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Evaluating PCR, culture & histopathology in the diagnosis of female genital tuberculosis

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***Background & objectives*: Genital tuberculosis (GTB) is one of the major causes for severe tubal disease leading to infertility. Unlike pulmonary tuberculosis, the clinical diagnosis of GTB is difficult because in majority of cases the disease is either asymptomatic or has varied clinical presentation. Routine laboratory values are of little value in the diagnosis. An absolute diagnosis cannot be made from characteristic features in hysterosalpingogram (HSG) or laparoscopy. Due to the paucibacillary nature of GTB, diagnosis by mycobacterial culture and histopathological examination (HPE) have limitations and low detection rate. The objective of this study was to evaluate the efficacy of PCR technique, culture and histopathological examination in the diagnosis of GTB in female infertility.**

***Methods*: This study included 72 infertile women who met the inclusion and exclusion criteria. After a detailed history and clinical examination all patients were subjected to investigations including pelvic sonogram, HSG and laparoscopy. Endometrial samples from were allocated for AFB smear, culture and HPE examination. Only 49 samples were available for PCR using IS 6110 and TRC_4 primers. In seven patients peritoneal fluid was also taken for culture and PCR. Based on the clinical profile and laparoscopic findings, a diagnostic criteria was derived to suspect GTB. Specific diagnostic tests were evaluated against this diagnostic criterion.**

***Results*: Laparoscopy was suggestive of tuberculosis in 59.7 per cent of cases, AFB smear was positive in 8.3 per cent, culture was positive in 5.6 per cent, HPE positive in 6.9 per cent and PCR was positive in 36.7 per cent of cases. Based on the diagnostic criteria, GTB was suspected in 28 of the 49 cases. On evaluating against the diagnostic criteria, the sensitivity of PCR, HPE and culture were 57.1, 10.7, 7.14 per cent respectively. The concordance of results between the clinical criteria and specific diagnostic tests were analysed by Kappa measure of agreement. The culture and HPE showed mild agreement with the clinical criteria, whereas PCR showed a moderate agreement. PCR was positive in Two of the 21 cases in whom GTB was not suspected. False positive PCR in these two cases were ruled out by multiple areas of sampling and re-sampling in one case. The PCR results were negative in 12 of the 28 cases. PCR using TRC_4 primers had a higher sensitivity (46.4%) than IS 6110 primers (25%) in detecting clinically suspected GTB.**

***Interpretation & conclusions*: Our results showed that conventional methods of diagnosis namely, HPE, AFB smear and culture have low sensitivity. PCR was found to be useful in diagnosing early disease as well as confirming diagnosis in clinically suspected cases. False negative PCR was an important limitation in this study.**

Key words Culture - female genital TB - histopathology - laparoscopy - PCR

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[1]. 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[2,3]. The disease often remains silent or may present with non-specific symptomatology. As a result, the prevalence of genital tuberculosis is largely underestimated. In developed countries, such as USA, Australia and Western European countries, the incidence of genital tuberculosis (GTB) is less than 1 per cent[4,5], but the incidence in some African countries is as high as 15-19 per cent[6,7]. Various Indian studies have shown that tuberculous endometritis and salpingitis account for 4-9 per cent of all infertility cases[8-11]. Due to the asymptomatic nature/varied clinical presentation, clinical diagnosis of genital tuberculosis is difficult[12,13].

A high degree of suspicion aided by intensive investigations is important in the diagnosis of the disease. Routine laboratory values are of little value. A positive chest X-ray for healed or active pulmonary tuberculosis, contact history, elevated ESR and positive tuberculin test may indicate the need for further investigations[14]. Though absolute diagnosis cannot be made from characteristic features in hysterosalpingogram (HSG) or laparoscopy, laparoscopy is a valuable procedure for obtaining tissue for culture and histopathological examination[15-17].

