

# Detection of Mycobacterium Tuberculosis among Infertile Patients Suspected with Female Genital Tuberculosis

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**Abstract** Female genital tuberculosis (FGTB) is a symptomless disease that evidences itself only when it is investigated for infertility. Demonstration of the etiologic agent by H & E staining or Z-N staining for acid fast bacilli, smear microscopy, culture of menstrual blood, urine and sputum were often unsuccessful. We therefore, proposed to use the endo-ovarian tissue biopsies and pelvic aspirated fluids for the detection of FGTB among infertile women by conventional versus genotypic methods. A prospective case-control study was undertaken. A total of 302 specimens were collected from 202 infertile women highly suspected of having FGTB on laparoscopic examination and from 100 control women of reproductive age. Out of these 302 specimens, 150 (49.67%) were premenstrual endometrial tissue biopsies (ETBs), 95 (31.46%) were ovarian tissue biopsies (OTBs) and 57 (18.87%) were pelvic aspirated fluids (PAFs). All specimens tested by conventional/ phenotypic methods were later compared with multi-gene/ multi-primer PCR (multi-gene PCR) method using four sets of primers for the detection of *Mycobacterium tuberculosis* (MTB) DNA in a single tube-single step reaction and correlated with laparoscopic findings. The presence of MTB DNA was observed in 49.5% of ETBs, 33.17% of OTBs and 5.44% of PAF specimens collected from highly suspected FGTB patients. All control women were confirmed as negative for tuberculosis. The conventional methods showed 99% to 100% specificity with a low sensitivity, ranging from 21.78% to 42.08% while H & E staining showed a sensitivity of 51.48%. Multi-gene PCR method was found to have a much higher sensitivity of 70.29% with MTB64 gene, 86.63% with 19kDa antigen gene at species and TRC4 element at regional MTB complex level and 88.12% with 32kDa protein gene at genus level (Pearson  $\chi^2 = 214.612$ , 1df, McNemar's test value  $< 0.0001$ ). The specificity of multi-gene PCR was 100%. We suggest site specific sampling, irrespective of sample type and amplification of the 19kDa antigen gene in combination with TRC4 element as a successful multi-gene PCR method for the diagnosis of FGTB and differentiation of mycobacterial infection among endo-ovarian tissue biopsies and PAFs taken from infertile women.

**Keywords:** endo-ovarian tissue biopsy processing, conventional/ phenotypic methods, multi-gene/ multi-primer polymerase chain reaction, female genital tuberculosis (FGTB), *Mycobacterium tuberculosis* (*M. tuberculosis*)

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## 1. Introduction

Female Genital Tuberculosis (FGTB) which is commonly implicated as a cause of infertility [1,2,3,4,5], is a symptom-less, rare disease with non-specific, mild clinical pictures and low index of clinical suspicion. There are no reliable confirmatory investigative procedures to ascertain the cause of infertility [4,5]. This is the most common form of extrapulmonary tuberculosis (TB), accounting for about 27% (range, 14 to 41%) worldwide [6]. The incidence of infertility in genital TB worldwide varies from 10-85% [5,6,7,8]; it is endemic in India, with an incidence of 58% [9] and majority are in the

reproductive age group (15–45 years) [10]. In 80-90% cases, it affects women with menstrual irregularities accounting for about 27% of manifestations of FGTB [11], even this rate can be higher among patients with tubal factor infertility (39- 41%) [12].

**Diagnosis and limitations:** *M. tuberculosis* (MTB) is a facultative intracellular acid-fast gram-positive pathogenic bacterium capable of producing both a progressive disease and an asymptomatic latent infection [13,14]. It generates worrying effects by causing irreversible damage to the fallopian tube consequential in infertility which is hard to treat both by medical and surgical methods [15,16]. It is estimated that at least 11% of the patients lack symptoms and FGTB is often detected in diagnostic workup of women attending infertility clinics [17]. Thangappah *et al*,

revealed that 57% of infertile women in whom the presence of TB was suspected on clinical grounds had a positive endo-TB-PCR test, whereas only 9.5% had a positive test with no clinical ground for suspicion [18]. Therefore, a high degree of suspicion assisted by intensive investigation is significant in the diagnosis of the disease. Though, the actual incidence may be under-reported due to asymptomatic, varied clinical presentations, diverse imaging, transforming laparoscopic results and a mixed bag of bacteriological and serological tests [4,5]. A positive chest X-ray for healed or active pulmonary tuberculosis, contact history, elevated erythrocyte sedimentation rate (ESR), positive tuberculin test and sampling by laparoscopy may specify the need for further investigations [19,20,21]. Studies such as serial sections of tissues need to be studied, because the lesions are frequently erratic and that a positive endometrial culture for TB, is found only in about 25% of cases of tuberculous endometritis as the endometrium is often focal and the functionalis layer is shed every four weeks (granulomas take two weeks to develop) [22,23]. Moreover, due to the cyclical shedding of the endometrium, granulomas do not have enough time to form, so the granulomatous endometrium may not show evidence of tuberculosis in all the cycles. Oophoritis is an inflammation of the ovaries, often seen in combination with salpingitis (inflammation of the fallopian tubes). It is a relatively rare condition, usually follows haematogenous spread and causes infertility. Since, there is no way to take the fallopian tubes out, sampling from the ovaries and endometrium was suggested for the detection of FGTB [24]. Use of menstrual blood for bacteriologic or molecular diagnosis has been recommended [25] but was reported to show low sensitivity [26]. Conventional/ phenotypic methods have slow and low detection rates and limited due to secondary nature of genital tuberculosis. Sampled sites may not represent the infected area and the infected site can be simply missed due to sparse number of paucibacillary nature of mycobacteria [18]. In addition, tissue reaction in those having tuberculosis may at times be atypical and bacteriologically mute [17]. These methods have poor sensitivity and specificity for the diagnosis of FGTB throughout the sub-clinical stages. On the other side, a range of PCR techniques have been mechanized for the detection of specific nucleic acid sequences of MTB and other mycobacteria [27]. Identification of genes encoding the virulence determinants, available targets in genome and highly expressing factors are current important markers in the detection of FGTB. Biological, molecular and immunological studies have resulted in identification of more than 33 different useful proteins, some of which are specific to MTB or MTB complex [28]. Out of which, four genes has been selected for our study considering their importance in the analysis of ethiopathogenesis and diagnosis of FGTB. In the present study, we report the efficacy of an amplification format based on the identification of the causative agents among highly suspected cases of FGTB in India at the genus (32kDa protein/ MPT59  $\alpha$  (alpha)-antigen gene), MTB complex (MPB64 gene), regional specific MTB complex (TRC4 element) and species levels (19kDa antigen) in determining the tubercular aetiology of female infertility. Further, we have attempted to evaluate the diagnostic competence of multi-gene/ multi-primer PCR (multi-gene

PCR) vis-a-vis laparoscopic findings, besides establishing the appropriateness of using multiple samples, namely endometrial tissue biopsies (ETBs), ovarian tissue biopsies (OTBs) and pelvic aspirated fluids (PAFs) for accurate diagnosis of FGTB.

