Correlation of different diagnostic techniques and laparoscopy with PCR in diagnosis of female genital tuberculosis
Chander Prakash Baveja1, Chinmoy Sahu4*, Usha Manaktala2, Vinay Kamal3, Vidya Nidhi G1, Himanshu Jha1
Departments of Microbiology1, Obstetrics and Gynecology2, Pathology3; Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi, Delhi, Pin code- 110002, India.
Department of Microbiology4, SGPGIMS, Raebareli Road, Lucknow- 226014, UP, India. *Email : csahu78@rediffmail.com

ABSTRACT

Aim: This study was done to evaluate polymerase chain reaction( PCR) and BACTEC culture in diagnosis of female genital tuberculosis. The findings of these diagnostic modalities were compared with that of direct microscopy and culture on Lowenstein-Jensen( LJ) medium and also with that of laparoscopy. Methods: Thirty clinically suspected cases of female genital tuberculosis were included in this study. Laparoscopy was done in each of the patient. Endometrial biopsy ( EB) was collected in each patient and sent to Microbiology and Pathology laboratory. Direct microscopy, culture on LJ medium and BACTEC were done for tuberculosis. PCR was done using two primers MPB64 and HupB for Mycobacterium tuberculosis. Histopathology was done in each sample to look out for granuloma and other features of chronic inflammation.

Results: 7 (23.3%) samples were positive by PCR using MPB64 and Hup B primers. Positivity in other diagnostic modalities were 1 (3.3%), 2 (6.7%) and 4 (13.3%) for direct microscopy, culture on LJ medium and BACTEC medium respectively. None of the samples were positive on either LJ medium or BACTEC culture and negative by PCR. 3 out of 3 cases (100%) showing suggestive findings of tuberculosis in laparoscopy were positive by PCR. 5 out of 5 cases (100%) showing granuloma in histopathology were positive by PCR. Conclusions: Polymerase Chain Reaction (PCR) showed higher sensitivity and specificity than conventional techniques and showed good correlation with laparoscopic findings in diagnosis of female genital tuberculosis.

Key words: female genital tuberculosis, culture, laparoscopy, PCR.

1. INTRODUCTION

Tuberculosis (TB), one of the oldest diseases known to affect humans, is caused by bacteria belonging to the Mycobacterium tuberculosis complex. Genital TB is a form of extrapulmonary TB which accounts for a large proportion of morbidity and mortality especially in developing countries [1]. The actual incidence of genital tuberculosis may be more because a large proportion of cases go unreported due to lack of sensitive and specific investigations till now. Definitive diagnosis of genital TB always depends on mycobacterial detection and isolation. While direct microscopy and culture Lowenstein- Jensen medium (LJ) are great success in pulmonary TB, it is of lesser value in genital TB. The reason being these methods have very low sensitivity as in the genital tissue the bacterial load is not much to be detected easily.

There is not much data about the role of PCR in the diagnosis of genital TB in
women in India. Because of obvious demand for a reliable and rapid means of diagnosing genital TB for public health reasons, this study has been done. In the present study, BACTEC and PCR have been applied to find out its usefulness in diagnosing genital TB of women. Correlation of the findings of laparoscopy with those of PCR as well as other conventional laboratory methods has been carried out.

2. MATERIALS AND METHODS
The present study was carried out in the Department of Microbiology, Maulana Azad Medical College, New Delhi in collaboration with Department of Obstetrics and Gynecology, Lok Nayak Hospital, New Delhi and Department of Pathology, Maulana Azad Medical College, New Delhi.

2.1. Inclusion criteria
Patients with symptoms of chronic pelvic inflammatory disease were screened. Informed consent was taken of each patient. Detailed clinical examination including pelvic examination was done. Among them, thirty clinically suspected patients of genital tuberculosis were included in this study.

2.2. Exclusion criteria
Patients with known diagnosis of other specific infections causing chronic pelvic disease were not considered for this study.

2.3. Laparoscopy
Laparoscopy was done for evidence of genital tuberculosis. The patients were divided according to Rattan et al who classified according to the following laparoscopic findings: (1) suggestive diagnosis of TB (presence of caseation, granuloma/tubercles and/or beaded/thickened tubes; (2) probable diagnosis of TB (hydrosalpinx, peritubal/periovarian adhesions, tubo-ovarian mass but without frank tubercles/caseation; (3) incidental findings (pelvic pathology other than pelvic inflammatory disease including fibroid uterus, endometriosis, polycystic ovaries); (4) normal findings [2].