A definite diagnosis can be made by positive mycobacterial culture and by demonstrating specific histopathological lesion in the specimen. However, these methods have low detection rates and limitations as GTB is paucibacillary. In recent years, polymerase chain reaction (PCR) technique has evolved as a useful and rapid technique for the diagnosis of pulmonary and extra-pulmonary tuberculosis. Any method that is used to diagnose GTB should be highly sensitive to diagnose the disease reliably in its early stage, so that treatment may improve the prospects of cure before the tubes are damaged beyond recovery[18]. The objective of the present study was to evaluate the efficacy of PCR technique in the diagnosis of GTB in female infertility in comparison to culture and histopathological examination.

Material & Methods

This study was conducted in the Fertility Research Centre of Institute of Obstetrics and Gynaecology, Chennai, in collaboration with Tuberculosis Research Centre (ICMR), Chennai, and included 72 infertile women who were enrolled over a period of 30 months (January 2005 to June 2007). Ethical committee approval was obtained to conduct the study and informed consent was obtained from each patient.

Sampling was done by convenience sampling technique. Women who presented with tubal factor infertility proved either by hysterosalpingogram (HSG) and/or laparoscopy, presence of adnexal mass diagnosed by ultrasound, cases presenting with recurrent pelvic inflammatory disease refractory to conventional therapy and those presenting with unexplained infertility were included in the study. Those cases in whom infertility was due to abnormalities of ovulation, male factors, endocrine problems, sexual disorders, endometriosis and peritoneal adhesions due to previous abdominal surgery were excluded. *Chlamydia trachomatis* and *Neisseria gonorrhoea* infections which are common causes of tubal factor infertility were ruled out by Amplicor CT/NG PCR test performed on endocervical swabs[19].

After a detailed history and thorough clinical examination, all patients were subjected to investigations such as Hb per cent, total count (TC), differential count (DC), ESR, tuberculin test, chest X-ray, HIV I and II and abdomen and pelvic sonogram. At the time of laparoscopy features suggestive of tuberculosis such as frank tubercles, caseation, granulomas and beaded tubes were noted. Evidence of past chronic infection in the form of thickened tubes, intraluminal caseation, and terminal hydrosalpinx with retort shaped tubes, tubo-ovarian masses, flimsy adhesions in the pouch of Douglas (POD) were also looked for.

The material for the study was collected from pre-menstrual endometrium and wherever possible pouch of Douglas fluid was also aspirated at the time of laparoscopy. In 23 of 72 cases, only smear, culture and histopathological examination were possible. In 49 cases, PCR was also carried out using IS6110 and TRC4 probes. Therefore, the results of specific tests were evaluated against clinical criteria only in these 49 cases. Culture and PCR were carried out at the Tuberculosis Research Centre, ICMR, Chennai.

For histopathological studies, a portion of the endometrial tissue/tissue from the lesion over the tube was fixed in 10 per cent formalin; routine processing was done and stained with haemotoxylin and eosin. Presence of caseating granulomas surrounded by

epitheloid cells, lymphocytes, plasma cells and giant cells were diagnostic of tuberculosis.

For microscopic examination of acid fast bacilli (AFB), biopsy material was ground well using homogenizer and the concentrated mix was taken for smear and was stained with Ziehl-Neilsen stain.

For culture, the tissue sample was ground well with 5 ml of sterile distilled water. The specimen was centrifuged and the supernatant fluid was discarded. The deposit was decontaminated by 5 per cent H_2SO_4 and added to selective Kirchner's liquid media and cultured for *Mycobacterium tuberculosis*. Since these extra-pulmonary lesions were paucibacillary in nature, their processing included milder decontamination and inoculation into multiple media.

Polymerase chain reaction technique:

(*i*) Processing of samples - The endometrial tissue was finely chopped using a sterile scalpel and homogenized manually in TE buffer (TRIS – EDTA – 10 mM Tris. Cl. *p*H 8.0; 1 mM EDTA *p*H 8.0) until the solution became turbid. This was centrifuged at 11200 *g* for 20 min. The supernatant was discarded and the pellet was processed for further studies. POD aspirate and urine samples were centrifuged at 700 *g* for 15 min. Supernatant was discarded and the pellet was used to extract DNA.