## 2. Statistical Analysis

The sensitivity, specificity, positive predictive values and negatives predictive values were calculated using standard formulae [29] for diagnostic accuracy. Comparison of quantitative variables (age, age at menarche age, body mass index and duration of infertility among infertile women highly suspected with FGTB and control women) was performed by means of ANOVA analysis with the F-test using SPSS v 20 for Windows (IBM® SPSS® Statistics 20, Chicago, USA). The data was presented as mean  $\pm$  SD. Comparison among qualitative variables (menstrual irregularities, laparoscopic findings, types of biopsies and findings of conventional versus molecular methods among infertile women highly suspected with FGTB and control women) was performed by Pearson Chi-square test or Fisher's exact test or McNemar's test, as needed. The significant differences in the positive rate among different methods were analysed. Data were considered statistically significant if p value was less than 0.05.

## 3. Ethical Approval

The study protocol was in compliance with the Declaration of Helsinki, approved by the ethic committee of MHRT Hospital and Research Centre, Hyderabad in India. Informed consent was taken by our Institution through Ethical Committee because patient's samples were obtained by Operative Laparoscopy. In this case, we presented the letter before to begin the study.

## 4. Materials and Methods

A prospective case-control study was set in the Zoology Modular Lab, CFRD, Osmania University, Hyderabad, India. During the period of our study (2006–2014), the samples from infertile women visiting the gynaecology clinics at two collaborating centres in Hyderabad were analysed. All patients met the **inclusion criteria** i.e. 18-40 years of age having irregular periods with laparoscopic finding indicating beaded, blocked and tubal adhesions, infertility, pelvic pain, scanty menstruation, amenorrhoea and frozen pelvic and histopathological evidence in biopsy of premenstrual endometrial tissues or demonstration of tubercle bacilli in culture of menstrual blood or pelvic aspirated fluids or endometrial curetting. **Exclusion criteria were the following:** Women above 40 years of age, symptoms suggestive of pulmonary tuberculosis (TB)/ extra pulmonary TB except infertility, with normal abdominal and vaginal examinations, with pregnancy or nursing, severe psychiatric dysfunctions, endocrine problems, sexual disorders, multiple sclerosis or other autoimmune disorders, pulmonary infections, HIV co-infection, women with diabetes, malnutrition and other medical disorders like hypertension, peritoneal adhesions

due to previous abdominal surgery, infertility due to male factors and abnormality in ovulations. Information on the general, obstetric and gynaecological details including family history, marital status, age at menarche, length of menstrual cycle, associated symptoms, duration and amount of blood loss, duration of infertility, and socio-demographic details like social status, occupation, lifestyle, age, body mass index (BMI), limited information on diet were obtained. Apart from routine examinations, laparoscopy and hysteroscopy was performed for infertility workup as and when needed. All subjects were HIV negative and negative for pulmonary TB on the basis of complete history, physical examinations; chest X-ray, lung plain X-ray and by appropriate tests such as tuberculin test [19]. Details of hystero-laparoscopy findings like unilateral or bilateral tubal block with hydrosalpinx, omental adhesions, frozen pelvis, tubo-ovarian masses, tubercular salpingitis, beaded tubes and tubercles were noted. Beaded appearance of tubes, frank

tubercles on uterus and pelvic mass in variable combination aroused a suspicion. Constitutional symptoms such as sweating, increase in temperature and weight loss were not major complaints while local organ dysfunction manifested in amenorrhea, omental adhesions and bilateral tubal blockage are seen on hysterosalpingographic study.

#### 4.1. Case Group

A total of 302 specimens were taken for study which included 202 infertile women highly suspected of having genital tuberculosis on laparoscopic examination and 100 control women (without TB) of reproductive age. All samples collected were examined by H & E staining [30,31,32], Z-N staining for AFB [32,33,34], cultured on L-J egg media [34,35,36] and MTB specific multi-gene PCR method [17,18,29,37-46] by which FGTB was confirmed and correlated with laparoscopic findings.

**Table 1. Socio-demographic details among infertile women highly suspected of FGTB and control women**

Characteristics	Case-Control Group (n = 302)	Mean $\pm$ SD	F-test values on ANOVA	Test of significant (Values on ANOVA)
Age (Years)	Case (n = 202)	28.54 $\pm$ 4.46	2.894	0.09
	Control (n = 100)	27.59 $\pm$ 4.62		
Age at menarche (Years)	Case (n = 202)	12.49 $\pm$ 1.02	0.965	0.327
	Control (n = 100)	12.37 $\pm$ 0.93		
Body mass index (kg/m <sup>2</sup> )	Case (n = 202)	24.36 $\pm$ 1.47	2.538	0.112
	Control (n = 100)	24.05 $\pm$ 1.68		
Duration of infertility (Years)	Case (n = 202)	3.92 $\pm$ 3.03	151.653	0.000
	Control (n = 100)	0.174 $\pm$ 0.184		

**Note:** Data were considered statistically significant if p value was less than 0.05; Degree of freedom (df) was one (1) for all calculations; n: number of patients; %: percentage; SD: Standard Deviation; Analysis of Variance (ANOVA); kg: kilogram; m: meters.

The diagnosis was done based on morphological [19] and molecular investigations. All specimens tested by conventional/ phenotypic methods were later compared with multi-gene PCR method using four sets of primers for detection of MTB in a single tube- single step reaction. *M. tuberculosis* (ATCC 35836) reference stain isolates provided by Dept of Microbiology, Nizam's Institute of Medical Sciences, Hyderabad (India) were used as controls in each assay. Out of 202 specimens taken for study, 123 (60.89%) were ETBs, 68 (33.66%) were OTBs and 11 (5.44%) were PAFs. The mean age of the subjects was 28.54  $\pm$  4.46 years, mean duration of infertility was

3.92  $\pm$  3.03 years, BMI was 24.36  $\pm$  1.47 and the difference had statistical significance with (F-test = 151.653, 1df, p <0.0001) different parameters. Table 1 depicts the demographic variables of the infertile women and control women. The majority of patients (77.23 %) presented primary infertility, 46 (22.77%) with secondary infertility and 69 (34.15%) women experienced abortion (Table 2). Apart from infertility, 125 (61.88%) patients had other menstrual complaints such as dysmenorrhoea in 94 (46.53%) women and abdominal/ pelvic pain in 31 (15.34%) women.

**Table 2. Type of infertility among Case-Control groups**

Characteristics	Infertile women suspected of FGTB (n=202) [n (%)]	Control group (n = 100) [n (%)]
Primary infertility	156 (77.23)	0
Secondary infertility	46 (22.77)	0
Proven fertility	0	100 (100)
Abortions	68 (33.66)	0
No dyspareunia & dysmenorrhoea	52 (25.74)	90 (90)

**Note:** %: percentage; n: number of patients.

63 (31.19%) patients had menstrual irregularities such as oligomenorrhea (12.87%), amenorrhea (8.91%), general malaise (4.95%) and menorrhagia (4.45%) among infertile women highly suspected with FGTB. The difference had statistical significance with (Pearson  $\chi^2$  = 67.565, 1df, Fisher's exact test value <0.0001; McNemar's test value = 0.720). Statistical analysis of menstrual

problems among infertile women highly suspected with FGTB and control women were described in Table 3. All patients were negative for chest X-ray and 42 (20.79%) patients were positive for Mantoux test. Erythrocyte sedimentation rate (ESR) was elevated in all the patients. All the patients were asymptomatic, resistant to all therapeutic treatments other than anti TB therapy.

