

Evaluation of Diagnostic Value of Pouch of Douglas Fluid in Comparison to Endometrial Biopsy Samples for the Diagnosis of Genital Tuberculosis by Real-time Polymerase Chain Reaction

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ABSTRACT

Objective: The aim of this study was to evaluate the benefits of using two types of samples: the endometrial biopsy (EB) and the pouch of Douglas (POD) fluid by real-time polymerase chain reaction (PCR) for an early and accurate diagnosis of female genital tuberculosis (FGTB).

Materials and methods: Two samples, one EB and second POD fluid, from each patient (total 173 patients) were subjected to IS6110-based real-time PCR assay for the diagnosis of FGTB.

Results: Among 173 patient samples received, a total of 32.94% (57/173) patients were positive for MTB by either or both sample types. Endometrial biopsy samples were positive in 19.60% (34/173) of all EB samples for MTB, and POD fluid samples were positive in 24.27% (42/173) of all POD fluid samples for MTB. Among the total MTB-positive patients, 26.31% (15/57) patients had only EB sample as positive, whereas in 40.35% (23/57) patients only POD samples were positive.

Conclusion: When genital tuberculosis is suspected clinically, it is better to opt for performing less invasive procedure like testing of POD fluid for MTB, and if it is negative, then further invasive procedure to obtain EB may be chosen for diagnosis.

Keywords: Endometrial biopsy, Genital tuberculosis, POD fluid.

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INTRODUCTION

Female genital tuberculosis (FGTB) is the most common form of extra-pulmonary tuberculosis (TB), accounting for about 27% cases (ranging from 14% to 41%) worldwide.¹ Among Indian females, incidence is 5–16%, and majority of cases come under the reproductive age group (15–45 years).² However, the actual incidence may be underreported as genital tuberculosis (GTB) patients usually remain asymptomatic and methods available to diagnose this are also less.

In our hospital, the specimens that are commonly used for the diagnosis of FGTB are endometrial biopsy (EB), endometrial aspirate, pouch of Douglas (POD) fluid, and menstrual blood used for diagnosing genital TB in females; out of these, EB is the specimen of choice. Although collection of menstrual blood sample is a noninvasive means and is done by patient herself, the reliability factor for testing with this is still questionable; the main reason is the cyclic shedding of the TB bacilli and the shortcomings of the tests available for the detection.

For the detection of genital TB, sampling is of utmost importance. In TB, genital tract gets involved either in localized or generalized forms. In case, we do the sample collection blindly, we can miss the infected areas.

Through laparoscopy the macroscopic changes such as peritubal adhesions, tubercles on the tubes, and small tubo-ovarian masses can be seen, but even if the peritoneum looks normal on laparoscopy, outpouring of bacilli through cornua and fallopian tubes cannot be ruled out.² Thus, there are various clinical presentations of the disease, and the diverse results of imaging, laparoscopy, histopathology, and the bacteriological tests further increase the confusion.³ Owing to limitations with respect to its diagnostic

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sensitivity and specificity; any method for the early diagnosis of the disease and cure before the tubal damage sets in, the test chosen for the diagnosis should be highly sensitive.³ We can increase the sensitivity of a test either by improving the type or quality of the test or by increasing the number or types of the samples.

We conducted the present study to determine the usefulness of IS6110 gene-based format in the diagnosis of FGTB. In addition, we also evaluated the importance of using two types of samples, namely, EB and fluid from the POD, by polymerase chain reaction (PCR) for an early and accurate diagnosis of FGTB.

MATERIALS AND METHODS

A total of 173 women, aged 18–50 years presenting with complaints of infertility and chronic pelvic inflammatory disease, during 2013,

were chosen for this study. Two samples, one EB sample in normal saline and POD fluid in sterile vials, collected from each patient were received in the Department of Microbiology, SMS Medical College, Jaipur, for investigation. Additionally, four EBs and four POD samples from patients having nontuberculous mycobacteria (NTM) infection were also taken in the study, to assess the specificity of PCR in these samples.

Sample Processing

Each EB sample received in a tube containing sterile normal saline was centrifuged for 20 minutes at 6,000 rpm, and the pellet (approximately 1 mL) was taken in a 1.5-mL Eppendorf tube containing fine glass beads up to one third of the capacity of the Eppendorf tube. The sample was homogenized in the tissue lyser (Bertin Technologies Pvt Ltd) for 1 minute. In total, 200 μ L of the completely lysed sample was taken in a different Eppendorf tube (1.5 μ L) for DNA extraction.