2.4. Sample collection and transport
In each patient, endometrial biopsy (EB) was collected in normal saline and formalin for microbiological and histopathological study respectively.

2.5. Histopathological examination
Sections of endometrial biopsy (EB) tissue were made. These were stained by haematoxylin and eosin stain. Microscopic examination was done for evidence of tuberculosis like chronic inflammation or granuloma [3].

2.6. Direct microscopy [4]
The specimens were grinded by tissue homogenizer and centrifuged. Two smears were made from sediments of each specimen. The smears were stained by Ziehl-Neelsen (ZN) staining. Acid fast bacilli (AFB) were observed as red, beaded and slightly curved rods against a bluish background. At least 300 fields were observed before declaring the slide as negative.

2.7. Culture
The sediments of the specimens were cultured on the following medium:
1. Lowenstein- Jensen medium
2. Bactec 12B vial medium.

2.8. Lowenstein-Jensen medium
The culture bottles were incubated at 37º C. These were examined within 5-7 days for detection of growth of any rapidly growing mycobacteria and contamination. All
cultures were examined weekly for 8 weeks, after which they were discarded. As soon as any growth was evident on culture medium, smear was made and stained by ZN stain for confirmation of acid fast bacilli (AFB) growth.

2.9. BACTEC culture
0.5 ml of processed sample was inoculated in BACTEC culture as described by BACTEC manual[5].

2.10. Polymerase Chain Reaction
DNA was extracted from each specimen, amplified and then detection of amplified product was done.

2.11. DNA extraction[6]
The DNA was extracted by phenol chloroform method. The DNA pellet was redissolved in 20 μl of 1X TE. The DNA was stored at -20º C until further use.

2.12. Amplification of extracted DNA
A 240 base pair (bp) region from the gene coding the MPB64 and a 645 bp region from the gene coding histone like protein gene (HupB) were selected for amplification. Amplification of the gene was done in 25μl reaction mixture containing 100 ng of genomic DNA, 10 mM Tris HCl (pH 8.0), 1.5 mM MgCl2, 50 mM KCl, 200 μM of each dNTP ( dATP, dCTP, dGTP, dTTP), 25 pmole of each oligonucleotide primer and 5 U of Taq DNA polymerase (Bio Labs, New England).

The sequences of primers used were:

For MPB64 gene:
Forward: 5’-TCC GCT GCC AGT CGT CTTCC-3’
Reverse: 5’-GTA TCC GTG TGT CTT GAC CTA TTT G-3’

The reaction mixture was overlaid with light mineral oil to prevent evaporation. The PCR amplification was done using DNA thermal cycler (MJ Research, USA). While using MPB 64 primer, DNA was initially denatured at 94º C for 4 mins then denatured at 94º C for 30 seconds, annealed at 55º C for 30 seconds and extended at 72º C for 30 seconds. In the last cycle the extension at 72º C was allowed for 5 minutes. Total 30 cycles were done[7].

In the process using Hup B primer, DNA was initially denatured at 94ºC for 10 minutes, then 35 cycles, each consisting of denaturation at 94ºC for 1 minutes 50 seconds, amplification at 60ºC for 1 minutes 50 seconds and extension at 72ºC for 1 minutes 50 seconds were done. Final extension was done at 72ºC for 30 minutes [8].

Amplified PCR products were electrophoresed on 3% agarose gel stained with ethidium bromide, along with Gene Rule 100bp DNA ladder molecular weight marker. The electrophoresis was carried out at a constant voltage of 50 V for 1 hour and a band of at either 645 bp ( Hup B gene) or 240 bp ( MPB64 gene) was taken as positive result. Positive and negative controls were also run along with samples. The bands in the gel were photographed under a gel documentation system. (Fig.1)

3. RESULTS
3.1. Clinical presentations
3.1.1. Age distribution
The age of the patients in the study group ranged from 17 to 44 years. All the patients were in reproductive age group. Age group 30 to 34 years contained the maximum
number of cases (36.7%). The mean age of the patients was 28 years.

3.1.2. Symptoms
In the present study, infertility was the main symptom (both primary and secondary). About 19 women out of 30 (63%) suffered from infertility. 17 out of 30 (56%) were having menstrual problems like menorrhagia, dysmenorrhea, amenorrhea, hypomenorrhea and irregular menstruation were the next common symptoms. They were Symptoms like pelvic pain (43%), vaginal discharge (13%), fever (20%), weight loss (20%), night sweat(16%), anorexia(20%) and abdominal mass(10%) were also seen.