(*ii*) Isolation of DNA - Pellets were re-suspended in 500 µl of TE buffer by repeated pipetting. Then 50 µl of 10 mg/ml of lysozyme was added, mixed well and incubated for one hour at 37°C. To this, 70 µl of 10 per cent SDS (sodium dodecyl sulphate) and 6µl of 10 mg/ml of proteinase K were mixed and incubated for 10 min at 65°C. After incubation 100 µl of 5 M NaCl was added and mixed thoroughly. The samples were further incubated with 80 µl of CTAB/NaCl (Cetyl trimethyl ammonium bromide in sodium chloride) solution for 10 min at 65^{0}C. To this prepared sample approximately equal volume (700 - 800 µl) of chloroform/isoamyl alcohol were added, mixed thoroughly and centrifuged for 10 min. All these chemicals were purchased from Sigma Chemical (St. Louis MO), USA. To the supernatant, 0.6 volume isopropanol was added to precipitate the nucleic acids and placed at -20^{0}C for 60 min. The resultant sample was spun at 16128 *g* for 20 min at 6^{0}C. The resulting DNA pellet was washed with 70 per cent ethanol to remove residual CTAB. The supernatant was carefully removed and the pellet was dried. The prepared pellet was re-dissolved in 25 µl of TE buffer (910mM TRIS and 1mM EDTA) and stored at 4^{0}C for future use.

(*iii*) Amplification of mycobacterial DNA - PCR was performed using Gene amplification 9700 Thermal cycler with standard 25 µl working volume (Gene Amplification PCR System 9700- Applied Biosystems, USA). Precautions were taken to avoid false positivity. Preparation of PCR reagents, addition of template DNA and analysis of amplified products were done in three different rooms to avoid carryover contamination. Reagents were aliquoted and each aliquot was used only once. Wax beads were added to minimize non-specific amplification.

DNAs from the samples were amplified using the following primers.

IS6110a (5' – CCT GCG AGC GTA GGC GTC GG – 3')

IS6110b (5' – CTC GTC CAG CGC CGC TTC GG – 3')

(PRIMER DESIGNER – Version 2.0 – copyright 90, 91 Scientific and Educational Software)

And TRC_4 primer 1 (5' – GAC AAC GAC GTG CGC CTA CT – 3')

TRC_4 primer 2 (5' – GAC CGA ATT AGC GTA GCT CC – 3')

(TRC4 nucleotide sequence has been assigned GenBank Accession No. µ 84405).

The IS6110 primers amplify a fragment with a length of 123bp, while the 18-mer TRC_4 primers amplify a fragment with a length of 173bp. DNA extraction chemicals and PCR chemicals were obtained from USB, Amersham Bioscience.

(*iv*) Cycling Parameters - The reaction was performed on ice to minimize non-specificity. The cycling parameter used was initial denaturation at 95^{0}C for 5 min, followed by denaturation at 94^{0}C for 30 sec, annealing at 58^{0}C for 30 sec, extension at 72^{0}C for 30 sec with 25 cycles and a final extension at 72^{0}C for 5 min. Detection of amplified products was done by agarose gel electrophoresis (2%) at 80 volts for 45 min. Gel was stained with ethidium bromide and viewed under UV transilluminator (VILBER-LOURMAT, France, TCP- 20.M).

The technician performing the PCR technique was blinded to the clinical impression of tuberculosis and the results of other investigations.

In order to rule out other organisms such as *C. trachomatis* and *N. gonorrhoea* which can also cause tubal block leading to infertility, the AMPLICOR CT/NG PCR Test (Roche Diagnostic Systems, Inc.,) was

performed on the endocervical swabs taken from 72 women[19]. The test was performed at the Venereology Department of Madras Medical College, Chennai.

(*v*) Evaluation of specific diagnostics - For the diagnosis of genital tuberculosis there is no absolute gold standard test available. Therefore, based on the clinical profile and laparoscopic evaluation of patients, a diagnostic criteria were derived to suspect tuberculosis. A woman was said to be suspected of having genital tuberculosis if she has had findings suggestive of tuberculosis at laparoscopy with one or more of the following findings: A definite past history of tuberculosis, in the presence of active extra-genital tuberculosis, characteristic features on HSG, elevated ESR, positive Mantoux test, evidence of calcification/ complex adnexal mass by scan.

