTB samples. Our study showed a low number of PCR positives among the 126 body fluid samples, *i.e.* 32 per cent in endometrial 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 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 (Van Embden *et al.*, 1993). These insertion elements are present in multiple copies on the genome of *M. tuberculosis*, with 16 copies of IS 6110, 6 copies of IS1081 and 2 copies each of IS1547 (Cole *et al.*, 1998). 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 (Small *et al.*, 1994).

The common problem raised during the PCR assays is the high risk of false positive results due to common laboratory contamination or presence of killed or

dormant bacilli in the patient specimens (Beige *et al.*, 1995; Connelly *et al.*, 1996). Proper control checks and good laboratory practice can minimize the chances of false positive results. There are several other possible reasons for false negativity *viz.*, the paucibacillary nature of the disease, possible hypersensitivity mechanisms, or the availability of only one small amount or volume of sample after it was distributed for various microbiological, pathological and biochemical investigations. The drawback with PCR assay is that it is not able to differentiate live from dead organisms. The advantages of IS6110 PCR are that it is very rapid, easy to perform method and result can be issued for early treatment and to prevent further transmission of tuberculosis infection. Further, IS 6110 PCR test proved to be more sensitive even when both smear examination and culture results were considered in conjunction (Makeshkumar *et al.*, 2014).

PCR techniques are highly sensitive and under optimum conditions may detect 1-10 organisms. A variety of PCR methods have been developed for detection of specific sequences of *M.tuberculosis* and other mycobacteria. These assays have been reported to be quite promising in confirming the diagnosis of different forms of tuberculosis. A PCR assay system for tuberculosis which is commercially available has been found and reported to be reproducible, sensitive as well as specific. These methods can also be adapted for in situ application for confirmation of histological diagnosis. Real time PCR has been investigated for rapid and specific detection of *M. tuberculosis* in the clinical specimens. This strategy can be used for confirming the diagnosis and also monitoring the progress. There has been a genuine concern of false positivity due to contamination occurring in clinics and laboratories. The problems of false positivity can be substantially reduced by Good Laboratory Practice (GLP). In case of false negative results several strategies can be used to improve the sensitivity. While there are individual problems of appropriate sample collection, extraction and assay design, very small number of organisms and inhibitors in paucibacillary specimens are specially important. It has been observed that by using immunomagnetic beads and capture resins, the sensitivity of PCR assays can be significantly improved.

Table.1 Endometrial samples on age wise

	Age wise Patients	Negatives (n)	Positives (n)	Total
Years	<20	1	2	3
	20-25	27	14	41
	25-30	33	11	44
	30-35	16	12	28
	35-40	5	1	6
	>40	3	1	4
Total		85	41	126

Table 2: Results of Conventional methods (L.J and MGIT)

		Culture Results				
		M.tuberculosis (MTB)	Not *M.tuberculosis* (NTM)	No Mycobacterial growth	Contaminated	Total
L.J	n	27	0	92	7	126
	row%	21.43%	0	73.02%	5.55%	
MGIT	n	32	0	85	9	126
	row%	25.40%	0	67.46%	7.14%	

Table 3: Results of Molecular methods (PCR)

		PCR Results				
		M.tuberculosis (MTB)	Not *M.tuberculosis* (NTM)	Not detected Mycobacterium	Invalid assay	Total
PCR	n	41	0	85	0	126
	row%	32.54%	0	67.46%		

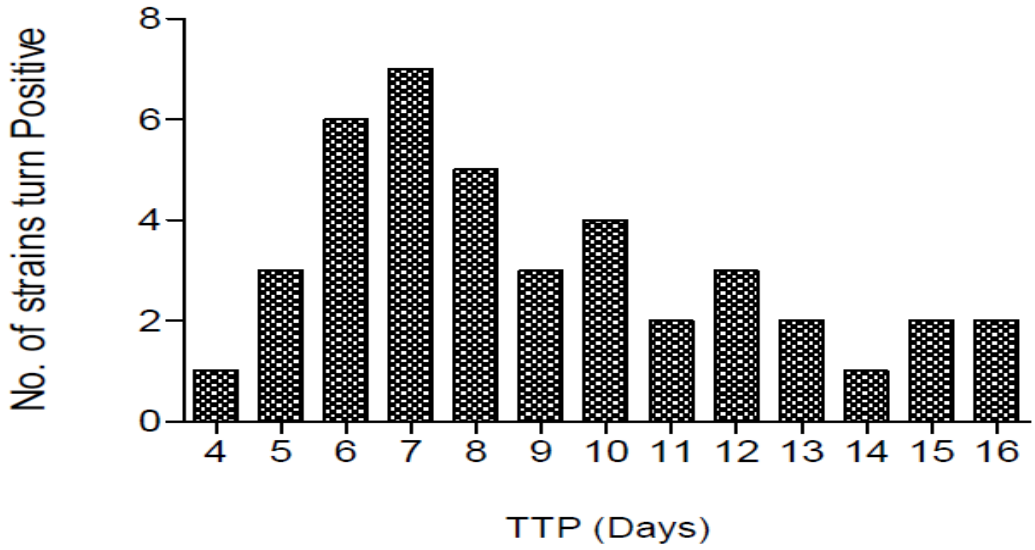

Figure 1: Number of days for turn to positive

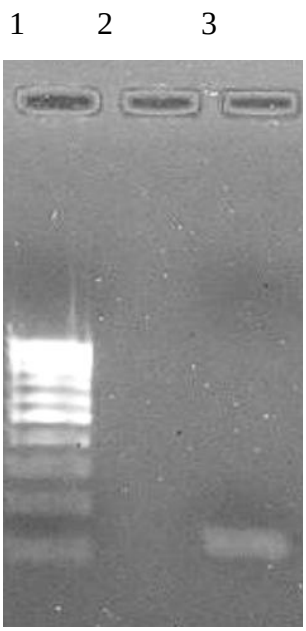

Figure 2: PCR Amplification for Species Identification. Lane 1: 100 bp DNA ladder, Lane 3:123 bp PCR amplified product.

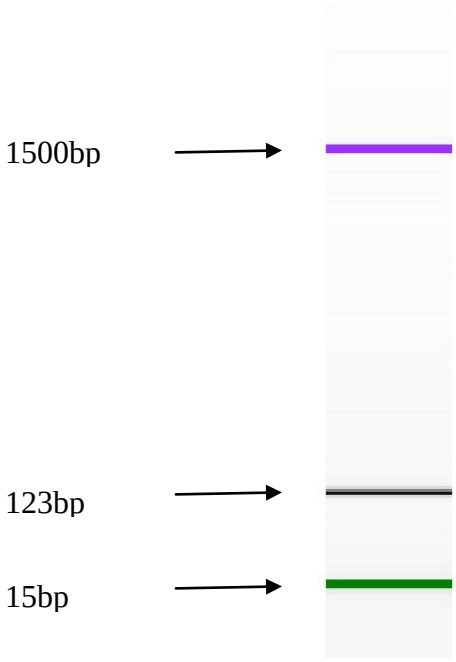

Figure 3: Electropherogram Analysis of PCR Amplified Products. Band 1: Lower Marker (15 bp), Band 2: PCR amplified product (123 bp) and Band 3: Upper marker (1500 bp)

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