Volume : 5 | Issue : 5 | May 2016 • ISSN No 2277 - 8179 | IF : 3.508 | IC Value : 69.48

Research Paper

Use of ESAT-6 Polymerase Chain Reaction for rapid diagnosis of female genital tuberculosis

Medical Science

KEYWORDS : Acid Fast Bacilli (AFB), ESAT-6, Female Genital TB (FGTB), Lowenstein-Jensen (LJ), Middle brook-7H9 (MB7H9), Mycobacterium tuberculosis (MTB), Polymerase Chain Reaction (PCR)

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ABSTRACT

Background: Female Genital Tuberculosis (FGTB) has been a diagnostic dilemma for clinicians due to the latency of the organism, asymptomatic and varied presentation in majority of the cases and paucity of an accurate diagnostic modality. Culture methods are still the gold standard in the detection of genital tuberculosis (TB) but slow growth of mycobacteria results in unacceptable delay in diagnosis and treatment. Moreover, acid fast bacilli (AFB) culture on Lowenstein-Jensen (LJ) medium has a low detection rate. Polymerase Chain Reaction (PCR) based molecular methods are rapid and sensitive in diagnosing TB infection.

Aims and Objectives: In the present study, we aimed to use ESAT-6 based PCR for detection of female genital TB and to compare the results with conventional methods.

Materials and Methods: Endometrium biopsy samples from 50 suspected cases of FGTB from June 2012 to August 2012 were processed by modified petroff's method and the deposit was used for detection of mycobacteria by AFB smear, AFB culture on both solid LJ and liquid Middle brook-7H9 (MB7H9) media and ESAT-6 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 MB7H9 liquid culture, 8% (4/50) by LJ solid culture and 20% (10/50) by ESAT-6 based PCR.

Conclusion: PCR is very rapid and sensitive technique for the diagnosis of genital tuberculosis and has utility in providing the results in clinically relevant time and thereby helping to initiate the treatment timely.

Introduction

Tuberculosis (TB) is an infectious disease that is a cause of considerable morbidity and mortality in India, affecting 14 million people, predominantly in the reproductive age group (15–45 years)1. Genital tuberculosis (GTB) is a type of extra pulmonary tuberculosis which affects the genital organs and its incidence is high amongst the patients with infertility. It is an important cause of infertility, being an etiological factor in 1-8% of the infertile cases as reported earlier 2-4.

Female Genital Tuberculosis (FGTB) is a symptom-less, rare disease with non-specific, mild clinical pictures and has been a diagnostic dilemma for clinicians. Culture methods are still the gold standard in the detection of genital TB but slow growth of mycobacteria results in unacceptable delay in diagnosis and treatment. *M. tuberculosis* may take up to 8 weeks to grow in Lowenstein-Jensen (LJ) medium1. Moreover, Acid Fast Bacilli (AFB) culture on LJ medium has a low detection rate.

Polymerase Chain Reaction (PCR) is reported to be the most sensitive and rapid method for the detection of genital tuberculosis that can rapidly detect even few copies of DNA (less than 10 bacteria/ ml specimen) with high sensitivity and specificity and results being available within 1-2 days5. 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 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 results6. Other targets like ESAT- 6 have also been used with varying results7.

Therefore in the present study, we planned to use *ESAT-6* PCR for detection of genital TB. We also comparatively evaluated AFB smear microscopy, culture on LJ, and *ESAT-6* based PCR to detect genital TB in infertility patients at our centre.

Materials and Methods

Study design: Hospital based observational study

Samples: 50 endometrium biopsy samples were collected from suspected cases of female genital tuberculosis from June 2012 to August 2012. The study were conducted at the Department of Microbiology, SMS Medical College, Jaipur for detection of mycobacteria by AFB smear, culture on LJ and conventional PCR using primers based on *ESAT-6* 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 5 minutes, the lysate was subjected to digestion and decontamination by 4% NaOH-NALC method (Modified Petroff's Method)8.

AFB smear

Smears were prepared from the above deposits and stained by

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routine 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 Brook 7H9 (MB7H9) broth medium, 100 µl of the deposit was inoculated in 1ml of MB7H9 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 MB7H9 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).

***ESAT-6* 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 *ESAT-6* 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 parameter included initial denaturation at 95°C for 2 min, followed by denaturation at 94°C for 15sec, annealing at 60°C for 30 sec, extension at 72°C for 1min with 25 cycles and a final extension at 72°C for 7 min. The primers sequences for *ESAT-6* amplification were the same as used earlier by Rozati *et al.*[7].

Detection was done by gel electrophoresis using 2% agarose gel and visualization of the specific gene fragment 300bp specific for MPB 64 under UV transilluminator.