The specific diagnostic tests culture, HPE and PCR were evaluated against the newly derived diagnostic criteria using bivariate two by two tables. Sensitivity, specificity and agreement by Kappa test were calculated.

Results

The patients were aged between 20 and 35 yr. Menstrual disturbances such as secondary amenorrhoea, oligomenorrhoea and menorrhagia were seen in 18 cases (25%). Oligomenorrhoea was the commonest menstrual abnormality. In 42 (58.3%) cases, other than infertility there were no other symptoms. In four patients (5.5%) there was past history of tuberculosis such as axillary adenitis, abdominal TB and pulmonary tuberculosis. In these four cases laparoscopy was suggestive of tuberculosis, Mantoux and ESR were elevated in three cases each. There was history of close contact with tuberculosis patients in 3 of the 72 cases. ESR was elevated in 11 cases (15%) and there was corroborative evidence of tuberculosis by other clinical parameters in 10 (90.9%) cases (laparoscopy was positive in 10 cases and Mantoux was positive in 5 cases). Positive tuberculin test was seen in 10 cases (13.8%) with a mean induration of 13 mm (range 11-16 mm). In these 10 cases, there was corroborative evidence of tuberculosis in nine (90%) (laparoscopy was positive in all nine and ESR was elevated in five). Evidence of calcification by ultrasound or X-ray abdomen was seen in five (6.9%) women.

Hysterosalpingogram was carried out in 59 cases. Characteristic features suggesting genital tuberculosis such as distorted endometrial cavity, beaded appearance of the tubes, retort shaped hydrosalpinx, calcified areas and cornual blocks were seen in 35 (54.2%). Among these, laparoscopy was positive in 33 cases (94.2%), ESR was positive in 10 and Mantoux was positive in six cases. Laparoscopy was carried out in all 72 patients and the findings were suggestive of tuberculosis in 43 (59.7%).

Only six endometrial samples (8.3%) revealed AFB on smear examination. Microbiological culture of the endometrium in multiple media showed that MTB was positive only in four samples (5.6%). On histopathological examination five of 72 (6.9%) endometrial samples were positive for tuberculosis. Of these five were positive by the conventional methods, one was positive by culture alone, two by histology alone and only in two patients both histology and culture were positive.

PCR on the endometrial samples was done in 49 women using both IS6110 and TRC_4 probes. Using either or both probes, PCR was positive in 18 endometrial samples (36.7%; Table I). In seven patients, at the time of laparoscopy, fluid from pouch of Douglas was also aspirated and sent for culture and PCR study. Two of these POD aspirate samples were positive by PCR and none were positive by culture.

Based on the diagnostic criteria developed in this study, the 49 cases were divided into two groups and diagnostic tests were evaluated in them. Group A consisted of 28 cases in whom genital tuberculosis was suspected. Group B consisted of 21 patients in whom genital tuberculosis was not suspected. In group A (n=28) two were positive by culture. In group B (n=21) all were negative by culture. The sensitivity of culture was 7.14 per cent and the specificity was 100 per cent. The concordance of results between clinical criteria and culture by Kappa measure of agreement was 0.062.

Evaluating diagnostic criteria and HPE showed that three in group A were positive by HPE, and all 21 cases in group B were negative by HPE. Correlating diagnostic criteria and HPE results by bivariate analysis, the sensitivity of HPE was 10.7 per cent and

Table I. Results of laparoscopy and specific diagnostic tests on endometrial samples

Test	No. of samples	Positive result (%)
AFB smear	72	6 (8.3)
Culture	72	4 (5.6)
HPE	72	5 (6.9)
PCR	49	18 (36.7)
Laparoscopy	72	43 (59.7)

the specificity was 100 per cent. The concordance of results between clinical criteria and HPE by Kappa measure of agreement was 0.093.

In group A, 16 (57%) were positive by PCR. In group B, only two were positive by PCR (9.5%). Correlating clinical criteria and PCR results by bivariate analysis, the sensitivity of PCR was 57.1 per cent and the specificity was 90.5 per cent. The Kappa measure of agreement between the clinical criteria and the PCR was 0.449 (moderate agreement).

