



## Detection of Female Genital Tuberculosis by using Endo-Ovarian Tissue Biopsy

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### ABSTRACT

Female genital tuberculosis is a symptomless disease accidentally revealed during inquiry for infertility. Inefficacy of costly tests and negligence have intended for underestimation of the disease. Therefore we have chosen to reevaluate the role of conventional and genotypic methods in the detection of female genital tuberculosis by using endometrial tissue biopsy, ovarian tissue biopsy and pelvic aspirated fluids taken as samples from infertile women during hysteroscopy or laparoscopy. It is a prospective case-control study. Premenstrual endometrial tissue biopsy, ovarian tissue biopsy and pelvic aspirated fluids were collected from 202 infertile women suspected of having genital tuberculosis on laparoscopic examination and from 100 normal women of reproductive age, suspected of having genital tuberculosis. All patients were subjected to laboratory examinations by the conventional/phenotypic methods to compare with multi gene/ multi-primer based PCR method using four set of primers for the detection of *Mycobacterium tuberculosis* in a single tube reaction. The conventional methods had 99% to 100% specificity with a low sensitivity, ranging from 21.78% to 42.08% while H & E staining had a sensitivity of 51.48%. In comparison, multi-gene PCR method was found to have a much higher sensitivity of 42.57% with MTB64 gene, 86.13% with TRC4 element, 99.01% with 19kDa antigen (131bp) and 100% with MPT59  $\alpha$ -antigen/32kDa protein (506bp) gene. The specificity of multi gene PCR was 100%. In conclusions, since 32kDa protein is encoded by *Mycobacterium* genus specific gene, we suggest 19kDa antigen in combination with TRC4 element could be a successful multi-gene/multi-primer PCR method in the diagnosis of FGTB.

### INTRODUCTION

Female genital tuberculosis (FGTB) is usually a symptom-less disease diagnosed during investigations for infertility (Namavar Jahromi et al., 2001). It is a form of extra pulmonary TB accounting for a large proportion of morbidity and mortality especially in developing countries, for about 27% (range, 14 to 41%) worldwide. The prevalence of

FGTB in women presenting with infertility varies from 1% in developed countries to 30% in developing countries (Singh et al., 2008). It is endemic in India, with a prevalence of 3%–39%. Therefore, WHO articulated its epidemiological situations and declared it as a global health problem (WHO, 2007). Detection of FGTB by conventional diagnostic methods is a major challenge. Exact diagnosis of FGTB with the collection, processing and storage of tissue biopsies

(Kapoor *et al.*, 2013) from endometrium, ovaries and aspirated fluids were found valuable procedures (William *et al.*, 2010) in the determination of existence or intensity of infections by performing certain tests including culturing and histopathological examinations. However, these methods have low detection rates and limitations due to the secondary nature of the genital tuberculosis, the infecting organisms are sparse in number and paucibacillary, the sampled site may not represent the infected area and the infected site can be easily missed, moreover, tissue reaction in those having tuberculosis may at times be atypical. Thus, it is only suggestive and not confirmatory unless acid fast bacilli (AFB) are demonstrated by Ziehl-Neelson's (Z-N) staining in the lesions. On other side, a range of PCR techniques have been mechanized for the detection of specific nucleic acid sequences of *M. tuberculosis* and other mycobacteria (CDCP, 1996). Due to various limitations of false negativity and danger of false positivity, there is no clear guidelines for application of gene amplification methods are available. Identification of genes encoding the virulence determinants, intensely available targets in genome and highly expressing factors may serve as new targets in the detection of FG TB, therefore this study was designed for the detection of 19 kDa antigen gene, TRC4 element, MPB64 antigen gene and 32-kDa protein (MTP-59  $\alpha$ -antigen) gene by using multi-gene/multi-primer based PCR method among endo-ovarian tissue biopsy and pelvic aspirated fluids taken from FG TB suspected patients.