DNA Extraction

DNA extraction was done as per the protocol of QIA Amp DNA mini kit (Qiagen Diagnostics Pvt Ltd). To the 200 μ L of the homogenized sample in the Eppendorf, 100 μ L of buffer ATL was added along with 20 μ L of Proteinase K, and the tube was vortexed well and incubated at 56°C for 1–3 hours (till tissue is completely lysed). Sample was tapped well or vortexed in-between the incubation period approximately 2–3 times per hour.

This step was not followed for the POD fluid sample. The POD fluids were centrifuged at 6,000 rpm for 20 minutes, and 200 μ L of the pellet was taken in an Eppendorf tube and 20 μ L of Proteinase K and 200 μ L of buffer AL were added to it.

Buffer AL was also added to the processed EB sample tube. Then, both the tubes were pulse vortexed and incubated at 70°C for 10 minutes. The samples were then briefly centrifuged to remove the droplets from inside of the lid and sides of the tube.

To the samples, 200 μ L of molecular grade ethanol (96–100%) was added, and pulse vortexing was done followed by brief centrifuging to remove the droplets from inside of the lid as well as from sides of the tube.

The whole volume was now applied to the QIAmp mini spin column in a 2-mL collection tube, taking care not to wet the rim. After closing the cap, the tube was centrifuged at 8,000 rpm for 1 minute. The tube containing the filtrate was discarded, and the mini spin column is placed in a new 2-mL collection tube. Then, 500 μ L of buffer AW1 was added to it, without wetting the rim; the cap was closed and centrifuged at 8,000 rpm for 1 minute. The mini spin column was then placed in a new 2-mL collection tube, and the old one containing the filtrate was discarded.

To it, 500 μ L of buffer AW1 was added without wetting the rim; the cap was closed and centrifuged at 8,000 rpm for 1 minute. The mini spin column was then placed in a new 2-mL collection tube, and the old one containing the filtrate was discarded. To it, 500 μ L of buffer AW2 was added; the cap was closed and centrifuged at 14,000 rpm for 3 minutes. Finally, the collection tube was replaced by a 1.5-mL Eppendorf, and 100 μ L of AVE was added to it and centrifuged at 8,000 rpm for 1 minute. The eluted out DNA was stored at –20°C.

DNA Amplification and Detection

For DNA amplification, first master mix was prepared by mixing 25 μ L of SYBR green master mix, which contained all the reagents (SYBR green I an intercalating dye, Taq DNA polymerase, and

deoxyuridine triphosphate) with 2.5 μ L of forward and reverse primers each and 15 μ L of nuclease free water in an Eppendorf tube up to a final volume of 45 μ L per sample. All the constituents were thawed and vortexed and spinned down well before use. The prepared master mix was mixed well 10–15 times by a micropipette. The master mix was then transferred in a K tube placed in K carrier. Finally, 25 μ L of sample DNA was added to it and mixed well by using a micropipette. The K carrier was then placed in the real-time PCR instrument. The primers used to amplify 123 bp fragment of IS6110 were 5'-CCTGCGAGCGTAGGCGTCGC-3' and 5'-CTCGTCCAGCGCCGCTTCGG-3'.

Cycle included initial cover heating at 100°C, precycle at 95°C for 600 seconds, 35 cycles of denaturation at 95°C for 90 seconds, annealing at 72°C for 16 seconds, then extension at 95°C for 30 seconds, followed by melting at 60°C for 0.3 seconds, final melting at 89.7°C for 0.3 seconds and post cycle at 40°C for 120 seconds. Internal positive and negative controls were also tested along with the samples. Autoclaved distilled water, four NTM-positive EBs and PODS each were used as negative controls (external) for testing the specificity of PCR.

Detection was done by measurement of the SYBR Green I fluorescence signal. SYBR Green I intercalates into the DNA double helix. Then, melting curve analysis was done. Melting point is a unique property dependent on product length and nucleotide composition. The melting curve of *Mycobacterium tuberculosis* DNA showed a peak at around 85°C, that is, it melted at 85°C. The sample was considered positive, which showed a melting peak at $\pm 1^\circ\text{C}$ to 85°C. The area under the peak curve, fluorescence level, and the crossing point of each sample were considered for reporting.

All the negative samples were subjected to spiking with known positive DNA to rule out the presence of PCR inhibitors.

