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Evaluation of Nested Polymerase Chain Reaction Technique in the Diagnosis of Female Genital TB in Endometrial Samples

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Mycobacterium tuberculosis has been identified as the etiological agent of 21st century's epidemic tuberculosis. India is the second-most populous country in the world and one fourth of the global incident TB cases occur in India annually. Genital Tuberculosis is among the top three killers of the women of reproductive age after HIV/AIDS and maternal cause. Genital tuberculosis is an upcoming chronic disease which often has low-grade symptomatology, and with very few specific complaints. The objective of this study was to evaluate the efficacy of PCR technique, in the diagnosis of GTB in female infertility. A case study was done at central India, in which Endometrial biopsies obtained from women suffering from infertility were subjected to PCR based method for diagnosis of genital TB resulting in 10.8% of positivity. Our studies concluded that Genital tuberculosis is difficult to diagnose due to restricted use of investigations and limited use of invasive procedures like pathological biopsy for extra-pulmonary TB, and therefore molecular tests like PCR can provide high sensitive and more rapid opportunity to identify Mycobacterium tuberculosis as compared to conventional techniques which are time consuming. Also genital TB can be linked with the fallopian tubes and, together with endometrial involvement, may cause infertility in patients.

Key words: GTB (Genital Tuberculosis), ET (Endometrial Tissue), BSL-II (Biosafety Level 2).

Tuberculosis (TB) is a chronic infectious disease and the morbidity associated with this condition has major health implications. The disease has a worldwide distribution, and the incidence is high in developing countries. When TB affects genital organs of young females, it produces devastating effects by causing irreversible damage to the fallopian tube resulting in infertility which is difficult to cure both by medical and surgical methods (Thangappah *et al.*, 2011). Genital tuberculosis is very rare in developed countries. Genital TB occurs mostly as secondary infection from a primary pulmonary tuberculosis infection, commonly by the haematogenous route in a manner similar to spread to other extra-pulmonary sites like urinary tract, bones and joints etc. The fallopian tubes are affected in almost 100% of the cases followed by the endometrium in 50%, ovaries in 20%, cervix in 5% and vagina and vulva in <1% (Arora *et al.*, 2003). However, a few reports have found endometrium to be the most commonly involved site. Various Indian studies have shown that tuberculos endometritis and salpingitis account for 4-9% of all infertility cases. The exact incidence of the disease remains unknown, as the majority of the cases remain undiagnosed due to asymptomatic presentation of genital TB and paucity of investigations. Conventional laboratory

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methods are of little value. Ziehl-Neelsen (ZN) staining, AFB Culture and microscopy are less sensitive and time consuming technique for the diagnosis of Mycobacterium tuberculosis. In recent year, Nucleic acid amplification (NAA) tests represent a major advance in the diagnosis of GTB. Advanced molecular methods such as polymerase chain reaction (PCR), has evolved have shown very promising results for early and rapid diagnosis of the disease due to its detection limit of one to ten bacilli in various clinical samples. The objective of the present study was to evaluate the efficacy of Nested PCR technique in the diagnosis of GTB in female infertility in comparison to culture and histopathological examination.

MATERIALS AND METHODS

The following studies were conducted in the BSL II facility of Dygnogene a PCR based diagnostic lab in collaboration with City Micro Lab and analyzed the endometrial sampling of 120 women collected from Bhopal Test Tube Baby Center over a period of 2 years (from May 2012-December 2014). Sampling was done by conventional techniques during menstrual and pre menstrual period.

Collection of Samples

Fresh biopsies of up to 5mm were collected and transferred aseptically into a sterile container without fixatives or preservatives. To keep the tissues moist, sterile saline was added and samples were refrigerated at -15°C and were transported to the lab for analysis.

Reagent preparation

2% N-Acetyl Cystein/NaOH (2g of NaOH, 1.45g of Sodium Citrate, 0.5g of N-acetyl in 100ml of sterile distill water was dissolved).1X phosphate buffer solution (8g of Sodium Chloride, 0.2g of Potassium Chloride, 1.44g of Disodium Phosphate, 0.24g of monopotassium phosphate in 800ml of sterile distill water was prepared, pH adjusted to 7.4 by HCl and makeup the final volume to 1 liter).