3.2. Laparoscopy
Laparoscopic findings were normal in 16.7% of patients. 10% of patients showed suggestive findings in laparoscopy like tubercles and thickened tubes. 40% had probability of tuberculosis like hydrosalpinx, peritubal adhesions. Pelvic pathology other than tuberculosis like fibroid, endometriosis, polycystic ovaries were seen in 33.3% of patients

3.3. Histopathology
The study showed normal endometrium in 15 (50%) patients. Chronic inflammatory cells without granuloma were observed in 10 (33.3%) and granulation tissues with epithelioid cells were observed in 5(16%) patients.

3.4. Microbiological study
7(23.3%) samples were positive by PCR using MPB64 primer. The same 7(23.3%) samples were also positive by PCR using Hup B primer. None of the cases showing normal or incidental findings in laparoscopy were positive by PCR. 3 out of 3 cases (100%) showing suggestive findings of tuberculosis were positive by PCR. 4 out of 12(33.3%) cases showing probable findings of tuberculosis were positive by PCR (Table 1).

None of the patients showing normal findings in histopathology were positive in PCR. Out of 10 cases showing chronic inflammatory cells, 2 cases (20%) were positive by PCR. 5 out of 5 cases (100%) showing granuloma were positive by PCR. (Table 2)

1(3.3%) sample was positive by both PCR and direct microscopy. 6(20%) samples were positive in PCR but negative in direct microscopy. 23(76.7%) samples were negative by both these methods. No sample was positive in direct microscopy and negative in PCR (Table 3).

2(6.7%) samples were positive by both LJ medium culture and PCR. 5(16.6%) were positive in PCR and negative in LJ culture. 23(76.7%) were both PCR and LJ culture negative. 4(13.3%) samples were positive by both PCR and BACTEC culture. 3(10%) sample was positive by PCR and negative by BACTEC culture. 23(76.7%) were both PCR and BACTEC culture negative. None of the patients showing normal findings in histopathology were positive in PCR. (Table 3)

4. DISCUSSION.
Female genital tuberculosis is often a clinical diagnostic dilemma because of its subtle presentations and lack of sensitive and specific diagnostic methods. In spite of normal physical examination, abnormalities can be detected in about 59% of patients. However laparoscopy is a useful tool in the diagnostic work-up of patients with genital tuberculosis.
Table 1: Correlation of PCR with laparoscopic findings

<table>
<thead>
<tr>
<th>Findings in laparoscopy (Number)</th>
<th>PCR positive (%)</th>
<th>PCR negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (5)</td>
<td>0</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Suggestive of TB (3)</td>
<td>3 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Probable TB (12)</td>
<td>4 (33.3%)</td>
<td>8 (67.7%)</td>
</tr>
<tr>
<td>Incidental (10)</td>
<td>0</td>
<td>10 (100%)</td>
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Table 2: Correlation of PCR with histopathology

<table>
<thead>
<tr>
<th>Findings in histopathology (Number)</th>
<th>PCR positive (%)</th>
<th>PCR negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (15)</td>
<td>0</td>
<td>15 (100%)</td>
</tr>
<tr>
<td>Chronic inflammatory cells (10)</td>
<td>2 (20%)</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>Granuloma (5)</td>
<td>5 (100%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3: Correlation of PCR with direct microscopy, culture on LJ medium and culture by BACTEC (n=30)

<table>
<thead>
<tr>
<th>Conventional methods (Number)</th>
<th>PCR positive (%)</th>
<th>PCR negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct microscopy positive (1)</td>
<td>1 (3.3)</td>
<td>0</td>
</tr>
<tr>
<td>Direct microscopy negative (29)</td>
<td>6 (20)</td>
<td>23 (76.7)</td>
</tr>
<tr>
<td>LJ culture positive (2)</td>
<td>2 (6.7)</td>
<td>0</td>
</tr>
<tr>
<td>LJ culture negative (28)</td>
<td>5 (16.6)</td>
<td>23 (76.7)</td>
</tr>
<tr>
<td>BACTEC culture positive (4)</td>
<td>4 (13.3)</td>
<td>0</td>
</tr>
<tr>
<td>BACTEC culture negative (26)</td>
<td>3 (10)</td>
<td>23 (76.7)</td>
</tr>
</tbody>
</table>

Fig 1. Amplified products on gel electrophoresis
According to laparoscopic findings, Rattan et al divided the patients in four groups like suggestive, probable, incidental and normal for tuberculosis [2] N Vijay Bhanu et al reported 100%, 60% and 33% and 25% positivity of PCR respectively in the different groups [9]. However our study showed 3 out of 3 (100%) and 4 out of 12 (33%) cases positive by PCR in suggestive and probable cases respectively while none of cases positive in incidental and normal group of laparoscopy.