**Table 3. Statistical analysis of menstrual problems among infertile women highly suspected of FGTB and Controls**

Symptoms	Case-Control Group (n = 302)	Observed Cases [n (%)]	Pearson Chi-Square Value	Exact Significance (2-sided) Values	
				Fisher's Exact Test	McNemar's Test <sup>a</sup>
Dysmenorrhoea	Case (n = 202)	94 (46.53)	67.565 <sup>b</sup>	0.000	0.720
	Control (n = 100)	0			
Abdominal pain	Case (n = 202)	31 (15.34)	17.102 <sup>c</sup>	0.000	0.000
	Control (n = 100)	0			
Oligomenorrhoea	Case (n = 202)	26 (12.87)	7.508 <sup>d</sup>	0.006	0.000
	Control (n = 100)	3 (3)			
Amenorrhoea	Case (n = 202)	18 (8.91)	9.476 <sup>e</sup>	0.001	0.000
	Control (n = 100)	0			
General malaise	Case (n = 202)	10 (4.95)	5.120 <sup>f</sup>	0.034	0.000
	Control (n = 100)	0			
Menorrhagia	Case (n = 202)	9 (4.45)	0.863 <sup>g</sup>	0.415	0.000
	Control (n = 100)	7 (7)			

**Note:** Statistical analysis showed 0 cells (0.0%) have expected count less than 5 among all methods, except for "f" 1 cell (25.0%) has expected count less than 5 and the minimum expected count is 3.31. The minimum expected counts for different methods were different, as the minimum expected count for "b" is 31.13; "c" is 10.26; "d" is 9.60; "e" is 5.96 and "g" is 5.30. "a": binomial distribution used. Degree of freedom (df) was one (1) for all calculations, %: percentage; n: number of patients. Data were considered statistically significant if p value was less than 0.05.

## 4.2. Control Group

A total of 100 specimens were collected control women of reproductive age (18-40 years). Of which 27 (27%) were ETBs, 27 (27%) were OTBs and 46 (46%) were PAFs. Women who attended the same clinic for other gynaecological disorders, tubal sterilization and laparoscopy for menorrhagia were selected as controls. All the women in this group were asymptomatic with mean age of  $27.59 \pm 4.62$  years, mean duration of infertility was  $0.174 \pm 0.184$  years and BMI was  $24.05 \pm 1.68$ . Menstrual irregularities such as oligomenorrhoea (3%) and mild menorrhagia (7%) were seen in the control women. 13 patients were positive to Mantoux test. All patients were fertile, negative for chest X-ray and laparoscopically confirmed to be without FGTB.

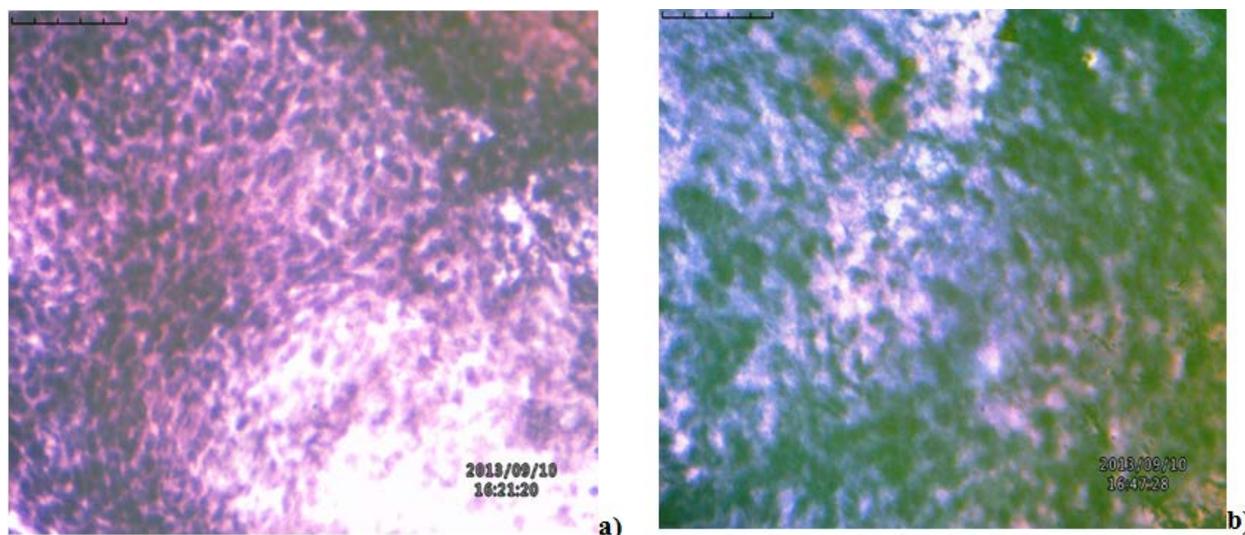
## 4.3. Processing of Tissue Biopsy

The specimens (like ETBs, OTBs and PAFs) collected from the lesions over the endometrium, ovaries and pelvic were mixed with sterile normal saline, transported in sterile vials to the laboratory and processed as per standard protocols [17,18,47,48,49,50]. Whole specimens were washed with saline and centrifugation at 6000 rpm for 10 min at 4°C. Supernatant were discarded and the

pellets were reconstituted with 1ml of sterile Tris Buffered Saline (1X TBS; pH-7.4). Each specimen (from TBS) was divided into two portions-one kept for decontamination and concentration for subsequent studies such as Z-N staining, culturing on L-J media and extraction of DNA for multi-gene PCR method, and the other portion kept was used for histopathology, RNA and protein extraction.

## 4.4. Decontamination and Concentration (DC)

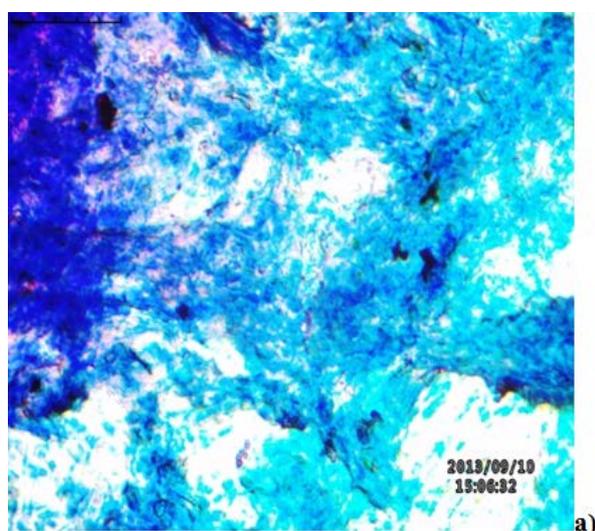
All specimens were decontaminated and concentrated by modified HS-SH procedure [51]. About 200µl of tissue biopsies or scrapings were minced and grinded well using tissue homogenizer and mixed with 200µl of 7% (w/v) NaCl and 200µl of 4% (w/v) NaOH in a sterile eppendorf centrifuge tube. Then, the tubes were incubated at 37°C for 30 min. The content was neutralized with sterile normal saline, to make the total volume to 2 ml. The mixture was mixed for 5 seconds and centrifuged at 12,000 rpm for 15 min at 4°C using aerosol proof shields. The supernatant was discarded into a splash-proof container with a tuberculous solution and the pellet was resuspended in 200µl of sterile TBS buffer and mixed for 5 seconds. Homogenised tissue sediments were used for culturing on L-J medium, Z-N staining and for the isolation of mycobacterial DNA.