## MATERIALS AND METHODS

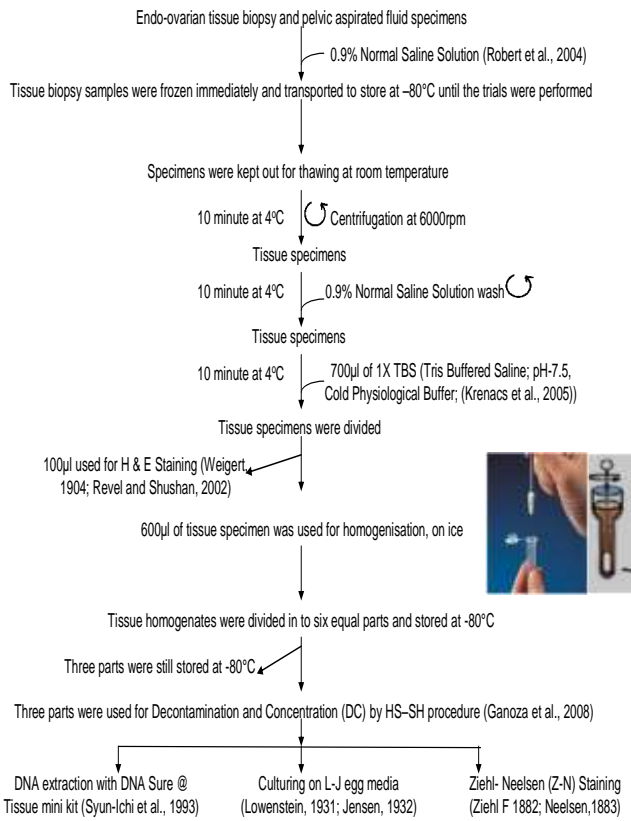
### Setting and Design of the Study

It is a prospective case-control study done in the Zoology Modular Lab, CFRD, Osmania University on 202 infertile women suspected of having genital tuberculosis on laparoscopic examination and from 100 normal women of reproductive age visiting the gynaecology clinics at two collaborating centres, Hyderabad which registers cases from all over Andhra Pradesh, India, complaining for infertility on clinical grounds. Single type of sample (either ovarian biopsy or endometrial biopsy) is collected from each patient. Only part of effected sites or washouts of pelvis were collected as a sample for this study from all case-control groups. Only, single type of sample is collected from each woman, therefore, 302 samples were collected. During the period of our study (2011-13), the samples from the consecutive women from these two centres were analysed. The ethical committee of

MHRT Hospital and Research Centre, Hyderabad, India approved the research protocol. There were no ethical issue related to this study.

Informed written consents were obtained from all the participants. Information on general characteristics for all patients was recorded in the medical chart. All patients met the **inclusion criteria**: 18-40 years of age having irregular periods with laparoscopic finding of beaded and blocked tubes, experiencing infertility (in >60% of cases), pelvic pain, scanty menstruation and amenorrhoea, histopathological evidences in the biopsy of premenstrual endometrial tissues, ovarian tissues or demonstration of tubercle bacilli in culture of menstrual blood or endometrial curetting.

**Exclusion criteria** were the following: Women above 40 years of age, symptoms suggestive of pulmonary TB/extra pulmonary TB except infertility, with normal abdominal and vaginal examinations, other chronic disease, pregnancy or nursing, severe psychiatric dysfunction, multiple sclerosis or other autoimmune disorders, pulmonary infections, HIV co-infection, women with diabetes, malnutrition and other medical disorders like hypertension. All subjects were HIV negative and normal 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 (Raut *et al.*, 2001). Surgically removed endometrial tissue biopsy, ovarian tissue biopsies and aspirated fluids were taken at the time of laparoscopy from both groups for laboratory examination, including the biopsy processing, (Robert *et al.*, 2004; Krenacs *et al.*, 2005; S.Chakravorty *et al.*, 2005) contamination and concentration (Figure 1), (Ganoza *et al.*, 2008) hematoxylin and eosin (H & E) staining, (Van Gieson, 1889; Weigert, 1904; Revel *et al.*, 2002) Z-N staining for acid fast bacilli (Sheehan D and Hrapchak B, 1980; NCCLS, 2001; Murray *et al.*, 2003) as well as culture on L-J egg media (Kent and Kubica, 1985) and *M. tuberculosis* specific multi-gene/multi-primer PCR using four set of primers for the detection of FG TB in a single tube reaction (Table 1 (Mustafa *et al.*, 1995; Sujatha *et al.*, 2001; Kidane *et al.*, 2002; Helen *et al.*, 2003; Barouni *et al.*, 2004; Bhanu *et al.*, 2005; Dil-Afroze *et al.*, 2008; Thangappah *et al.*, 2011)), by which tuberculosis was confirmed and correlated with each other among all methods. Details of Laparoscopic / Hysteroscopy findings of infertile patients with suspected female genital TB versus controls are given in Table 2. Beaded appearance of tubes, tubercles on uterus and pelvic mass in variable combination aroused a suspicion (Figure 2). Constitutional symptoms such as sweating, increase in temperature and weight loss were not major complaints while local

