Original Article

# PCR-Based Evaluation of Tuberculous Endometritis in Infertile Women of North India

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## Abstract

**Background:** Tuberculous endometritis is often a diagnostic dilemma for gynecologists in the evaluation of infertility. This study evaluated and compared different diagnostic methods in tuberculous endometritis.

**Methods:** 100 infertile women were investigated for tuberculous endometritis. The endometrial samples obtained by curettage were evaluated by Ziehl-Neelsen (ZN) staining of the smears for acid-fast bacilli (AFB), the samples were also cultured on Lowenstein-Jensen medium, and histopathological examination and nested PCR targeting '*hupB'* gene *(Rv 2986c* in *M. tuberculosis* and *Mb3010c* in *M. bovis)* which can differentiate between *M. tuberculosis* and *M. bovis* were performed. Antibodies against 38-kDa and 16-kDa mycobacterial antigens were detected in serum using ELISA. Statistical analysis was done by online GraphPad Prism software, version 5.0. McNemar's test was applied and Kappa agreement coefficient was calculated for agreement between various methods. A p-value $< 0.05$ was considered significant.

**Results:** Among the 100 evaluated endometrial samples, one was AFB smear positive, none was positive by culture, four were positive by histopathology and 13 were positive by PCR. Of the 13 PCR-positive cases, 38.4% were positive for *M. tuberculosis*, 23.07% for *M. bovis*, and 38.4% showed co-infection with both species. 40% of the patients had raised IgG against *M. tuberculosis* 38-kDa antigen. McNemar's test was applied to PCR and the conventional methods of TB diagnosis (AFB, Culture and histopathology) and the p-value was $< 0.001$ (highly significant) for PCR. Detection by PCR showed a fair agreement with detection by Mantoux test and ELISA.

**Conclusion:** In paucibacillary endometrial tuberculosis, the positive detection rate was found to be significantly higher for PCR compared to other methods. The 'in-house' nested PCR assay targeting the *hupB* gene and used in this study, can serve as a rapid diagnostic aid for tubercular endometritis. It can also differentiate between members of the *Mycobacterium tuberculosis* complex, namely *M. tuberculosis* and *M. bovis.*

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**Received:** Oct. 9, 2010
**Accepted:** Feb. 14, 2011

**Keywords**: Infertility, *M. bovis, M. tuberculosis*, PCR, Tuberculous endometritis.
**To cite this article:** Kohli MD, Nambam B, Trivedi SS, Sherwal BL, Arora S, Jain A. PCR-Based Evaluation of Tuberculous Endometritis in Infertile Women of North India. J Reprod Infertil. 2011;12(1):9-14.



## Background

Genital tuberculosis is one of the commonest causes of infertility due to chronic pelvic inflammatory disease in women of developing countries (1-5). Due to nonspecific signs and symptoms, the disease often poses a diagnostic dilemma for gynecologists (4,5). Endometrial tuberculosis is often associated with a poor prognosis with respect to conception and hence there is a need for early diagnosis and management to restore fertility (5). Effective chemotherapy for tuberculosis is crucial in decreasing the spread of the disease; however the prevalence is still increasing in developing countries due to increasing population and HIV infection (6).

Although *M. tuberculosis* has been the main causative agent, recent studies (7-9) indicate the increasing incidence of *M. bovis* infection in humans. Thus, rapid and less cumbersome, newer

diagnostic approaches that can differentiate between *M. tuberculosis* and *M. bovis* need to be evaluated. Aside from the epidemiological relevance, it is also clinically important to differentiate between the two species as *M. bovis* is intrinsically resistant to pyrazinamide, a first line anti-tubercular drug.

Diagnosis of tuberculosis employs the identification of acid-fast bacilli (AFB) in smears stained by Ziehl-Neelsen technique and culture of the organism on Lowenstein-Jensen medium (10). However, in paucibacillary, extra-pulmonary endometrial samples, AFB smears are almost always negative besides the long period required for their culture.

Prabhakar et al. (11) discovered a histone-like protein and the gene encoding it was named the 'hlp' gene. This gene has been given the name *'hupB'* gene by Cole et al. (12). The present study utilizes a nested PCR targeting the '*hupB*' gene [*Rv2986c* in *M. tuberculosis* and *Mb3010c* in *M. bovis*] (7,8,11-15) in endometrial biopsy samples as a rapid diagnostic aid. This gene has 645bp in *M. tuberculosis (Rv2986)* and 618bp in *M. bovis (Mb3010c)* due to the deletion of 27 base pairs (9 amino acids) after codon 128 in the C-terminal part of the *'hupB'* gene in *M. bovis*. Based on this difference, nested PCR can differentiate between *M. tuberculosis and M. bovis* (7,8,10-15).