**Figure 1.** Histopathological Examination of Endo-ovarian Tissue Biopsy: a) Lymphocytic infiltrations and beaded cells were observed, b) Granulomatous cells were observed in endometrial tissue biopsies

#### 4.5. Histopathological Examination

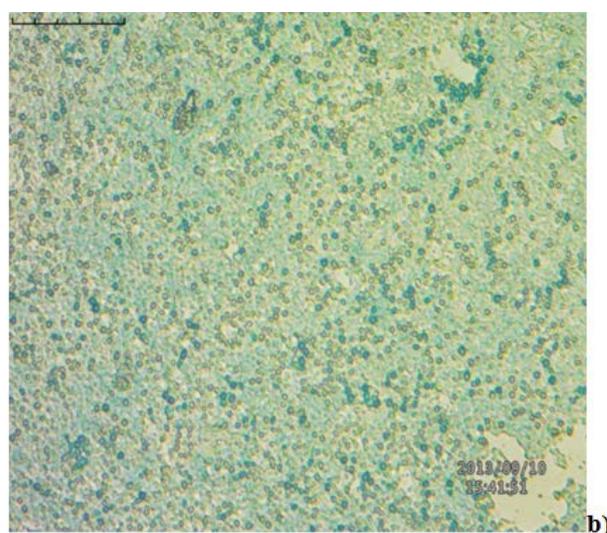
Thin slices of the processed tissue biopsies and PAFs were placed onto the slides and kept for air drying at room temperature. Tissue specimens were fixed with buffered formalin (10%), cleaned with xylene and dehydrated with absolute alcohol (100%). The slides were again fixed with ethyl alcohol (95%), washed with water and dried. Then, the slides were stained with Weigert's iron hematoxylin, washed with water, differentiated with acid alcohol (1%) and alcohol (95%) and washed with water. Followed by, the slides were counterstained with eosin [30,31,32], dehydrated with increasing gradients of alcohol, cleaned with xylene and mounted. Mounted slides were viewed under bright field (40x), Inverted Biological Microscope (BLM-290, BestScope, China). The presence of caseating granulomas surrounded by epithelioid cells, malignant lymphocytic infiltrations, beaded plasma cells and giant



polymorphonuclear cells were diagnostic of FGTB (Figure 1).

#### 4.6. Ziehl-Neelson's (Z-N) Staining of Tissue Sediments for AFB

For about 50µl of the decontaminated and concentrated endo-ovarian tissue sediments were spread onto the slides, kept air drying for 10 minutes at 60°C and heat-fixed for 10 minutes at 90°C. The slides were then stained with carbolfuchsin, decolorized by acid alcohol and counterstained by methylene blue and rinsed with water to remove excess methylene blue. Stained slides were viewed under Inverted Biological Microscope. The portion of smear that stained pink/red on pale blue background was noted as *Mycobacterium* [32,33,34] (Figure 2).



**Figures 2.** Z-N staining of Endo-ovarian Tissue Biopsy and Cultures for the detection of Acid fast bacilli (AFB): **a)** Pink/ Red colour rod like beaded structures were observed in the tissue biopsy; **b)** Pink/ Red colour rod like structures were observed on pale blue background in the cultures

#### 4.7. Culture of Tissue Sediments

About 50µl inoculum of the decontaminated and concentrated endo-ovarian tissue biopsy sediments were taken and applied rapidly on L-J egg medium slants using sterile micropipette. Slant and bottled culture media were incubated in a horizontal plane until the inoculum was absorbed. The culture tubes were then incubated at 37°C for 6-8 weeks until heavy growth was obtained. Cultures were stained by Z-N staining for confirmation of AFB growth [34,35]. Standard Precautions" [35,36] and Institutional guidelines have been followed in handling all items contaminated with blood and other body fluids.

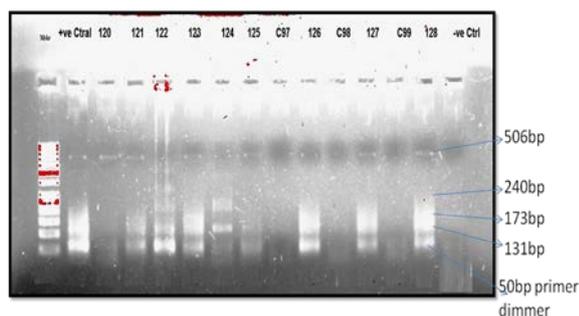
#### 4.8. Quality Control

Reagents were aliquoted and each aliquote was used only once. Sterile microcentrifuge tubes and PCR tubes were used for the PCR assay. Reagent preparation, DNA extraction, DNA amplification and detection were performed in separate rooms to avoid cross-contamination of amplicons. A positive control was included in each test and distilled water was included as a negative test control. The Clinical and Laboratory Standards Institute (CLSI, 2001 [36]) recommendations and regulations were followed for quality control and standards.

#### 4.9. DNA Extraction and Purification

All the decontaminated and concentrated endo-ovarian tissue sediments were centrifuged at 8,000 g for 10 minutes and 180 µl of 2X TE buffer (20 mM Tris-HCl, 2 mM EDTA, pH 8.0) containing 1% sodium dodecyl sulfate (40µl from 10% SDS), 2.4% Triton X-100 was added to the pellet for lysis of gram-positive bacteria [52], 20 mg/ml of lysozyme was subsequently added, mixed and incubated for 30-60 min at 37°C. 25µl of Proteinase K (20mg/ml) was added and mixed by vortexing and incubated in dry bath at 56°C for 1 to 3 hours until complete lysis was achieved (provided by DNASure @ Tissue mini kit from Genetix Biotech Asia Pvt. Ltd, New Delhi, India). Then, appropriate condition for binding of DNA to the silica membrane in the DNASure @ Tissue Mini Kit Columns was achieved by the addition of chaotropic salts and ethanol to the lysate. The binding process is reversible and specific to nucleic acids. Contaminations were removed by subsequent washing with two different buffers according to manufacturer instructions. Pure genomic DNA was finally eluted under low ionic strength conditions in a slightly alkaline elution buffer. The purity of DNA was checked on 0.8% agarose gel electrophoresis, incorporated with ethidium bromide.

The bands in the gel were photographed under a Bio-Rad gel documentation system and quantified.