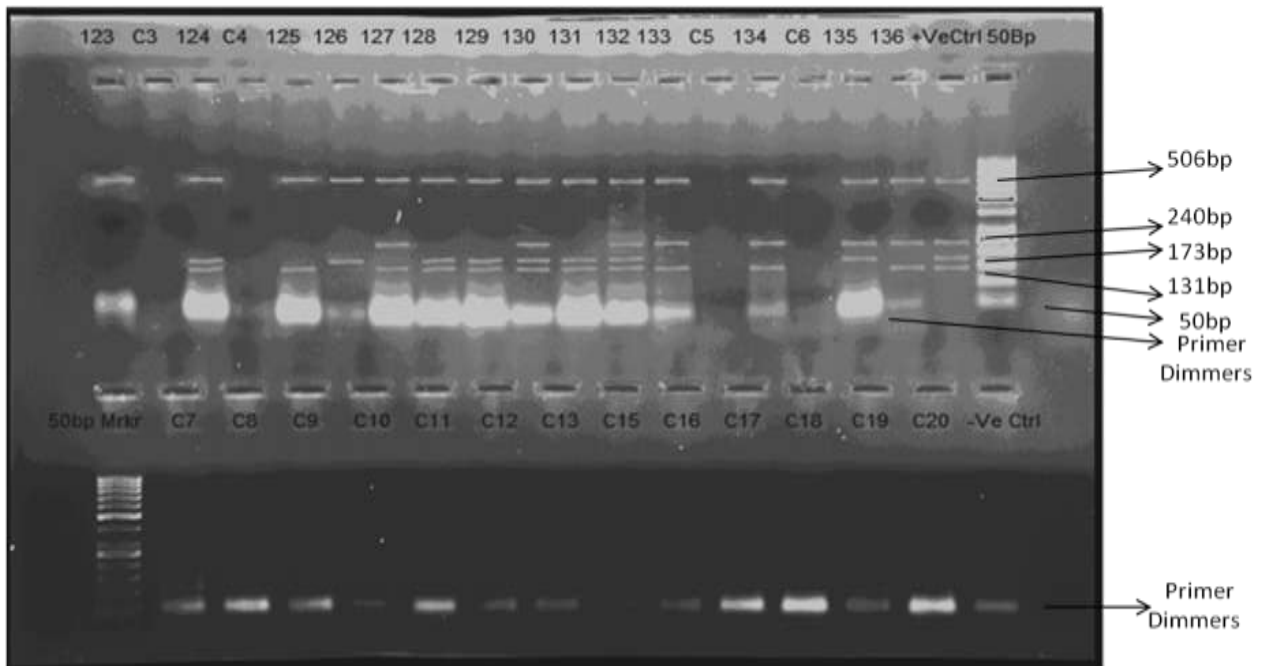


**Fig 1:** Tissue Biopsy Processing Protocol

organ dysfunction manifested in amenorrhea, omental adhesions and bilateral tubal blockage seen among case-controls on hysteroscopy. All cases were asymptomatic, resistant to all therapeutic treatments, medicals and surgically involved IVF. Tissue specimens were examined for granulomatous reactions and fibrosis cysts suggestive of *Mycobacterium* infection. Control groups (n=100) attended the same clinics with other gynaecological disorders and came for tubal sterilization and underwent laparoscopy for menorrhagia.