In this study, by Kappa statistics PCR showed moderate agreement with the clinical criteria and a sensitivity of 57.1 per cent which was higher compared to the other diagnostic tests. Given the low sensitivity of conventional methods, and PCR showing moderate agreement with clinical criteria and higher sensitivity compared to the other tests, false positive and false negative results of PCR were analysed.

In two PCR positive cases in group B POD aspirates were also positive by PCR. Re-sampling was possible in one case and PCR was consistently positive. In these two cases, retesting of samples was carried out and the positivity was confirmed.

The PCR results were negative in 12 of the 28 patients in group A. This could indicate the possibility of false negative PCR results. Re-testing was done on 5 of the 12 negative samples and all were found to be negative. Repeat sampling was possible only in three and all were reported negative. When comparing the PCR results with the conventional methods of diagnosis, of the 49 cases, three were positive by histology and PCR was positive in only one. Similarly, out of two samples which were positive by mycobacterial culture only one was positive by PCR.

We also looked at the agreement between clinical diagnosis of endometrial tuberculosis and PCR results using two probes (Table II). Of the 28 clinically positive samples, seven were (25%) positive by PCR using IS6110 probes, whereas 13 (46%) were positive by PCR using TRC_4 probes. Of the 21 clinically negative samples, 19 (90%) were negative by using IS6110 probes, and all were negative by TRC_4 probe. The sensitivity of TRC_4 when compared to clinical criteria is 46.4 per cent and the specificity is 100 per cent. The sensitivity of IS6110 is 25 per cent and the specificity is 90.5 per cent . The Kappa measure of agreement between clinical criteria and IS6110 was 0.139 (slight agreement), whereas that of TRC4 was 0.426 (moderate agreement). However, in the two cases in whom the clinical criteria were negative, PCR has detected an early disease.

Table II. Agreement between clinical criteria and PCR results using two probes

	Clinical criteria		Total 49
	Positive 28 (group A)	Negative 21 (group B)	
IS6110			
Positive	7	2	9
Negative	21	19	40
TRC4			
Positive	13	0	13
Negative	15	21	36

In our study genital tuberculosis was seen in relatively young females as 83.3 per cent were less than 30 yr of age. In 58.3 per cent of cases, other than infertility there were no other symptoms. Similar observations have been made by other authors as well[13]. Menstrual disturbances such as secondary amenorrhoea, oligomenorrhoea and menorrhagia were seen in 25 per cent of cases. Oligomenorrhoea was the commonest menstrual abnormality noted in this group of women.

In an earlier study[20] 34 per cent of patients presented with menstrual disturbances. In this study, a definite past history of tuberculosis was available only in four cases and they were treated with anti tuberculous treatment (ATT) 2-15 years earlier. In all the four cases, either one or more of the other diagnostic parameters were positive; therefore, a definite past history of tuberculosis was taken as one of the parameters to arrive at a diagnostic criteria to suspect genital tuberculosis. Among the most cases with elevated ESR, the other diagnostic parameters were also positive. Therefore, an elevated ESR was also included as one of the components of diagnostic criteria to suspect tuberculosis.

The tuberculin test can be positive, when there is infection with non–tuberculous mycobacterial (NTM) organisms[21] or when the patient has had a past infection. Because of the corroborative evidence of tuberculosis from other test results, a positive Mantoux test was also taken as an important criterion to suspect genital tuberculosis.

As the other common organisms (*C. trachomatis* and *N. gonorrhoea*) which cause tubal block have been ruled out, characteristic features on HSG and laparoscopy were also included in the criteria to suspect genital tuberculosis.

In extra-pulmonary lesions the bacilli are sparse in number. In our study, only 8.3 per cent samples revealed AFB on smear examination. Namavar *et al*[23] reported AFB in direct smears of tissue biopsies in 12.19 per cent of cases.

Accurate identification of *M. tuberculosis* through culture is presently the yardstick for diagnosis and remains the gold standard. However, in spite of inoculation into multiple media, only 5.6 per cent samples yielded microbiological proof of *M. tuberculosis*. Similar detection rates of *M. tuberculosis* by culture have been reported earlier[23-25]. The possible reasons for the low incidence of culture positivity in endometrial tissue could be due to paucibacillary nature and a substantial number of TB lesions of the genital tract are bacteriologically mute[23]. The low rate of positivity in culture may also be due to the presence of a bacteriostatic substance which inhibits the growth of the bacilli[26].