**Figure 3:** 2.5% Agarose Gel Electrophoresis was carried out for Multi-gene/ Multi-primer PCR products: Lanes in the first row (120 to 128) were loaded with PCR products of FGTB patients; Lanes in the second row (C97 to C99) were loaded with PCR products of control patients; Lane +Ve Ctrl was loaded with positive Reference Strain (*M. tuberculosis*, ATCC 35836); Lane -Ve Ctrl was loaded with negative control (H<sub>2</sub>O); Lane 50bp marker was loaded with 50 base pair (bp) molecular weight ladder (50bp size of product starts from the bottom side of gel and ends with 650bp product on the top/upper side of the gel), The band corresponding to 131bp was noted as 19kDa antigen gene, 173bp was noted as TRC4 element, 240bp was noted as MPB64 gene, 506bp was noted as 32kDa protein/ MPT59  $\alpha$ - antigen gene. Primer dimmers were also noted at the bottom during the end of sample run

#### 4.10. Multi-gene/ Multi-primer PCR Method

Multi-gene PCR was performed with 20-30ng extracted DNA, and DNA amplification with 5U Taq DNA polymerase (Bangalore Genie, Bangalore, India), 10 mM deoxyribonucleoside triphosphates (Bangalore Genie, Bangalore, India) and 13.5 pmol each primer (Bioserve Biotechnologies (India) Pvt Ltd, Hyderabad, AP, India) in a final volume of 50 $\mu$ L. Two looped touchdown multi-gene PCR program; each with 25 cycles was followed. In the first loop, the template DNA was initially denatured at 95°C for 5 minutes then denatured at 94°C for 45 seconds, annealed at 68°C for 45 seconds, extended at 72°C for 45 seconds and continued for total of 25 cycles. In 2<sup>nd</sup> loop; DNA was denatured at 94°C for 45 seconds, annealed at

58°C for 45 seconds, extended at 72°C for 45 seconds and continued for total of 25 cycles with final extension at 72°C for 15 minutes. The PCR amplification was done using master cycler gradient PCR system (Eppendorf, Hamburg, Germany). PCR products were subjected to electrophoresis in a 2.5% agarose gel incorporated with ethidium bromide, along with Gene Rule 50bp DNA ladder/ molecular weight marker. The electrophoresis was carried out at a constant voltage (110 V) for one hour. The bands in the gel were photographed under a Gel Doc-XRT with image lab software (Molecular Image, Bio-Rad, Hercules, CA, United States of America). The results of case-control group were compared with reference strain (Figure 3).

## 5. Results

There were no particular gold standard methods followed for the detection of FGTB. However, one should use varied combinations of tests to overcome the limitations such as the false positivity by way of contaminations, false negativity and dead bacilli or past infection or asymptomatic TB at another site. Even technical considerations, such as the use of suitable controls, standard strains and the retesting of doubtful positive samples, considerably influence the sensitivity and specificity. Therefore, this investigation has been conducted meticulously with appropriate controls, replications, large sample size and with different combination of experiments. The present study assesses the role of multi-gene PCR in the diagnosis of FGTB using ETBs, OTBs and PAF samples and correlated with laparoscopic findings. Primer selection of present study was done based on local characteristics of strains and their importance in the diagnosis of the disease (tabulated in Table 4). The nucleotide sequences of primers used for the detection of *Mycobacterium* in this study were described and validated as diagnostic markers in the past [17,18,29,37-46].

**Table 4.** Details of genes of *Mycobacterium tuberculosis* Complex

S.No	Gene	Forward primer	Reverse primer	Length & Size (bp)	Reported annealing temp.	Function	Conclusion	Ref.
1	19 kDa Antigen gene	5'TCTTT CCGA TGTTCA AGCA 3'	5'TGAC GTTCTG GTCCTT ACC3'	20; 131	58, 68	It acts as an antigen for cellular and humoral arms of the adaptive response. Involved in suppression of growth and apoptosis of infected cells.	Secreted nature can contribute to its serological immunodominance by enhancing its accessibility in a native form for B-cell recognition.	37, 38
2	TRC4 Element	5'GACA ACGAC GTGCG CCTACT 3'	5'GACC GAATT AGCGT AGCTC C 3'	20; 173	57, 58	It is from a very essential region of <i>M. tuberculosis</i> genome participating in recombination	Ideal target for PCR assays to identify <i>M. tuberculosis</i> ; especially in strains carrying no copies of IS6110 in extra pulmonary patients	18, 39, 40
3	MPB64 Antigen gene	5'TCCG CTGCC AGTGC TCTTC 3'	5'GTCC TCGCG AGTCT AGGCC A 3'	20; 240	55,60	Highly immunogenic antigen and found in active cultures	This polymeric epitopes can be a good candidates for serodiagnosis	17, 29, 41
4	32kDa Protein	5'TTCC TGACC AGCGA GCTGC CG 3'	5'CCCC AGTAC TCCCA GCTGT GC 3'	21;506	68, 71	Abundantly secreted, catalyses in formation of mycobacterial cell wall assembly	This antigen would provide a target, which is universally present.	42-46

T: thymine; A: adenine; G: guanine; C: cytosine

None of the patients in our study reported family history of tuberculosis. Laparoscopy usually detects macroscopic changes such as peritubal adhesions, tubercles on the tubes and small tubo-ovarian masses that are commonly seen in chronic cases. FG TB also presents distinctive diagnostic challenges including subtle clinical manifestations that were over looked in laparoscopy during early stages of infection [8]. Clinical presentations such as dysmenorrhoea in 94 (46.53%) women, beaded tubes in 139 (68.81%) women, tubal block with hydrosalpinx in 119 (58.91%), tubercular salpingitis in 97 (48.01%), omental adhesions in 78 (38.61%) and multiple tubercles in 72 (35.64%) women were recorded in the case group. Only thickened tubes and tubal adhesions were observed in 4 (4%) control women varied clinical presentations among infertile women with FG TB were significantly dissimilar from that of the control women (Pearson  $\chi^2 = 97.219$ , 1df, Fisher's exact test value <0.0001). The difference was due to over representations

of clinical symptoms in infertile women with FG TB (Pearson  $\chi^2 = 127.492$ , 1df, McNemar's test value = 0.014). Table 5 shows varied clinical variables among infertile women with FG TB and control women. A standard protocol of investigations revealed a number of causes for fertility deprivation. The multi-gene PCR detection rate for the disease was the highest of all the other tests as it detected, 178 (58.94%) out of the 302 endo-ovarian tissue specimens, 104 (34.43%) specimens collected from infertile women and 1 (0.33%) ETB from control woman was positive on hematoxylin and eosin (H & E) staining (Pearson  $\chi^2 = 75.167$ , 1df, Fisher's exact test value <0.0001, McNemar's test value =0.779) [17,18]. Acid fast bacilli (AFB) were positive in 44 (14.57%) decontamination and concentration (DC) tissue specimens collected from infertile women and 1 (0.33%) OTB from control woman was positive by cultivation on Loewenstein-Jensen (L-J) egg media.