**Fig 2:** Bilateral tubal blockage and tubercular salpingitis



**Fig 3:** 2% Agarose Electrophoresis of Multi-primer PCR: Lane 123 -136 for FG TB patients; Lane C3-C20 for control group; Lane +Ve Ctrl for Reference Strain (NIMS, HYD), Lane -Ve Ctrl is Negative control (H<sub>2</sub>O), lane 50bp Marker is 50 base pair molecular weight ladder (Band towards bottom or lower side starts with 50bp pair and ends with 650bp at top or upper side), The band corresponding to 131bp is noted as 19kDa antigen gen, 173bp is noted as TRC4 element, 240bp is noted as MPB64 gene, 506bp is noted as 32kDa protein/MPT-59 alpha antigen gene. At the bottom Primer Dimmers were also seen.

### Extraction of DNA and Multi-gene/ multi-primer PCR

The extraction of DNA was carried out by using commercially available DNA Sure @ Tissue mini kit (Genetix, India) and reagents with few modifications in the procedures (Syun-Ichi *et al.*, 1993) according to manufacturer instructions. The purity of DNA was checked on 0.8% agarose gel electrophoresis and quantified. Two looped touchdown multi-gene/ multi-primer PCR method was designed for the amplification of *Mycobacterium* specific genes each loop with 25 cycles at two different annealing temperatures. Reaction conditions were optimized with respect to concentration of MgCl<sub>2</sub>, annealing temperatures, denaturation temperatures and number of cycles.

The amplified PCR products were electrophoresed on 2% agarose gel incorporated with ethidium bromide, along with Gene Rule 50bp DNA ladder/ molecular weight marker. The bands were photographed under a Bio-Rad gel documentation system as shown in **Figure 3**. The results of case-control group were compared with reference strain.

### Statistical analysis

The sensitivity, specificity, positive predictive values and negatives predictive values were calculated using standard formulae (Kusum *et al.*, 2013).

**Table 1:** Details of genes of *Mycobacterium tuberculosis* complex

S.No	Gene	Forward Primer	Reverse Primer	Length & Size (bp)	Reported Annealing Temp.	Function	Conclusion
1	19 kDa Antigen gene	5'TCTTCC GGATGTT CAAGCA 3'	5'TGACGTTC TGGTCCTTA CC 3'	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.
2	TRC4 Element	5'GACAAC GACGTGC GCCTACT 3'	5'GACCGAAT TAGCGTAGC TCC 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
3	MPB64 gene	5'TCCGCT GCCAGTC GTCTTCC 3'	5'GTCCTCGC GAGTCTAGG CCA 3'	20; 240	55,60	Highly immunogenic antigen and found in active cultures	This polymeric epitopes can be a good candidates for serodiagnosis
4	32kDa Protein gene	5'TTCCTG ACCAGCG AGCTGCC G 3'	5'CCCCAGTA CTCCCAGCT GTGC 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.

