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Rapid Diagnosis of Female Genital Tuberculosis by MPB64 Polymerase Chain Reaction

KEYWORDS: Acid Fast Bacilli (AFB), Female Genital TB (FGTB), Lowenstein-Jensen (LJ), Middle brook-7H9 (MB7H9), MPB64 , Mycobacterium tuberculosis (MTB), Polymerase Chain Reaction (PCR)

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ABSTRACT *Background: Female Genital Tuberculosis (FGTB) is the main cause of infertility in about 5–16% of cases among women in India. However the exact incidence of FGTB remains unknown, as the majority of the cases remain undiagnosed due to asymptomatic presentation and paucity of investigations. Though various options are available for confirming the diagnosis of tuberculosis (TB), conventional techniques lack the sensitivity and are often time consuming. Polymerase Chain Reaction (PCR) based molecular methods are rapid and sensitive in diagnosing TB infection.*

Aims and Objectives: We aimed to use MPB64 PCR for detection of female genital TB and to compare the results with conventional methods.

Materials and Methods: 50 endometrium biopsy samples from suspected cases of FGTB from June 2012 to August 2012 were processed by modified petroff's method. The deposit was used for detection of mycobacteria by Acid Fast Bacilli (AFB) smear, AFB culture on solid and liquid media and MPB64 based PCR. Results of PCR were compared with those of conventional methods.

Results: Out of 50 samples received, a total of 36% (18/50) were positive by either of the methods used. Overall positivity by AFB smear microscopy was 2% (1/50) and 10% (5/50) by Middle brook-7H9 (MB7H9) liquid culture, 8% (4/50) by Lowenstein-Jensen (LJ) solid culture and 24% (12/50) by MPB64 based PCR.

Conclusion: PCR is very rapid and sensitive technique for the diagnosis of genital tuberculosis. Due to the short turnaround time of PCR, it has utility in providing the results in clinically relevant time and thus helps to initiate the treatment timely.

Introduction

Female genital tuberculosis poses serious concern throughout the world especially in developing countries. Female genital TB (FGTB) is a form of extra-pulmonary tuberculosis (EPTB) which affects the female genital organs, with fallopian tubes being the most commonly affected (90%), followed by the endometrium (50%) and the ovaries (10–30%)[1]. FGTB is the main cause in about 5–16% of cases of infertility among women in India. However the exact incidence of FGTB remains unknown, as the majority of the cases remain undiagnosed due to asymptomatic presentation and paucity of investigations [2].

Genital tuberculosis (GTB) has been a diagnostic dilemma for clinicians for decades due to the latency of the organism, asymptomatic and varied presentation in majority of the cases and paucity of an accurate diagnostic modality but with the advent of newer diagnostic modalities, there have been an increase in the number of cases of genital tuberculosis that are being recognized.

Early diagnosis of tuberculosis (TB) is still based on the demonstration of acid-fast bacilli (AFB) by microscopy and/or their isolation by culture. Microscopy has poor sensitivity (requires 10^4 bacilli/ml) for the diagnosis of TB[3]. Culture methods are still the gold standard in the detection of genital TB but slow growth of most pathogenic mycobacteria (3 to 6 weeks) results in unacceptable delay in diagnosis and treatment. However traditional AFB culture on Lowenstein-Jensen (LJ) medium has a low detection rate. Colonies are seen if the bacillary count is >1000 bacilli and it also takes a longer time to give positive results[4,5].

Polymerase Chain Reaction (PCR) is reported to be the most sensitive and rapid method for the detection of tuberculosis that can rapidly detect even few copies of DNA (less than 10 bacteria/ ml specimen) with high sensitivity and specificity and results are available within 1-2 days[6]. Its sensitivity is so high that it can detect even <10 microorganisms in clinical specimens to achieve a positive report and this is an important feature since genital TB is paucibacillary.

IS6110 has been most commonly used for detection of *Mycobacterium tuberculosis* (MTB) but has certain limitations as it is reported that MTB isolates from certain parts of India may have no copy or very low copy numbers of *IS6110* element and this may lead to false negative results[7]. Other targets like *MPB64* have also been used with varying results[8].

Therefore in the present study, we planned to use *MPB64* PCR for detection of genital TB. We did comparative evaluation of AFB smear microscopy, culture on Lowenstein-

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Jensen (LJ), Middle brook 7H9 liquid media (MB7H9) and *MPB64* based PCR to detect genital TB in infertility patients at our centre.

Materials and Methods
Study design: Hospital based observational study

Samples: A total of 50 endometrium biopsy samples were collected from suspected cases of FGTB from June 2012 to August 2012. The samples were processed in the Department of Microbiology, SMS Medical College, Jaipur for detection of mycobacteria by AFB smear, culture on LJ, Middle brook 7H9 liquid media and conventional PCR using primers based on *MPB64* gene.

Sample Processing: Tissue samples obtained in sterile saline were centrifuged at 3000g for 15 min. Supernatant was decanted and deposit was crushed with glass beads by means of electric tissue homogenizer for 1 minute, the lysate was subjected to digestion and decontamination by 4% NaOH-NALC method (Modified Petroff's Method)[9].

AFB smear
Smears were prepared from the above deposits and stained by Ziehl Neelson (ZN) staining for AFB smear microscopy.

AFB culture
For AFB culture on solid media, LJ medium slants were inoculated with 5mm loopful of the digested *and* decontaminated deposit and incubated at 35-37°C. Cultures were examined 48-72 hours after inoculation to detect gross contaminants. Thereafter cultures were examined weekly for growth up to 8 weeks on a specified day of the week.