Processing of Samples

Based on the consistency and size of the endometrial tissues following process was undertaken for pre processing. The endometrial tissue samples had some amount of RBC's they were removed by giving 1-2 washings with PBS (500-800µL) followed by vigorous vortexing and centrifugation at 8000rpm for 1-2 minutes, some endometrial tissues were viscous in consistency so NALC-NaOH treatment was followed ie (to the sample 2% of NALC-NaOH was added in the 1: 1 ratio) and incubated at 37°C for 20-30 minutes with simultaneous vortexing. After the NALC-NaOH treatment 500-800µl of PBS was added followed by vigorous vortexing and centrifugation at 8000rpm for 8-10 minutes followed by final wash of the obtained pellet with sterile water. The pellet obtained was then further processed for DNA extraction using 3B Speed tools Tissue DNA extraction kit.

Extraction of DNA

Pre-Lyse sample

The preprocessed sample were resuspended in 180µl of BT1 lysis buffer provided in the kit with addition of 25µl of Proteinase K solution (10mg/ml) and heated at 56°C for 30-60 minutes in a waterbath.

Lyse sample

200µl of Buffer BB3 was added and vortexed vigorously and kept for incubation at 70°C for 10 minutes in a water bath.

Adjusting DNA binding conditions

Then 210µl of 100% ethanol was added to the treated sample followed by gentle vortexing.

Binding of DNA

The ethanol added sample solution was loaded onto MTB DNA spin column into a collection tube and was centrifuged at 13,000rpm for 1 minute. The flow through obtained was discarded and column containing binded DNA was transferred to a fresh sterile column.

Washing to remove salts and other PCR inhibitors

500µl of BBW buffer ie wash buffer 1 was loaded onto the column and centrifuged at 13,000rpm for 1 minute. The flow through obtained was discarded and the column was kept back in the collection tube. A 2nd washing was given by loading 600µl of wash buffer II ie BB5 followed by centrifugation at 13,000rpm and the flow through was discarded.

Drying of silica membrane

The column was placed back in the collection tube and was kept for spin at 13,000rpm for 3 minutes.

Elution of DNA: MTB DNA spin column was placed in a 1.5ml Micro centrifuge Tube and 50µl of pre-heated elution buffer was added onto

the column followed by centrifugation at 13,000rpm for 1 minute. The DNA obtained was stored at -20°C for further use.

Amplification of DNA

Samples as described were analyzed using PCR which was carried out using 3 B BioTUB nested kit for MTB detection.

Primers Used

Two primer pair set were selected which target IS6110 gene of *Mycobacterium* thus ensuring a *Mycobacterium* infection.

First PCR

The 1st amplification reaction mixture was of 30µl consisting of a pair of primers hybridizing with conserved sequence specific to *M. tuberculosis* ie targeting IS6110 gene of the bacterium.

The PCR reaction conditions followed to carry out 1st PCR were as follows:

Nested PCR

Initial denaturation	94°C for 1 5 minutes
Denaturation	94°C for 20 seconds
Annealing	60°C for 20 seconds
Extension	72°C for 30 seconds
Post cycling extension	72°C for 5 minutes
Number of cycles	30

Was done using 2nd set of primers and taking 1.5µl of 1st amplification product as template in a 30µl reaction, the PCR conditions that were followed are as follows.

No Template Control (NTC) was used as

Initial denaturation	94°C for 1 5 minutes
Denaturation	94°C for 20 seconds
Annealing	62°C for 30 seconds
Extension	72°C for 30 seconds
Post cycling extension	72°C for 5 minutes
Number of cycles	35

sterile water to avoid cross contamination.

A known positive sample was used as Positive Control (PC) and Internal Control (provided in the kit) was also used to screen out for PCR inhibitors in the extracted DNA sample.

Result Analysis

The results were analyzed by a 2% Agarose Gel electrophoresis.