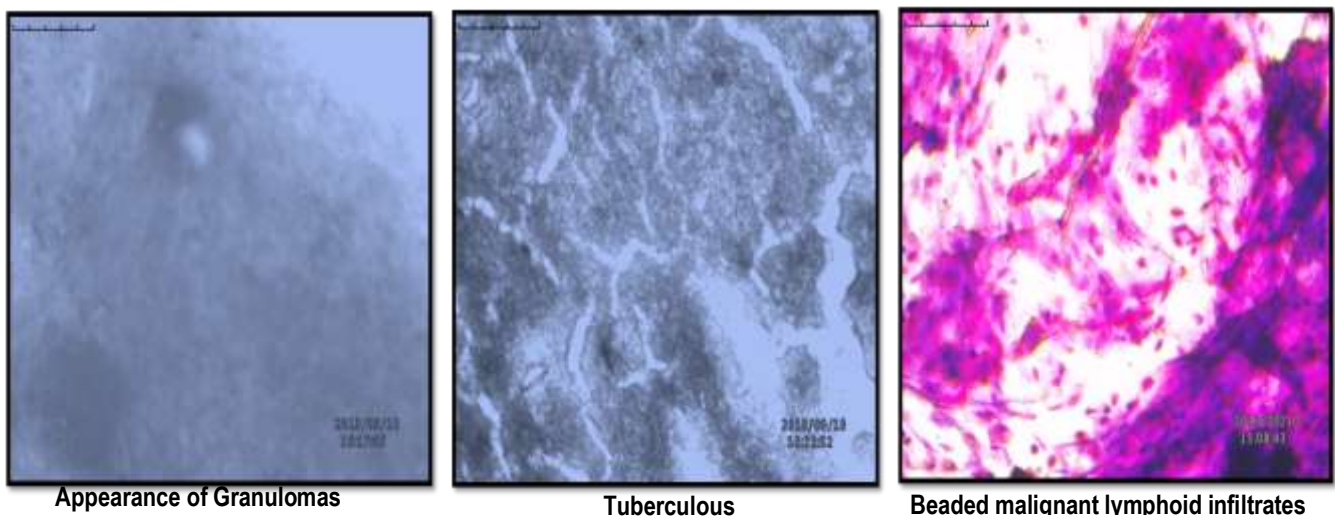
T: thymine; A: adenine; G: guanine; C: cytosine.

## RESULTS AND DISCUSSION

Symptoms are found mild and local, such as abdominal pain or menstrual irregularities, tubal blockage, beaded tubes, tubercular salpingitis, tubercles in tube and infertility are the most common consequences, (Namavar Jahromi *et al.*, 2001) clinical signs of the FGTB patients (case group) versus control groups were given in the **Table 2**.

Once fibrosis is established, fertility is generally difficult to restore even with appropriate treatment (Lamba *et al.*, 2002).

Disparity among the results for detection of AFB positive, histopathological evidence of tuberculosis infection, isolation by culture, and detection of *M. tuberculosis* complex by multi-gene/multi-primer PCR by using endometrial tissue biopsies, ovarian tissue biopsies and aspirated fluids are tabulated in **Table 3**.



**Figure 4:** H&E stained endo-ovarian tissues biopsies (Bright field Inverted Microscopy, 40X)



ZN stained tissues

Culture growth on LJ egg Media

ZN stained culture

**Figure 5:** Z-N stained endo-ovarian tissues sediments and cultures (Bright field Inverted Microscopy, 40X)

**Table 2:** Laparoscopic / Hysteroscopy findings of infertile patients with suspected of Female Genital TB versus Controls

Characteristics	Infertile patients with suspected of Female Genital TB (n=202)	Control group (n=100)
Tubal block with Hydrosalpinx [n (%)]	119 (58.91)	NA
Tubo-ovarian mass [n (%)]	59 (29.21)	NA
Tubercular salpingitis [n (%)]	97 (48.01)	NA
Beaded Tubes [n (%)]	139 (68.81)	NA
Bilateral Opening of tubes [n (%)]	50 (24.75)	NA
Thicken tubes & Tubal Adhesions [n (%)]	34 (16.83)	4 (4)
Tubercles on Uterus [n (%)]	19 (9.4)	NA
Omental Adhesions [n (%)]	78 (38.61)	NA
Frozen pelvis [n (%)]	55 (27.23)	NA
Multiple Tubercles [n (%)]	72 (35.64)	NA
Small Ovaries and lower abdominal mass [n (%)]	37 (18.31)	NA

**Note:** Some patients had more than one abnormal finding, Data are presented as percentages, NA: not applicable

66.88% (n=202) of FG TB patients were confirmed to have *M. tuberculosis* infection by either AFB smear microscopy, or positive culture, or histopathology, or multi-gene/multi-primer PCR or a combination of these. 33.11% (n=100) of women with normal endometrium, ovaries and pelvic fluids. Chronic inflammation or lesions such as proliferative solid epithelioid granulomas, (Revel *et al.*, 2002) giant cells, tuberculous salpingitis, dense polymorphonuclear and lymphocytic infiltrations were observed in 104 (34.43%) infertile women with FG TB and 1 (0.33%) control woman as observed by H & E staining (**Figure 4**). Out of the 302 tissue sediments collected, AFB was positive in 44 (14.57%) infertile women with beaded tubes, tubercular salpingitis and with menstrual dysfunctions. *Mycobacterium* growth was observed in 85 (28.14%) specimens collected from FG TB patients and 1 (0.33%) sample was positive in control woman when cultured on L-J egg medium.