For culture on liquid media, Middle Brooke 7H9 broth medium, 100 µl of the deposit was inoculated in 1ml of Middle Brook 7H9 broth in which 100 µL of ADCC was added beforehand. Cultures were incubated at 35-37°C and examined weekly up to 6 weeks by ZN staining.

The growth obtained on LJ slants *and* MB 7H9 broth was identified primarily by colony appearance and was biochemically confirmed by niacin, nitrate test *and* susceptibility to *p*-nitrobenzoic acid.

Molecular identification
DNA extraction
DNA was extracted from the processed endometrial biopsy tissue samples by using QIAamp DNA Mini Kit (Qiagen Gmg H, Hilden, Germany).

***MPB64* PCR amplification and detection**
For DNA amplification, master mix was prepared in a total reaction volume of 25 µl containing 2µl of dNTPs (10mM), 0.25 µl Taq DNA polymerase (1.25 U), 2.5µl PCR buffer (10X), 2µL primers specific for *MPB64* gene (10picomol/µl) and 11.25 µl of nuclease free water. 5µl of the extracted DNA was added to it and placed in thermal cycler. The cycling parameters included initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94 °C for 2 min, annealing at 60 °C for 2 min and extension at 72 °C for 2 min, with final extension at 72°C for 5 min. The primers sequences for *MPB64* amplification were the same as used earlier by Bhanu *et al*.[8] Detection was done by gel electrophoresis using 2% agarose gel and visualization of the specific gene fragment 240bp specific for MPB 64 under UV transilluminator.

Results
The present study was conducted on suspected cases of female genital tuberculosis, whose samples were received for routine diagnosis in the Advanced Research Laboratory (ADRL), Department of Microbiology *and* Immunology, SMS Medical College, Jaipur. The study subjects included females (n=50) having genital tuberculosis and presenting with complaints like primary infertility, secondary infertility, pain abdomen, abortions, ectopic pregnancies, and menstrual irregularities etc , from age group 18-45 yrs. The age distribution of these FGTB suspects is shown in Table1.

Table 1 : Age wise distribution of FTGB cases

Age	Percentage
18-25 Yrs	33.67
26-30 Yrs	41.33
31-35 Yrs	14.67
36-40 Yrs	7.00
>40 Yrs	3.33
Total	100.00

"Age wise distribution of FTGB cases" here
Out of 50 samples received, a total of 36% (18/50) were positive by either of the methods used. Overall positivity by AFB smear microscopy was 2% (1/50) and 10% (5/50) by MB7H9 liquid culture, 8% (4/50) by LJ solid culture and 24% (12/50) by *MPB64* based PCR. All the smear-positive samples were positive either by culture or PCR. 8/50 (16%) samples were positive by PCR alone and negative by all other techniques used.

All the smear-positive samples were positive either by culture or PCR. 1/5 (20%) isolate was identified as nontubercular mycobacteria (NTM) by biochemical tests. 4/5 (80%) were confirmed as *Mycobacterium tuberculosis* by MB7H9 broth liquid culture and 3/4(75%) by LJ solid media. 3/50 (6.00%) samples got contaminated in MB7H9 broth liquid culture and out of these 3 samples, 1/3 (33.33%) were positive by PCR. None of the samples were contaminated on LJ culture.

Discussion
In the present study, we compared the conventional diagnostic modalities: smear microscopy, culture on solid and liquid media with PCR targeting *MPB64* gene for diagnosis of MTB infection. Majority of these suspected cases were in the 18-30 years age group in our study and this shows that genital tuberculosis affects women in relatively young age group. This is in agreement with the earlier studies [8,10,11].

Smear microscopy is a simple and easy to perform and interpret thus making it the more chosen technique but it has a poor sensitivity. In our study, 2% (1/50) cases were reported positive by AFB smear microscopy. In our study, LJ culture positivity was 8% (4/50). All but one isolates were identified as MTB by biochemical tests. The low ZN smear and culture positivity in endometrial tissue is due to the paucibacillary nature of the endometrial sample[8]. LJ culture plays an important role in distinguishing tubercular and non tubercular mycobacteria[12].

In our study, liquid culture was positive in 10% (5/50) cases as compared to 8% (4/50) by culture on solid media. Bhanu *et al*[8] reported a low positivity of 3.2% as com-

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pared to our study while the positivity rate in the other studies varied from 3.3 to 10.6% [13-15]. The use of liquid culture also reduces the delay in obtaining results.

In our study, the positivity PCR was higher than other conventional methods. Out of 50 samples received, 24% (12/50) were positive by *MPB64* PCR. Some of the earlier studies have also used *MPB64* as gene target for PCR amplification. These authors have reported a positivity rate of 23.30%[17], 26.50%[13], 42.57%[18], 56%[8]. The use of *MPB64* PCR may be better than IS6110 PCR which has limitation of low or zero copy number in some isolates[19-20].

In our study, PCR was found to be most sensitive method for the detection of mycobacteria among all the three methods used. PCR was found to be around 12 fold more sensitive than the AFB smear, 2.5 fold more sensitive than the liquid culture and 3 fold more sensitive than the solid culture. Bhanu *et al*[8] had reported 14 fold more sensitivity of the PCR in comparison to the smear examination. Higher sensitivity of PCR reported by Bhanu *et al*[8] as compared to our study may be attributed to the lower smear positivity in their study.

Conclusion

PCR is very rapid and sensitive technique for the diagnosis of genital tuberculosis. Due to the short turn-around time of PCR, it has utility in providing the results in clinically relevant time and thus helps to initiate the treatment timely.

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