The present study was planned to develop, evaluate and compare different diagnostic methods for tuberculous endometritis.

## Methods

***Selection of Study Subjects:*** In this prospective study carried out in a tertiary care hospital in New Delhi, 100 cases of primary/ secondary infertility attending the infertility clinic of the hospital were enrolled after signing an informed consent form. They were investigated for tuberculous endometritis as one of the causes of infertility. All patients with polycystic ovarian disease, hormonal imbalances or any known cause of infertility were excluded from the study. The patients were subjected to detailed history and clinical examination. Routine biochemical and hematological investigations, Mantoux test and chest X-rays were done for all patients.

Serum sample was preserved (500 *µL)* at -20° *C* for the detection of IgG antibodies against 38-kDa and 16-kDa antigens of *M. tuberculosis* by ELISA. Endometrial biopsy/ curettage samples were taken from the patients under aseptic conditions. The study was approved by the relevant Institutional Ethical Committee.

***AFB Staining, Culture and Histopathology:*** Endometrial tissue samples were examined microcopically after Ziehl-Neelsen staining of the smears for acid-fast bacilli. A part of each sample was inoculated on Lowenstein-Jensen culture medium and examined after 3-8 weeks for mycobacterial growth (10). Histopathological examination of tissue sections was done for the presence of TB granuloma.

***ELISA for IgG Antibodies:*** Estimation of IgG was done by Pathozyme-TB Complex Plus kit. The test utilizes the 38-kDa and 16-kDa antigens (which are both highly specific recombinant antigens for the *Mycobacterium tuberculosis* complex) to test the serum for the presence of antibodies.

***Two-step PCR Assay for hupB Gene:*** Mycobacterial DNA was extracted by a method introduced by Chakravorty et al. (16) in a (grade 3) pathogenic laboratory. The PCR assay was carried out utilizing a 40-*µl* reaction mix (2,10,11) with two sets of primers -S and C terminal F-R. Positive, negative and controls were set up with each reaction. The first amplification step utilized N-S primers and was subjected to an initial denatureation at 95° *C* for 10 minutes followed by cyclic denaturation at 94° *C* for one minute 30 seconds (1′30″) annealing at 60° *C* for 1′30″, and extension at 72° *C* for 1′50″. 35 cycles were carried out followed by a final extension at 72 °*C* for 30′. The 645-bp and 618-bp products, respectively, were for *M. tuberculosis* and *M. bovis* which were then used as templates for the nested PCR to amplify the C-terminal portion of the *hupB* gene (8,11,12). The products – 118-bp (*M. tuberculosis*) and 89-bp (M. *bovis*) – were resolved on 8% PAGE[1], stained with ethidium bromide and viewed in a gel documentation system.

***Statistical Evaluation:*** Statistical analysis was done by the online GraphPad Prism software, version 5.0. McNemar's test was applied and Kappa agreement coefficient was calculated for agreement between various methods. A *p*-value

1- Polyacrylamide Gel Electrophoresis

smaller than 0.05 was considered significant, whereas a *p*-value smaller than 0.01 was considered highly significant.

## Results

***Clinical Profile of the Patients:*** Of the 100 infertile women included in the study, 82% were in the age group of 21-30 years. The majority of patients (71%) presented with primary infertility and 29% with secondary infertility. Along with infertility, 28 patients presented with menstrual complaints, six with abdominal/pelvic pain, three with dysmenorrhea and two with dyspareunia. Among the menstrual irregularities, oligomenorrhea was the most common complaint.

The enrolled women were screened for any evidence of pulmonary tuberculosis. 20% of the patients either gave a history of pulmonary tuberculosis or were on anti-tubercular therapy. Three patients gave a history of prior contact with a patient with pulmonary tuberculosis. Four patients had chest X-rays suggestive of pulmonary tuberculosis. Out of 100 patients, 30 patients had a positive Mantoux test.