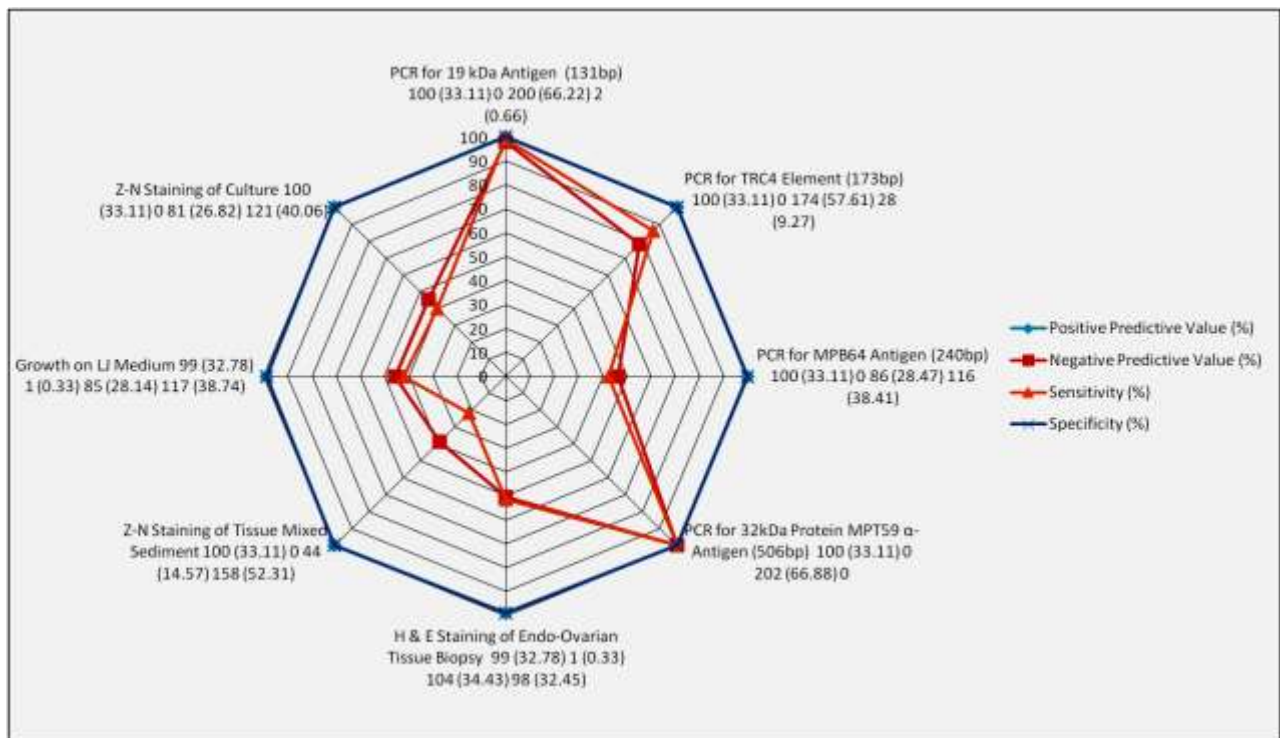
Out of 85 cultures positive cases, 81 (26.82%) FG TB patients are found AFB positive by Z-N staining when compared with reference strains given by Dept of Microbiology, NIMS, Hyderabad as shown in **Figure 5**. Two hundred (66.22%) specimens were positive for 19 kDa antigen (131bp) gene, 174 (57.61%) were positive for TRC4 (173bp) repetitive

element, 86 (28.47%) were positive for MPB64 antigen (240bp) gene and 202 (66.88%) of endo-ovarian tissue biopsies and aspirated fluid specimens were positive for 32 kDa protein/MPT59  $\alpha$ -antigen (506bp) gene by multi-gene/ multi-primer PCR.