**Table 5. Laparoscopic findings and their statistical association with infertile women highly suspected of FG TB and Controls**

Clinical Symptoms	Case-Control Group (n = 302)	Observed Cases [n (%)]	Pearson Chi-Square Value	Exact Significance (2-sided) Values	
				Fisher's Exact Test	McNemar's Test <sup>1</sup>
Tubal block with hydrosalpinx	Case (n = 202)	119 (58.91)	97.219 <sup>a</sup>	0.000	0.224
	Control (n = 100)	0			
Tubo-ovarian mass	Case (n = 202)	59 (29.21)	36.300 <sup>b</sup>	0.000	0.001
	Control (n = 100)	0			
Tubercular salpingitis	Case (n = 202)	97 (48.01)	70.741 <sup>c</sup>	0.000	0.887
	Control (n = 100)	0			
Beaded tubes	Case (n = 202)	139 (68.81)	127.492 <sup>d</sup>	0.000	0.014
	Control (n = 100)	0			
Bilateral opening of tubes	Case (n = 202)	50 (24.75)	29.664 <sup>e</sup>	0.000	0.000
	Control (n = 100)	0			
Thicken tubes & Tubal adhesions	Case (n = 202)	34 (16.83)	10.012 <sup>f</sup>	0.001	0.000
	Control (n = 100)	4 (4)			
Tubercles on uterus	Case (n = 202)	19 (9.4)	10.037 <sup>g</sup>	0.001	0.000
	Control (n = 100)	0			
Omental adhesions	Case (n = 202)	78 (38.61)	52.060 <sup>h</sup>	0.000	0.115
	Control (n = 100)	0			
Frozen pelvis	Case (n = 202)	55 (27.23)	33.291 <sup>i</sup>	0.000	0.000
	Control (n = 100)	0			
Multiple tubercles	Case (n = 202)	72 (35.64)	46.802 <sup>j</sup>	0.000	0.039
	Control (n = 100)	0			
Small ovaries and lower abdominal mass	Case (n = 202)	37 (18.31)	20.874 <sup>k</sup>	0.000	0.000
	Control (n = 100)	0			

**Note:** %: percentage; n: number of patients; statistical analysis showed 0 cells (0.0%) have expected count less than 5 for all methods. The minimum expected count for different methods were different, as the minimum expected count for "a" is 39.40; "b" is 19.54; "c" is 32.12; "d" is 46.03; "e" is 16.56; "f" is 12.58; "g" is 6.29; "h" is 25.83; "i" is 18.21; "j" is 23.84 and "k" is 12.25; "l" denotes binomial distribution used. Degree of freedom (df) was one (1) for all calculations. Data were considered statistically significant if p value was less than 0.05.

Out of 85 cultures positive cases, 81 (26.82%) FG TB cases were found AFB positive by Ziehl-Neelson's (Z-N) staining. All control women (n = 100) were found negative for tuberculosis with endometrium, ovaries and pelvis. Disparity among detection of FG TB by Z-N staining for AFB positive, histopathological evidence of tuberculosis infection, isolation by culture, and detection of *M. tuberculosis* complex by multi-gene PCR within infertile women highly suspected of genital TB and

control women were found to have statistical significance. (tabulated in Table 6). Out of 123 (60.89%) ETBs, 57 (28.21%) were positive on H & E staining, 37 (18.31%) by culturing on L-J egg medium and 35 (17.32%) were AFB positive on Z-N staining of culture. Of the 123 ETBs, 97 (48.02%) were positive for 19kDa antigen (131bp) gene and 100 (49.50%) were positive for TRC4 (173bp) repetitive element and MPT59  $\alpha$ -antigen (506bp) gene (32kDa protein) by multi-gene PCR.

**Table 6. Statistical analysis and validation of the results obtained by conventional verses molecular methods among case-control groups (n=302)**

Conventional verses Molecular Methods	Case-Control Group (n = 302)	Observed Cases [n (%)]	Pearson Chi-Square Value	Exact Significance (2-sided) Values	
				Fisher's Exact Test	McNemar's Test <sup>a</sup>
H & E Staining	Case (n = 202)	104 (51.48)	75.167 <sup>b</sup>	0.000	0.779
	Control (n = 100)	1 (1)			
AFB +Ve on Z-N Staining of tissue sediments	Case (n = 202)	44 (21.78)	25.497 <sup>c</sup>	0.000	0.000
	Control (n = 100)	0			
L-J Egg medium	Case (n = 202)	85 (42.08)	55.418 <sup>d</sup>	0.000	0.338
	Control (n = 100)	1 (1)			
AFB +Ve on Z-N Staining of culture	Case (n = 202)	81 (40.1)	55.725 <sup>e</sup>	0.000	0.208
	Control (n = 100)	0			
19kDa (131bp)	Case (n = 202)	175 (86.63)	206.011 <sup>f</sup>	0.000	0.000
	Control (n = 100)	0			
TRC4 (173bp)	Case (n = 202)	175 (86.63)	206.011 <sup>g</sup>	0.000	0.000
	Control (n = 100)	0			
MPB64 (240bp)	Case (n = 202)	142 (70.3)	132.686 <sup>h</sup>	0.000	0.008
	Control (n = 100)	0			
32kDa (506bp)	Case (n = 202)	178 (88.12)	214.612 <sup>i</sup>	0.000	0.000
	Control (n = 100)	0			

**Note:** "a" denotes binomial distribution used. Statistical analysis showed 0 cells (0.0%) have expected count less than 5 for all methods. The minimum expected counts for different methods were different, as the minimum expected count for "b" is 34.77; "c" is 14.57; "d" is 28.48; "e" is 27.15; "f" is 42.05; "g" is 42.05; "h" is 47.02; and "i" is 41.06. Degree of freedom (df) was one (1) for all calculations. Data were considered statistically significant if p value was less than 0.05. Some patients had more than one abnormal finding; n: number of patients; %: percentage. **Abbreviations:** Female Genital Tuberculosis (FGTB); Endometrial tissue biopsies (ETBs); Ovarian tissue biopsies (OTBs); Pelvic aspirated fluids (PAFs); Hematoxylin and Eosin (H & E) staining; Lowenstein Jensen (L-J) Egg medium; AFB +Ve, Acid fast bacilli positive; Ziehl-Neelson's (Z-N) staining; Polymerase Chain Reaction (PCR); 19kDa antigen gene: 19kDa (131bp); TRC4 element: TRC4 (173bp); MPB64 antigen gene: MPB64 (240bp); 32kDa protein (MTP-59  $\alpha$ -antigen) gene: 32kDa (506bp).

Of the 68 (33.66%) OTBs, 42 (20.79%) were positive with H & E staining, 41 (20.29%) by culturing on L-J egg medium and 39 (19.3%) were AFB positive with Z-N staining of culture. Subsequently, 67 (33.17%) were positive for 19kDa antigen (131bp) gene and 32kDa protein (506bp) gene by multi-gene PCR. Present study also reports, 5 (2.47%) out of 11 (5.44%) PAFs, were positive with H & E staining, 7 (3.46%) were positive on L-J egg medium and with Z-N staining after culturing. 11 (5.44%) samples were positive for 19kDa antigen (131bp) gene and 32kDa protein (506bp) gene by multi-gene PCR (Table 7). Amongst, the 202 specimens collected from

highly suspected cases of FGTB, 175 (86.63%) specimens were positive for 19kDa antigen (131bp) gene (MTB species-specific gene) and TRC4 (173bp) repetitive element (south Indian MTB complex specific). 142 (70.3%) specimens were positive for MPB64 antigen (240bp) gene (MTB complex specific). 178 (88.12%) endo-ovarian tissue biopsies and pelvic aspirated fluid specimens were positive for 32kDa protein (506bp) gene by multi-gene PCR method (Pearson  $\chi^2 = 214.612$ , 1df, Fisher's exact test value <0.0001, McNemar's test value <0.0001).