RESULTS AND DISCUSSION

It was found that out of 120 endometrial samples collected from women who were suffering from a common cause of infertility, positive PCR was found in 13 cases ie (contributing to 10.8% positivity). The data collected covered a time frame of two years and the representative sample of women confirmed their reproductive time period was between 21-40 years confirming that women are highly vulnerable in fertile age to this rare form of genital TB. The data also correlates with the findings of Parikh *et al.*, linking the disease profile to incidence and nature of ever increasing infertility the results clearly show need for an extensive survey and screening of women across India with special focus on rural women for building up of morbidity data which could be linked to development and planning of women health and reproduction.

The bands of the amplified products on the agarose gel revealed very sharp, on 219bp with the positive control, to detect the positive and negative specimens clearly.

Endometrial Tuberculosis is becoming a major health problem in developing country like India and presents difficult diagnostic challenge because it is asymptomatic and shows varied clinical manifestations in affected women. Early diagnosis and detection of the disease plays an important role in correct treatment. Acid Fast Bacilli culture technique remains the gold standard for detection of genital TB, but due to paucibacillary nature of the disease in early stages which may

Table 1. Table denoting specificity and time taken by Conventional & Molecular Technique

Test	Specificity	Time
AFB Culture	100%	5-6 Weeks
PCR	95%	24 hours

Table 2. Total number of samples processed

Endometrial Biopsy	No. of Specimen	PCR	
	120	Positive 13 (10.8%)	Negative 107 (89%)

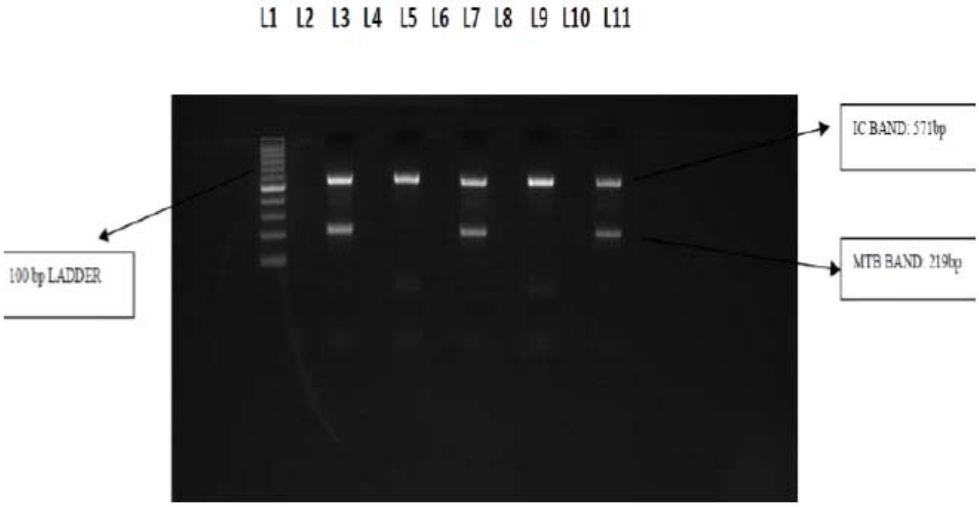

Image 1. Shows the gel electrophoresis of the nested PCR products. Lane 1:100 bp Ladder, Lane 3: MTB Marker (provided in the kit, Lane 5: NTC, Lane 7: Positive Sample, Lane 9: Negative Sample, Lane 11: Positive Control.

result in false negativity of AFB culture therefore early stages can only be detected in PCR.

In our study, out of total of 120 patients investigated for unexplained infertility, 10.8% ie 13 out of 120 cases were found to be harboring MTB DNA identified by PCR. The disease remains undiagnosed in its early stages by techniques like laparoscopy; such techniques prove to be effective only when the disease has taken to chronic clinical condition.

Rapid molecular techniques like PCR are highly sensitive and under optimum conditions can prove to be an excellent tool in the field of diagnosis but proper precautions must be taken to avoid cross contamination of samples while using PCR to avoid false positivity also it is important to consider PCR inhibitors thereby improving the clinician's ability to initiate proper treatment.

CONCLUSION

Our results showed that GTB may be a cause for the infertility in affected females and conventional methods of diagnosis namely AFB culture is time consuming and may provide with false negativity whereas PCR was found to be useful in diagnosing early disease as well as confirming diagnosis in clinically suspected cases. Therefore PCR under standard conditions may act as a potential molecular tool for the early diagnosis of GTB.

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