The Z-N smear of endo-ovarian tissue biopsy sediments had a sensitivity of 21.78% and a specificity of 100%, resultant positive predictive value of 100% and a negative predictive value of 38.75%. Whereas sensitivity was doubled up to 40.1% with culture positive samples and a specificity of 100%, resulting in a positive predictive value of 100% and a negative predictive value of 45.24%. L-J egg media culture had a sensitivity of 42.08% and a specificity of 99%, ensuing in a positive predictive value of 98.83 % and a negative predictive value of 45.83%. H & E staining of endometrial tissue biopsy, ovarian tissue biopsy and aspirated specimen (i.e., histopathological studies) demonstrates a sensitivity of 51.48% and a specificity of 99% positive predictive value of 99.04 % and a negative predictive value of 50.25%. Multi-gene/ multi-primer PCR method was found to have a much higher sensitivity of 99.01% with 19 kDa antigen (131bp) gene and a specificity of 100%, resulting in a positive predictive value of 100% and a negative predictive value of 98.03%. The results clarifies that TRC4 (173bp) element has a sensitivity of 86.13% and a specificity of 100%, with a positive predictive value of 100% and negative predictive value of 78.12%. The MPB64 antigen (240bp) gene has a sensitivity of 42.57% and a specificity of 100%, subsequent in a positive predictive value of 100% and a negative predictive value of 46.29%. Even MPT-59  $\alpha$ -antigen/32kDa protein (506bp) gene has higher sensitivity of 100% and a specificity of 100%, with a positive predictive value of 100% and a negative predictive value of 100% which might be due to *Mycobacterium* genus specific gene. 19 kDa antigen (131bp) gene and TRC4 (173bp) element by multigene PCR method, H & E staining of endometrial tissue biopsy, culture and Z-N staining for AFB positive specimen were found to be practically important in the detection of FG TB, though, there is much difference in the sensitivity and specificity of these methods. Out of four different methods, histopathology studies and culture on LJ egg

media showed false positive in 1 (0.33%) tissue sediment collected out of 100 normal women. It is unlikely that the false positive samples of control women were entirely negative by multigene PCR. On other side, multigene/multi primer PCR method for 19 kDa antigen (131bp) gene, TRC4 (173bp) element and MPB64 antigen (240bp) gene within single tube reaction gave false negative in 2 (0.66%), 28 (9.27%) and 116 (38.41%) tissue specimens respectively and nil for MPT59  $\alpha$ -antigen/32kDa protein (506bp) gene.

98 (32.45%) false negative by H & E staining, 158 (52.31%) false AFB negative by Z-N staining of tissue specimens, 117 (38.74%) false negative by culture on LJ egg medium and 121 (40.06%) false AFB negative by Z-N staining of cultures might be due to sparse number of paucibacillary bacterium. In the non TB control group, all the tests were negative. These results showed that molecular method is more promising than conventional methods (Kusum *et al.*, 2013).



**Fig. 6:** Comparison and Validation of four characteristics among conventional versus molecular methods by using Radar Plots

**Table 3:** Findings of Conventional methods versus Molecular methods among infertile patients with suspected of Female Genital TB and Controls

Characteristics	Conventional Method				Molecular Method			
	H & E Staining of Endometrial Biopsy [n (%)]	Z-N Staining of Tissue Sediment [n (%)]	Growth on LJ Medium [n (%)]	Z-N Staining of Culture [n (%)]	19 kDa Antigen (131bp) [n(%)]	TRC4 gene (173bp) [n (%)]	MPB64 Antigen (240bp) [n (%)]	32kDa protein (506bp) [n (%)]
<b>FGTB Cases (n=202)</b>	104 (51.48)	44 (21.78)	85 (42.07)	81 (40.09)	200 (99.01)	174 (86.13)	86 (42.57)	202 (100)
<b>Control group (n=100)</b>	1(1)	NA	1(1)	NA	NA	NA	NA	NA