***Detection of Tuberculous Endometritis:*** The PCR detection rate for the disease was the highest of all the other tests (Table 1) as it detected 13 out of the 100 infertile cases, followed by histopathology, AFB and Culture. McNemar's test was applied to the above four tests and the *p*-value was highly significant ($p<0.001$). Of the 13 PCR positive cases, five were positive for *M. tuberculosis* and three for *M. bovis*, while five showed co-infection with both species (Figure 1).

In our study, 40 patients (40%) had elevated IgG against *M. tuberculosis* 38-kDa and 16-kDa antigens on ELISA. Out of these 40 patients, 28 showed only a positive Mantoux reaction but none of them showed a positive ZN stain, culture or

**Table 1.** Comparison of diagnostic modalities for endometrial tuberculosis (n=100)

| Method | Result | |
|---|---|---|
| | Positive | Negative |
| ZN smear for AFB | 1 | 99 |
| Culture on LJ medium | 0 | 100 |
| Histopathology | 4 | 96 |
| ELISA for IgG Antibody | 40 | 60 |
| PCR for *hupB* gene | 13 | 87 |

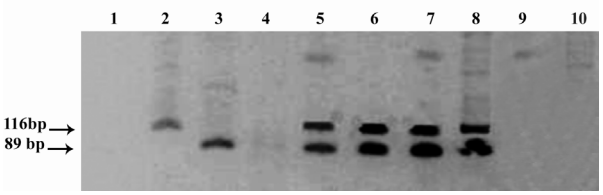


**Figure 1.** Nested PCR products for *hupB* gene resolved on 8% polyacrylamide gel
Lane 1: Negative control
Lane 2: *M. tuberculosis* positive control
Lane 3: *M. bovis* positive control
Lanes 4, 9 and 10: negative samples
Lanes 5, 6, 7 and 8: Dual infection with *M. tuberculosis* and *M. bovis*

positive histopathological finding. Clinical presentation and positivity rate of other diagnostic modalities used for tuberculosis were compared in the 13 cases with positive PCR (as shown in Table 2).

92.3% of the PCR positive cases had a positive Mantoux reaction too (Kappa coefficient = 0.399) and raised serum IgG antibodies against *M. tuberculosis* 38-kDa antigen (Kappa coefficient = 0.319). Thus, a fair strength of agreement was found between detection of tuberculous endometritis by PCR, Mantoux test and ELISA. However, routinely used diagnostic methods for pulmonary/ genital tuberculosis (Chest X-ray, smear examination for AFB and hysterosalpingography) were positive in only 7.7% of the cases (one out of 13 PCR positive cases).

## Discussion

The prevalence of genital tuberculosis in women presenting with infertility varies from 1% in de-

**Table 2.** Clinical findings and investigations suggestive of tuberculosis in PCR positive infertile cases.

| Clinical findings | Patients (n=13) | |
|---|---|---|
| | Number | Percentage (%) |
| Hx of menstrual irregularities | 9 | 69.23 |
| Hx of pulmonary TB | 6 | 46.2 |
| Hx of contact | 2 | 15.4 |
| Chest X-ray | 1 | 7.7 |
| Mantoux skin test | 12 | 92.3 |
| ZN smear for AFB | 1 | 7.7 |
| Culture on LJ medium | 0 | 0 |
| Histopathology | 3 | 23 |
| Hysterosalpingography | 1 | 7.7 |
| ELISA | 12 | 92.3 |

veloped countries to 30% in developing countries (6). Further, genital tuberculosis is a paucibacillary form of the disease in which smears and cultures are usually negative leading to the underdiagnosis of this clinical problem (2,4). Early detection is crucial because, once the infection has damaged the tubes; restoring tubal patency is very difficult. The increasing incidence of *M. bovis* infection in humans also necessitates the differentiation between *M. bovis* and *M. tuberculosis* during diagnosis.

In this study evaluating 100 infertile women, only one patient's endometrial biopsy was found positive by ZN stain. When cultured, none of the endometrial biopsies showed any growth even after eight weeks (Table 1). In paucibacillary, extra-pulmonary genital tuberculosis, the efficacy of detection by smears and cultures is rather low. Mani et al. found that diagnosis of genital tuberculosis by ZN stain could be achieved only in 2% of cases (5). Another study by Srivastava et al. detected only 5% of suspected genital tuberculosis cases by culture (17). Biswas et al. detected 4.8% of suspected genital tuberculosis cases by culture and none by ZN stain (18).