**Table 7. Type of samples and findings of conventional versus molecular methods among infertile women suspected of FGTB and Controls**

Characteristics	Type of samples	n (%)	Conventional/ Phenotypic methods				Molecular method (Multi-gene/ multi-primer PCR)			
			H & E Staining [n (%)]	AFB +Ve on Z-N Staining of tissue sediments [n (%)]	L-J Egg medium [n (%)]	AFB +Ve on Z-N Staining of culture [n (%)]	19kDa (131bp) [n (%)]	TRC4 (173bp) [n (%)]	MPB64 (240bp) [n (%)]	32kDa (506bp) [n (%)]
Suspected Female Genital Tuberculosis (FGTB) cases	ETB	123 (60.89)	57 (28.21)	12 (5.94)	37 (18.31)	35 (17.32)	97 (48.02)	100 (49.50)	71 (35.14)	100 (49.50)
	OTB	68 (33.66)	42 (20.79)	29 (14.35)	41 (20.29)	39 (19.30)	67 (33.17)	66 (32.67)	63 (31.18)	67 (33.17)
	PAF	11 (5.44)	5 (2.47)	3 (1.48)	7 (3.46)	7 (3.46)	11 (5.44)	9 (4.45)	8 (3.96)	11 (5.44)
	Total	202 (100)	104 (51.48)	44 (21.78)	85 (42.08)	81 (40.1)	175 (86.63)	175 (86.63)	142 (70.3)	178 (88.12)
Controls	ETB	27(27)	1(1)	NA	NA	NA	NA	NA	NA	NA
	OTB	27 (27)	NA		1 (1)					
	PAF	46 (46)	NA		NA					
	Total	100 (100)	1(1)		1(1)					

**Note:** Some patients had more than one abnormal finding; n: number of patients; %: percentage; NA: not applicable. **Abbreviations:** Endometrial tissue biopsies (ETBs), Ovarian tissue biopsies (OTBs), Pelvic aspirated fluids (PAFs).

The conventional methods showed 99% to 100% specificity with a low sensitivity, ranging from 21.78% to 42.08% while H & E staining showed a sensitivity of 51.48%. However, multi-gene PCR method was found to have a much higher sensitivity of 70.29% for MTB64

gene, 86.63% for 19kDa antigen gene and TRC4 element and 88.12% for 32kDa protein gene [43,53]. The specificity of multi-gene PCR was 100% (tabulated in Table 8). The conventional/ phenotypic methods had 98.83% to 100% positive predictive value with a lower

negative predictive value, ranging from 38.75% to 45.83% whereas H & E staining had a negative predictive value of 50.25%. Multi-gene PCR method was found to have much varied negative predictive value of 62.5% with MTB64 gene, 78.74% with 19kDa antigen gene and TRC4 element and 80.64% with 32kDa protein gene. The positive predictive value of multi-gene PCR was 100%. Of four different methods used, histopathology and culture on L-J egg media showed false positive in 1 (0.33%) tissue sediment. On other side, 24 (7.95 %) specimens showed false negative with 32kDa protein (506bp) gene and 27 (8.94%) tissue specimens were false negative with 19kDa antigen (131bp) gene and TRC4 (173bp) element and 60 (19.86%) with MPB64 antigen (240bp) gene by multi-gene PCR method. 98 (32.45%) tissue specimens were false negative by H & E staining, 158 (52.31%) tissue sediments were false negative by Z-N staining, 117 (38.74%) specimens were false negative with cultures on L-J egg medium and 121 (40.06%) cultures were false negative by Z-N staining. All false negative samples evidenced by every conventional and molecular methods were likely to represent negative for tuberculosis as multi-gene PCR was repeatedly proven negative even though, it belongs to highly suspected cases of FGTB on

laparoscopic examination. In the non TB control group, all the tests were negative for TB. These results showed that the molecular method is more accurate than the conventional methods [17,18,29].

## 6. Discussion

Female genital tuberculosis is an important cause of infertility and may simulate advanced ovarian malignancy [54]. It is an indefinable diagnosis and a high index of suspicion is very essential in the routine diagnostic process. However laparoscopy is invasive, expensive procedure by which absolute diagnosis of FGTB cannot be made, although laparoscopy can pick up the signs of disease and provides an opportunity to take samples for laboratory investigations from various suspicious sites [20,21]. Several PCR techniques have been developed for the direct detection of *M. tuberculosis* [17,18,43,55] and for the detection of *Mycobacterium* spp [43,45,56]. However, PCR using single target gene alone is not sufficient in the detection of all strains of *M. tuberculosis*, therefore use of multiple target genes is more appropriate [29,39].

**Table 8. Comparison of Specificity and Sensitivity among methods (n = 302)**

Methods versus Characteristics	True Negatives [n (%)]	False Positives [n (%)]	True Positive [n (%)]	False Negatives [n (%)]	Positive Predictive Value (%)	Negative Predictive Value (%)	Sensitivity (%)	Specificity (%)
PCR for 19 kDa Antigen (131bp)	100 (33.11)	0	175 (57.95)	27 (8.94)	100	78.74	86.63	100
PCR for TRC4 Element (173bp)	100 (33.11)	0	175 (57.95)	27 (8.94)	100	78.74	86.63	100
PCR for MPB64 Antigen (240bp)	100 (33.11)	0	142 (47.02)	60 (19.86)	100	62.5	70.29	100
PCR for 32kDa Protein/MPT59 $\alpha$ -Antigen (506bp)	100 (33.11)	0	178 (58.94)	24 (7.95)	100	80.64	88.12	100
H & E Staining of endo-ovarian tissue biopsy	99 (32.78)	1 (0.33)	104 (34.43)	98 (32.45)	99.04	50.25	51.48	99
Z-N Staining of tissue sediment	100 (33.11)	0	44 (14.57)	158 (52.31)	100	38.75	21.78	100
Growth on L-J Egg medium	99 (32.78)	1 (0.33)	85 (28.14)	117 (38.74)	98.83	45.83	42.08	99
AFB Z-N Staining of culture	100 (33.11)	0	81 (26.82)	121 (40.06)	100	45.24	40.1	100

**Note:** Some patients had more than one abnormal finding; n: number of patients; %: percentage. **Abbreviations:** Multi-gene/ multi-primer Polymerase Chain Reaction (Multi-gene PCR), Hematoxylin and Eosin (H & E) Staining, Ziehl-Neelsen (Z-N) Staining, Growth on Loewenstein-Jensen (L-J) Egg medium. The following terms were described based on socio-demographic details, clinical and hysteroscopic/ laparoscopic findings of infertile women highly suspected of female genital tuberculosis (FGTB) and control women (without tuberculosis): **True Negatives:** 19kDa antigen (131bp) gene negative/ TRC4 (173bp) element negative/ MPB64 (240bp) gene negative/ 32kDa protein (MPT-59  $\alpha$ -antigen; 506bp) gene negative on multi-gene/ multi-primer PCR method or H & E staining negative/ AFB negative on Z-N staining/ absence of growth on L-J egg media/ without TB/ remains fertile/ healthy/ absence of abnormal clinical findings on laparoscopic examination; **True Positive:** 19kDa antigen (131bp) gene positive/ TRC4 (173bp) element positive/ MPB64 (240bp) gene positive/ 32kDa protein (MPT-59  $\alpha$ -antigen; 506bp) gene positive on multi-gene/ multi-primer PCR method or H & E staining positive/ AFB positive on Z-N staining/ presence of growth on L-J egg medium/ highly suspected of FGTB/ remains infertile/ presence of abnormal clinical findings on hysteroscopic/ laparoscopic examination; **False positive:** Multi-gene/ Multi-primer PCR positive or Conventional/ phenotypic methods positive among negative TB patients (control) group/ remains fertile/ healthy/ laparoscopically negative clinical findings; **False negative:** Multi-gene/ Multi-primer PCR or Conventional/ Phenotypic methods negative among women with highly suspected of FGTB/ remains infertile/ women with abnormal clinical findings on hysteroscopic/ laparoscopic examination.