**Note:** Some patients had more than one abnormal finding; Data are presented as percentages, NA: not applicable

Female genital tuberculosis is an important cause of infertility, rarely diagnosed in developed countries. It is an elusive diagnosis and a high index of suspicion is very essential in the routine diagnostic process. PCR using one target gene alone cannot detect all strains of *M. tuberculosis* however the use of multiple targets can improve detection of pathogen. Previous studies have suggested that the sensitivity of PCR can be increased by using different sets of targets to detect tuberculous meningitis and tubercular pleuritis (Parandaman *et al.*, 2000; Sujatha *et al.*, 2001; Kusum *et al.*, 2013). The increase in PCR sensitivity and decrease in false negative results were also reported using dual targets for *M. tuberculosis* detection (Barani *et al.*, 2012). 32kDa protein/MPT-59 alpha antigen is encoded by *Mycobacterium* genus specific gene, so it revealed wide sensitivity and specificity (100%). Hence, all infections due to more than one mycobacterial species and nontuberculous mycobacterium may be identified; as a result treatment and disease management might become a difficult task. Percentages or values are often plotted as a particular shape on a radar graph that emerges from the multivariate outputs of conventional versus molecular methods on which several measured parameters (Joan Saary, 2008) were explained (Figure 6). It is evident that the conventional methods represents 99% to 100% specificity with almost similar positive predictive values and 100% specificity with multi-gene/ multi-primer PCR method were found to satisfy the ideal condition.

In addition, multi-gene/ multi-primer PCR method have much higher sensitivity of 86.13% with TRC4 element, 99.01% with 19kDa antigen (131bp) and 100% with MPT59  $\alpha$ -antigen/32kDa protein (506bp) gene with different negative predictive values of 78.12%, 98.03% and 100% respectively compared with conventional methods where there is greater dispersion. Conventional methods had very low sensitivity ranging from 21.78% to 42.08% even H & E staining had a sensitivity of 51.48% compared to molecular methods where there is poorer dispersion. 19kDa antigen showed better sensitivity of 99.01%, specificity of 100% and found to be a future diagnostic marker in the identification of FG TB. MPB64 gene has been reported promising in the detection of *M. tuberculosis* complex, but due to mutations within the MPB64 gene leading to the production of an incomplete protein as a result of a deletion of the C-terminal region of the protein (Kazue Hirano *et al.*, 2004). The results of this study inveterate that TRC4 element is universally detected, especially among so called south Indian strain of *M. tuberculosis*. Therefore, our study suggested the combination of

19kDa antigen gene with TRC4 element could be a better choice in the detection of Female Genital Tuberculosis using endo-ovarian tissue biopsy and aspirated fluids as a sample and the positive multi-gene/ multi-primer PCR results should be given due importance. The main advantage of the multigene amplification is the minimal detection limits without the use of radioisotopes. In our set up, multi gene PCR was standardized to detect even single bacilli thus increased the sensitivity of detection (Madhavan *et al.*, 2000). Therefore, there is an urgent need to increase the awareness and importance of incorporating multiple genes, targeting different characteristics of infectious agents using multigene/ multiprimer PCR method in the detection of *M. tuberculosis*. The PCR method should be utilized not only from bacteriological but also in clinical points of view as a rational approach towards a definite diagnosis of FG TB. Technical considerations, including the use of suitable controls and the retesting of doubtful positive samples considerably influence the sensitivity and specificity. But a well standardized PCR method in combination with epidemiological and clinical factors, could offers the benefit of rapid and reliable results within a short time. Thereby removes a significant amount of clinical diagnostic uncertainty.

## CONCLUSION

Our results concluded that *M. tuberculosis* is present in enough density to be detected in samples by multi-gene/ multi-primer PCR and also highlights the simulated characteristics of the clinical distinctions between infection and disease. It seems improbable that the detection and reporting of FG TB would results in a spurious over-representation of women with infertility. It is more likely that biological or immunological factors along with genetic susceptibility either in the host or pathogen may be responsible for this consistent association. Since 32kDa protein is encoded by *Mycobacterium* genus specific gene, we recommend 19kDa antigen gene in combination with TRC4 element could be a successful multi-gene/multi-primer PCR based method in the diagnosis of FG TB among infertile patients using endo-ovarian tissue biopsy and aspirated fluids as a sample.

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