Results

The present study was conducted on suspected cases of FGTB, 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 Figure1.

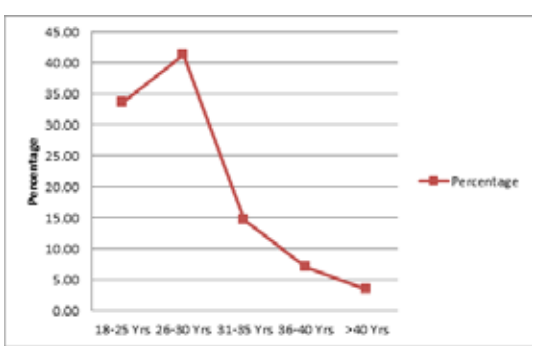

Figure 1: Age distribution of study subjects

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 20% (10/50) by *ESAT-6* based PCR. Molecular confirmation of *Mycobacterium tuberculosis* infection by *ESAT-6* based PCR was based on the presence of a 300 bp band corresponding to the *ESAT-6* gene amplification after electrophoresis (Figure 2).

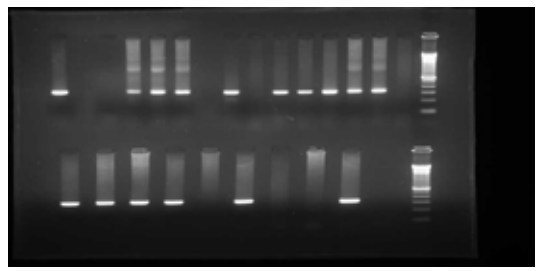

Figure 2: PCR based detection of *M. tuberculosis* targeting *ESAT-6*

All the smear-positive samples were positive either by culture or PCR. 1/5 (20%) isolates were 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

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. This poses diagnostic challenges mainly because the primary symptoms of the disease are usually non-characteristic[9].

In the present study, we compared the conventional diagnostic modalities: smear microscopy, culture on solid and liquid media with PCR targeting the ESAT6 gene, for diagnosis of MTB infection. The study was carried out at Mycobacteriology and Advanced Research Laboratory in Department of Microbiology at SMS Medical College, Jaipur on 50 suspected cases of FGTB whose specimen were received for routine diagnosis.

Although the smear microscopy has a very poor sensitivity, it remains a specific and economical mode of diagnosis and the results are available immediately. In our study, 2.0% (1/50) cases were tested positive by AFB smear microscopy. This low positivity rate observed in our study might be due to the fact that the positivity rate in AFB smear is low in extrapulmonary disease as the disease being paucibacillary is difficult to visualize in smear microscopy. Earlier studies have also reported similar findings1,7,10-15. Therefore, AFB smear alone is not an adequate criteria and must be followed by AFB culture to determine the species of mycobacteria16.

LJ culture is still considered to be the gold standard, but 10-100 viable bacilli are required for culture to be positive. Moreover, long period of time (of about 6-8 week) is required for reports to be available17. In our study, culture was positive in 8% (4/50) cases. All except one cultures isolates were identified as MTB by biochemical tests. Previous studies have also reported similar findings with regard to the low sensitivity of culture in the diagnosis of female genital tuberculosis1,7,10,12,15.

In our study, liquid culture was positive in 10% (5/50) cases as compared to 8% (4/50) by culture on solid media. Our results are in agreement to the study done by Malhotra *et al*11 in which

a positivity rate of 8.28% in liquid culture was reported. Bhanu *et al*1 reported a low positivity of 3.2% as compared to our study while the positivity rate in the other studies varied from 3.3% to 10.6%19-21.

In our study, we found a positivity rate of 20% (10/50) by *ESAT-6* based PCR. This was comparatively lower than reported in an earlier study by Rozati *et al.*7. These authors used *ESAT-6* PCR and reported a positivity rate of 43.1% by *ESAT-6* PCR amplification and found PCR as a method of choice for identifying the infection in FGTB samples.

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 10 fold more sensitive than the AFB smear, 2 fold more sensitive than the liquid culture and 2.5 fold more sensitive than the solid culture. Malhotra *et al*11 found PCR to be 9 fold sensitive than AFB smear and 3 fold more sensitive than liquid culture.

Conclusion

Based on the results of our study, we conclude that PCR tests for DNA specific to *M. tuberculosis* may be of hope for a rapid and accurate diagnostic test for FGTB and it helps where conventional diagnosis fails and provisional diagnosis of tuberculosis is made on the basis of clinical presentation and histology/cytology examination without evidence of AFB. PCR based methods may have a great potential for rapid diagnosis, treatment and prevention of FGTB. 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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