Senol et al., using the Pathozyme-TB Complex Plus kit (19) analyzed 179 tuberculous patients (143 smear-positive, 19 smear negative, eight lymphadenitis and nine pleuritis) and reported a very good specificity (93.3%), positive predictive value (95.9%) and an acceptable level of sensitivity (52.5%). According to another study utilizing the same kit by Demkow et al., the specificity, sensitivity, positive and negative predictive values were 99%, 56%, 94% and 88%, respectively in the detection of bone and joint tuberculosis (20). However, no data is available for genital tuberculosis. In our study, 40% of the patients had raised IgG against *M. tuberculosis* 38-kDa and 16-kDa antigens. However, serology-only diagnosis should be done with caution in endemic countries like India. Though, most of the serological tests have high specificity, their sensitivity is poor. In addition, they may be influenced by factors such as age, prior BCG vaccination and exposure to environmental mycobacteria. The serological tests may not be able to differentiate between infection and disease. Hence, ELISA can only be used as an adjunct to other diagnostic techniques.

The nested PCR assay targeting the *hupB* gene was positive in 13% (13 out of 100) of the patients in the study (Table 1). Thus, it has a higher positivity rate (13%) as compared to the conventional methods (smear and culture and histopathology). The high detection rate obtained in this study and those of previous works (8,14-16) emphasizes the accuracy and potential of this assay as a rapid diagnostic aid for routine use in the future. Of the 13 PCR positive cases, five were positive for *M. tuberculosis* and three for *M. bovis*, while five showed co-infection with both species. As the study was conducted on a small sample size it may not be indicative of the general population and could be the proverbial 'tip of the iceberg'.

A study by Abebe et al. also reported that 4% (1/25) of the clinically suspected patients of genital tuberculosis were positive by AFB staining, 12% (3/25) by culture, 28% (7/25) by histopathology and PCR gave the highest detection rate of 48% (21).

Another study by Manjunath et al., targeting the MPB64 protein coding gene in endometrial tissues, showed that 9/30 were positive, while culture was positive in only one case (22). Similar findings were also reported by Vishnevskaia et al. (23) and Mirlina et al. (24).

Among the PCR positive patients, the diagnosis was supported by histopathological evidence of tuberculosis in three patients and by smear microscopy in one patient (Table 2). The results of ELISA and Mantoux test correlate well with PCR. Out of these 13 patients, 12 patients had raised IgG against *M. tuberculosis* 38-kDa antigen and 12 patients had a reactive Mantoux test. The PCR results were further supported by the fact that six patients had a history of tuberculosis and two patients gave a history of contact with a diagnosed case. The PCR positive cases also remained positive even on repeated testing. Cross-contamination was ruled out, since all negative controls remained negative throughout the study and the samples were not processed as a batch. The clinical history and supporting laboratory investigations make it unlikely that the PCR positive but culture negative cases represent false positivity. On the other hand, it is reasonable to assume that these cases were detected by PCR assay because of its high sensitivity (ability to

diagnose the disease before clinically relevant manifestations appear).

Based on the results obtained in our study and of previous works (8,14-16) on the same gene target, it would not be presumptuous to say that this PCR assay can be used for the rapid diagnosis of extrapulmonary, paucibacillary cases where the conventional techniques are seldom positive. Another advantage is its ability to differentiate between *M. tuberculosis* and *M. bovis* infections, which cannot be differentiated clinically or by the routinely available diagnostic means.

## Conclusion

Extrapulmonary tuberculosis is mainly caused by *M. tuberculosis,* though; *M. bovis* has been recognized as a potential human pathogen. In paucibacillary extra-pulmonary endometrial tuberculosis, the efficacy of detection by conventional methods (smear and culture) is considered as the gold standard but the efficacy for histopathology is low.

The nested PCR assay targeting the *hupB* gene in this study should be further evaluated as a possible rapid diagnostic aid for the detection of tuberculous endometritis. Nested PCR can differentiate between members of the *Mycobacterium tuberculosis* complex, namely *M. tuberculosis* and *M. bovis* which otherwise have similar clinical presentations.

ELISA can be used as a corroborative investigation for the diagnosis of genital tuberculosis.

## Acknowledgement

The authors declare that there are no conflicts of interest and the study was funded by the Institution (Lady Hardinge Medical College) itself.

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