RESULTS

Out of total 173 patient samples tested, 32.94% (57) patients were positive for *M. tuberculosis*.

Out of 173 EB samples, 19.60% (34) were positive for *M. tuberculosis* and 24.27% (42) were positive out of 173 POD fluids.

Out of total positive cases (57), the percentage positivity with EB samples alone was 59.64% (34) and that of POD fluid was 73.68% (42).

In total, 19/57 (33.33%) patients had both the samples (EB and POD fluids) positive for *M. tuberculosis*.

In total, 15/57 (26.31%) patients were positive for *M. tuberculosis* from their EB samples, whereas their corresponding POD fluids were negative for that.

In total, 23/57 (40.35%) patients were positive for *M. tuberculosis* from their POD samples, whereas their corresponding EBs were negative for the same.

DISCUSSION

Female genital tuberculosis may cause irreversible infertility in many cases as it may affect all the reproductive organs. It also leads to lower pregnancy rate in assisted reproductive technology program. Endometrial infestation of tubercular bacilli (mere presence of MTB on the endometrial surface) has been found to affect fertility as well.⁴

Female genital tuberculosis usually affects younger females, that is, of reproductive age group. This could be because, after puberty the blood supply to the pelvic organs is increased due to

which, more bacilli could reach there and infect the reproductive organs.⁵ The most commonly affected organ is fallopian tube, if multiple samples are collected from either end of the tubes, shows promising improvement in the diagnostic yield.³

In our study, percentage positivity of POD fluid samples was 73.68% (42/57). This was more in comparison to the EB samples: 31/57 (54.38%). In contrast, 15/57 (26.31%) of the POD samples were negative, whereas their corresponding EB samples were positive. In the case of EB samples, lower positivity may be attributed to the facts that areas from where the sample has been collected may not actually represent the infected area and the site can be missed due to sparse number of *Mycobacteria*,¹ and the granulomas are often focal and the functionalis layer is shed every 4 weeks and moreover granulomas also take at least 2 weeks' time to develop,⁶ so the granulomatous endometrium may not show evidence of TB in all the cycles. In addition to the aforementioned reasons, tissue reaction in those having TB may at times be atypical and bacteriologically mute.¹

Out of the total samples tested (173), 19.65% (34) of EB samples were positive in our study, which is lower than that quoted as 49.5%,¹ 53.3%,² and 48%⁷ by others. A positivity of 24.27% (42) with POD fluid samples in our study was much higher in comparison to the studies who have reported 16%² and 5.44%.¹ Endometrial samples are always mixed with blood; this could possibly explain the false-negative results. A negative PCR in such cases may result in missing the diagnosis in a few cases. Also, it is so important that the sample should be collected from actual suspected lesions (tubercles) seen on laparoscopy. Unfortunately, many gynecologists do not do this and prefer treating patients empirically or leaving them untreated once they receive the negative report of TB PCR. Other reasons for false-negative results include nonrepresentative tissue samples, technical failure in processing biopsy, period of specimen collection in relation to disease stage, and effect of HIV coinfection.⁷

Regarding POD PCR positivity, we had centrifuged whole amount of the fluid received as such and taken the pellet (200 µL) for further extraction procedure. This could be the reason of higher positivity in our study in comparison to others.

Above all, proper sampling, testing of multiple types of samples, and repeat sampling from the patient enhance the sensitivity of PCR as a diagnostic tool for FGTB.³ Our study also endorses the studies done by Bhanothu et al.¹ and Bhanu et al.² on the importance of multiple types of samples in aiding the diagnosis of GTB by PCR.

All PCR-negative samples were spiked with 100 pg H37Rv DNA, and reamplification was done to exclude the possibility of amplification inhibition. Inhibition was indicated by the presence of IS6110 gene on reamplification in three EB samples but none in POD fluids. As endometrial samples are always mixed with blood, this could possibly explain the false-negative results with EB samples, whereas there is no such complication with POD samples.² Diagnosis of FGTB may be missed in some cases due to false-negative PCR result, if EB samples alone are used for diagnosis. Besides this, sample collection for POD fluid is easier and less invasive than the collection for endometrial biopsies.

CONCLUSION

When GTB is suspected clinically, it is better to opt for performing less invasive procedures such as testing of POD fluid for MTB, and if it is negative, then further invasive procedure to obtain EB may be chosen for diagnosis.

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