Histopathology is another useful method for detection of active infection by tuberculosis. But the result depends upon proper sampling. N Vijay Bhanu et al reported 0% positivity [9]. Abebe et al and Sharma et al reported 28% and 18.8% positivity in their studies respectively [10,11]. The present study showed 50% positivity of pathological lesions overall.

Definitive diagnosis of genital tuberculosis depends on the isolation of Mycobacterium tuberculosis from the lesions. Conventional methods like direct microscopy, culture on LJ medium lack sensitivity and specificity because they require high bacterial load in the tissue. PCR, on the other hand requires as little as 25 pg (5 organisms) of DNA to give positive results. Various primers like IS6110, MPB64 can be used in PCR [12]. To the best of our knowledge it is the first time that both MPB 64 and HupB primers in EB samples have been used. Studies have been done using the gene for histone –like protein HupB and findings claim that it is more specific for Mycobacterium tuberculosis and can differentiate it from Mycobacterium bovis [13]. In the present study, 23.3% of the cases were positive by PCR using both the primers. This indicated exclusive infection by Mycobacterium tuberculosis in genital tuberculosis cases.

Though the positivity is slightly on the lower side, its sensitivity is more than the conventional methods.

Various studies have been done to evaluate the effectiveness of PCR in the diagnosis of female genital tuberculosis. Our result was close to the Indian study by Gupta et al who reported 22% positivity by PCR as compared to culture (2%) [14]. N Vijay Bhanu et al studied 25 women aged 20-40 years presenting with infertility [9]. They found the sensitivity of PCR to be 53.3% which was more than that found in direct smear microscopy (1.6%) and culture (3.2%). Abebe et al collected biopsy or curettage samples from 25 women suspected to have genital tuberculosis in Ethiopia [15]. They found the sensitivity of PCR to be of 48% as compared to direct smear microscopy (4%), culture on LJ medium (12%). Cheng V C et al found sensitivity of PCR to be 78.3% taking culture as the gold standard [16]. Better sensitivity by PCR over conventional methods were also found by Sharma et al in a study on 85 suspected cases of genital tuberculosis [17]. They have reported 64.7% positivity by PCR as compared to microscopy (2.3%) and culture on LJ medium (2.3%).

Although PCR results were slightly on lower side than other studies, it showed more sensitivity and specificity than the other conventional methods. It also correlated well with other clinical and radiological methods. Some cases may be negative due to: (a) the presence of inhibitors not detected by the control amplification. (b) Absence of bacilli in the biopsy specimen because of non-homogenous distribution and involvement of reproductive organs other than uterus.

Although studies of BACTEC in female genital tuberculosis are scarce, it was
reported that it had double the sensitivity than that of culture on LJ medium. Malik et all reported the sensitivity of BACTEC culture to be about 17.6% in the diagnosis of female genital tuberculosis [18]. In the present study, 13.4% positivity was found by BACTEC method. It indicated BACTEC culture had more sensitivity than conventional culture in isolating the bacilli. It also took lesser time for mycobacterial growth (average 11 days as compared to 23 days by LJ medium in our study).

All the four (4) samples that were positive by BACTEC culture were also positive by PCR. Three (3) samples were positive by PCR and negative by BACTEC culture. It was very unlikely that these were false positive because a rigorous protocol was followed to prevent carry over contamination and negative control samples yielded consistently negative results. Also these cases showed positive results in laparoscopy and histopathology. It indicated that PCR had a better sensitivity than BACTEC culture.

5. CONCLUSIONS
PCR and culture in BACTEC medium have better sensitivity than direct microscopy and culture on LJ medium in diagnosing female genital tuberculosis where the bacterial load is less in sample. PCR has also better specificity and corresponds well with radiological and histopathological findings. Its results are also rapid. So PCR and BACTEC culture will be very much useful in diagnosing female genital tuberculosis.

6. REFERENCES