Histopathological examination is easy, quick and cheap and provides characteristic features of *M. tuberculosis*. But due to the secondary nature of the genital tuberculosis, the infecting organisms are sparse in number, the sampled site may not represent the infected area and the infected site can be easily missed. The fallopian tube is the initial site of involvement, affected in almost all cases, followed by endometrium in 50-90 per cent of cases[11,27]. In as many as 50 per cent of cases infection may be limited to the fallopian tube[15]. Moreover, due to the cyclical shedding of the endometrium, granulomas do not have enough time to form, so the endometrium may not show evidence of tuberculosis in all the cycles. The specimen obtained by biopsy may be small and the blood flow may be scanty. Due to these reasons, to ensure maximum yield multiple specimens from several sources should be collected.

In our study 6.9 per cent endometrial samples were positive by histology, similar to that, reported earlier[20,23,28] Chhabra *et al*[29] suggested that histology and bacteriology both are complementary and neither is completely dependable.

Bhanu *et al*[23] have reported the sensitivity of PCR in both endometrial biopsy and endometrial aspiration samples to be 76.9 per cent. Compared to others, the sensitivity of PCR in diagnosing GTB was low in this study. However, Gupta *et al*[30] reported only 22.5 per cent PCR positivity in endometrial biopsy samples from 40 infertile women.

False positive results were found in 9.5 per cent cases only in this study. Positivity to PCR with negative clinical findings could be due to the early disease with low number of bacilli or with latent infection which are picked by PCR when women are still asymptomatic and before the structural damage to the tube has taken place. However, as there is no gold standard method in diagnosing genital tuberculosis and to compare PCR, one should be cautious about the false positivity by way of contamination, dead bacilli or previous infection or asymptomatic TB at another site. During this study stringent precautions were taken to avoid the problem of false positivity.

The PCR results were negative in 12 of the 28 patients who had positive clinical and laparoscopic features of genital TB. This could indicate the possibility of false negative PCR results. Also PCR has failed to detect 2 cases who were positive by conventional methods (culture & histology). Similar observation was made by Rozati *et al*[31]. The possible explanation for these false negative results of PCR could be due to paucibacillary nature of the specimen, and the portion of the specimen taken for PCR would not have had any *M. tuberculosis*. The analyzed specimen may also contain inhibitors of PCR. Restrepo *et al*[32] have shown that mycobacterial DNA amplification was compromised when the human: bacterial genome ratio was at least 190:1. As endometrial samples are always mixed with blood, this could possibly explain the false negative results in this study[32].

The most widely used primers to detect *M. tuberculosis* in clinical specimens by PCR are from the insertion element IS 6110. It has already been reported that 40 per cent of the strains of *M. tuberculosis* isolated from patients in Chennai had only a single copy of IS 6110 and 4 per cent did not carry even a single copy of IS 6110[33]. Evaluating the PCR by two sets of primers, 4 samples were positive and 31 samples were negative by both probes. Thus, there was agreement between the results of two primers in 71 per cent cases. The sensitivity of TRC_4 when compared to clinical criteria was 46.4 per cent and that of IS6110 was 25 per cent. This low positivity by IS 6110 compared to the TRC_4 probe could be due to the so called south Indian strain of *M. tuberculosis* which carries only a single copy of IS 6110.

Based on the results of PCR, our study has shown that a positive PCR result should be given due importance. Therefore, in clinically suspected cases, in the presence of positive PCR results, an infertile woman should be considered as having GTB and should be treated.

The high false negative result is an important limitation in this study. A negative PCR may result in missing the diagnosis in a few cases. Therefore, when GTB is suspected clinically, but the PCR results are negative, it indicates the need for further evaluation using other diagnostic tests and repeat testing to confirm/exclude diagnosis. This study also shows that the sensitivity of PCR can be improved by using more than one set of primers in the detection of genital tuberculosis.

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