It has great potential in the laboratory diagnosis of FGTB, particularly in latent and paucibacillary conditions as well as in active tuberculosis. Improvement in the sensitivity of PCR was reported by using different set of targets in the detection of extrapulmonary tuberculosis [39,40,44]. The increase in PCR sensitivity and decrease in false negative results was achieved using dual targets for the detection of *M. tuberculosis* [53]. Furthermore, the high endemicity of TB in India raises the possibility of this patient harbouring a latent infection. A broad range of mycobacterial species are involved in causing such

infections, but the type of complexes, species or type of strains that causes disease are not clear. Identification of nontuberculous *Mycobacterium* and treatments based on inconclusive findings involving different strains may not be adequate to control the disease. Therefore, this prospective large case-control cohort study was commenced for the detection of FGTB in Indian population for the first time. In the present study, we have demonstrated the use of a multi-gene PCR system based on the simultaneous amplification of the species-specific 19kDa antigen gene, MTB complex specific TRC4

element and MPB64 antigen gene and genus specific 32kDa protein gene in a single step reaction, by which MTB can be identified and distinguished from other nontuberculous mycobacteria among endo-ovarian tissue biopsies and PAFs. This study supporting the use of endometrial tissue biopsy, ovarian tissue biopsy, endometrial aspirations, pelvic aspirated fluids and fluid samples from the pouch of Douglas (POD), therefore endorses the study by Bhanu *et al* on the importance of multiple sampling in aiding the diagnosis of FGTB [17,18]. At present, almost all genes of multi gene PCR were in the use for routine examinations of FGTB. However, this method may be limited in the diagnosis, because TRC4 element is present in all south Indian specific MTB complexes and it may not be suitable for other population. Further, a huge number of clinical isolates of *M. tuberculosis* from south India had either a single copy (40%) or no copy (4%) of IS6110 [39,40]. But, the use of the multi-gene PCR system would not have the pitfalls caused by the absence of TRC4 element and IS6110 element from the particular region and mycobacteria, since DNA fragments corresponding to the amplification of 19kDa antigen gene, MPB64 antigen gene and 32kDa protein gene would still be present. The advantage of discriminating *M. tuberculosis* from nontuberculous mycobacteria in a single tube-single step reaction would lie in the possibility of using 19kDa antigen gene and the TRC4 element, to identify the particular type of mycobacteria under the study. 32kDa protein gene showed higher sensitivity and specificity than any other gene due to the presence of infectious organisms representing *Mycobacterium* genus. Even 19kDa antigen gene and TRC4 element showed better sensitivity (86.63%) and specificity (100%) [43,53]. Thus, these genes could be emerging future diagnostic biomarkers in the detection of FGTB (Pearson  $\chi^2 = 206.011$ , 1df, Fisher's exact test (p value) = 0.000, McNemar's test value <0.0001) [38,39]. These results are similar to those at *Mycobacterium* genus and *M. tuberculosis* complex levels obtained in fine needle aspirates in 35 (87.5%) of the 40 patients with clinical and cytological diagnosis of tuberculous lymphadenitis [17,43] and others by using single step PCR [29,43]. MPB64 gene could be appropriate or useful in the detection of *M. tuberculosis* complex but our results revealed that the role of MPB64 gene is limited to the detection of *M. Bovis* among patients with FGTB. This may suggest mutations within the MPB64 gene, leading to the production of an incomplete protein as a result of deletion in the C-terminal region of the protein [57]. The results of this study inveterate that TRC4 element might be universally detected, especially among so called south Indian strain of *M. tuberculosis* [56]. Hence, the combination of 19kDa antigen gene with TRC4 element could be a better choice in the detection of FGTB using endo-ovarian tissue biopsy and pelvic aspirated fluids and the positive multi-gene PCR results can be given due importance (Table 6). Increasing the awareness and importance of incorporating multiple genes targeting different characteristics of infectious agents in a single tube-single step reaction using multi-gene PCR method is needed. Information of this investigation will be made available online, particularly in cases, where the data is not published. Culturing of decontaminated and concentrated (DC) tissue sediments on L-J egg media and

subsequent staining with Z-N stain have been showed significant increase in AFB positive cases than direct staining of DC tissue specimens. Thus, culturing of tissue specimen was recommended prior to report the samples as AFB negative.

## 7. Conclusion

Our results clearly demonstrated that multi-gene PCR showed a significant advantage over the conventional techniques, in that the minimal detection limits of bacilli without the use of radioisotopes, without the use of costly and complex equipments and more than that three target genes can be studied at a time (Pearson  $\chi^2 = 214.612$ , 1df, Fisher's exact test value <0.0001, McNemar's test value <0.0001) [52]. As, we find clear and accurate banding patterns by agarose gel electrophoresis, sequencing of the PCR product was not suggested. This multi-gene PCR method also reveals that mycobacterial DNA is consistently observed more in ETBs (49.5%) and OTBs (33.17%) and very low in PAFs (5.44%). Suggesting that, the presence of localized and latent tuberculosis infection such as FGTB can be detected by taking the site specific sampling, irrespective of sample types. This method can be utilized not only for bacteriological presence but also in the clinical findings of host in response to infectious agents. Exclusion criteria such as severe psychiatric dysfunction, multiple sclerosis or other autoimmune disorders, women with diabetes, malnutrition and other medical disorders like hypertension have been reported early to have a stimulated influence on the host immune response and can also increase the risk of developing tuberculosis [58,59]. This could mask the immune reaction produced by TB infections, may cause infertility and role of subclinical symptoms may not be conclusive. The results mentioned here demonstrate that *M. tuberculosis* was present in sufficient density among samples and can be detected surely by multi-gene PCR. It seems improbable that reporting of FGTB would result in a spurious over-representation of women with infertility. Multi-gene PCR was found to be a powerful technique for diagnosis and differentiation of mycobacterial infection. Since 32kDa protein is encoded by *Mycobacterium* genus specific gene, we suggest amplification of the 19kDa antigen gene in combination with TRC4 element as a successful multi-gene PCR method for the diagnosis of FGTB among infertile patients using both cultured and uncultured endo-ovarian tissue biopsies and pelvic aspirated fluids.

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