Improved diagnostic value of PCR in the diagnosis of female genital tuberculosis leading to infertility

N. Vijaya Bhanu,1,3 Urvashi B. Singh,1 Milan Chakraborty,1 Naga Suresh,1 Jyoti Árora,1 Tanu Rana,1 D. Takkar2 and Pradeep Seth1

1,2Departments of Microbiology1 and Gynaecology2, All India Institute of Medical Sciences, New Delhi, India
3Molecular Medicine Branch, NIDDK, National Institutes of Health, Bethesda, MD 20892, USA

Histopathological and mycobacteriological examinations have limited utility in the diagnosis of genital tuberculosis. In this double-blind study, 61 samples, consisting of endometrial aspirates (EAs), endometrial biopsies (EBs) and fluid from the pouch of Douglas (POD), from 25 women suffering from infertility were investigated for the presence of the mpt64 gene of Mycobacterium tuberculosis by PCR and correlated with laparoscopic findings. PCR demonstrated M. tuberculosis DNA in 14 out of 25 patients (56.0 %), compared to one smear with acid-fast bacilli (1.6 %) and two culture-positive samples (3.2 %). The presence of M. tuberculosis DNA was observed in 53.3 % of EBs, 47.6 % of EAs and 16.0 % of POD fluid samples. All patients with laparoscopy suggestive of tuberculosis, 60 % of those with a probable diagnosis and 33 % of those with incidental findings were positive by PCR. However, one EA sample from an infertile patient with normal laparoscopy was also positive. Multiple sampling from different sites and amplification of the mpt64 gene segment by PCR offered increased sensitivity in determining tuberculous aetiology in female infertility.

INTRODUCTION

Tuberculosis (TB) is an infectious disease that causes considerable morbidity and mortality. It is a major socio-economic burden in India, afflicting 14 million people, mostly in the reproductive age group (15–45 years). It is involved in about 5–16 % of cases of infertility among Indian women (Krishna et al., 1979; Parikh et al., 1997; Roy et al., 1993), though the actual incidence may be under-reported due to asymptomatic presentation of genital tuberculosis (GTB) and paucity of investigations.

Traditionally, the laboratory diagnosis of TB depends on demonstration of the causative organism, Mycobacterium tuberculosis, by acid-fast staining and/or growth of the organism on Lowenstein-Jensen (LJ) medium. Microscopic examination of acid-fast bacilli (AFB) requires the presence of at least 10 000 organisms ml−1 in the sample, while culture is more sensitive, requiring as little as 100 organisms ml−1 (Bates, 1979). However, M. tuberculosis may take up to 8 weeks to grow in LJ medium. Besides technical drawbacks in demonstrating M. tuberculosis in the laboratory, a substantial number of TB lesions of the genital tract are bacteriologically mute.

PCR is a rapid, sensitive and specific molecular biological method for detecting mycobacterial DNA in both pulmonary and extra-pulmonary samples from suspected TB patients. PCR assays targeting various gene segments, including a 65 kDa protein-encoding gene (Brisson-Noel et al., 1991), the IS6110 element (Eisenach et al., 1990; Kolk et al., 1992) and the mpt64 gene (Manjunath et al., 1991; Seth et al., 1996; Dar et al., 1998), have abbreviated the turnaround time for definitive mycobacteriological detection in the laboratory to 1–2 days, besides being more sensitive than conventional methods.

We report the usefulness of an amplification format based on the mpt64 gene in determining the tubercular aetiology of female infertility. Further, in a double-blinded fashion, we evaluated the diagnostic competence of PCR vis-à-vis laparoscopic findings, besides establishing the appropriateness of using multiple samples, namely endometrial biopsy (EB), endometrial aspirate (EA) and fluid from the pouch of Douglas (POD), in this assay system for an accurate diagnosis of GTB.
METHODS

Patients and samples. Twenty-five women, aged 20–40 years, presenting to the Infertility Clinic, All India Institute of Medical Sciences (AIIMS), New Delhi, with chronic pelvic inflammatory disease of non-chlamydial aetiology during 1999 were enrolled for this study after informed consent. Sixty-one coded samples, consisting of 15 EBs, 21 EAs and 25 samples of fluid from the POD, were sent to the Tuberculosis Laboratory, Department of Microbiology, AIIMS, for investigation. Additionally, five EBs, seven EAs and 12 POD samples from patients with a diagnosis other than TB were collected and evaluated for the specificity of PCR in these gynaecological samples.

Sample processing. Samples of EB collected in normal saline, and EAs and fluid from the POD transported in sterile vials were received in a blinded fashion in the laboratory. The samples were decontaminated in 2% NaOH employing modified Hank’s flocculation method (Shrinivas & Bhattacharya, 1973). About 200 μl of the concentrated sediment was used each for microscopy by Zielh–Neelsen’s (ZN) stain, growth on LJ medium at 37 °C and PCR. Growth was monitored for 8 weeks and the mycobacterial species identified in positive cultures (Kent & Kubica, 1985).

DNA extraction. For extraction of DNA, the decontaminated sediment was incubated in a lysis buffer containing 20 mM Tris/HCl (pH 8.3), 0.5% Tween 20 and 1 mg proteinase K ml−1 for 16 h at 56 °C. The DNA was purified in phenol and phenol/chloroform/isooamy alcohol (25:24:1), followed by precipitation in 0.3 M sodium acetate and absolute ethanol overnight at −20 °C.

PCR. A 240 bp region of the mpt64 gene of M. tuberculosis was amplified using primers MPT1 (5’-TCCGTCGGCCATCTGTCCTCC-3’; nt 460–479) and MPT2 (5’-GTCTCCTCGGAGTCTAGGCCA-3’; nt 700–681). The PCR was carried out in 50 μl volumes consisting of 10 mM Tris/HCl (pH 8.3), 50 mM NaCl, 1.5 mM MgCl2, 0.01% (w/v) gelatin, 200 μM of each of the four deoxynucleotide triphosphates (Perkin-Elmer), 0.4 μM of each primer, 1.25 U Taq DNA polymerase (Perkin-Elmer) and 10 μl of extracted DNA. Using an Amplitron thermocycler (Barnstead/Thermolyne), amplification was carried out for 30 cycles, each consisting of denaturation at 94 °C for 2 min, annealing at 60 °C for 2 min and extension at 72 °C for 2 min.

The sensitivity of detection of the mpt64 gene was determined by PCR using DNA from the avirulent reference strain of M. tuberculosis, H37Rv (ATCC 25177), in a 10-fold serial dilution range between 100 ng and 1 fg, as reported previously (Seth et al., 1996). Extracts of DNA from gynaecological samples known to be negative for M. tuberculosis were amplified in order to determine the specificity of the PCR assay. The sensitivity of the PCR assay was calculated as the percentage of the results that were positive amongst patients with disease (defined as belonging to groups A and B). The specificity was determined as the percentage of the results that were negative amongst patients who did not have disease (defined as belonging to groups C and D).

Each PCR series had one positive control (100 pg H37Rv DNA) and several negative controls (sterile distilled water) interspersed with the samples to monitor cross-contamination. The amplified products were fractionated along with the molecular mass marker φX174 DNA digested with HaeIII (Promega) in 1.5% agarose gel and electrophoresed in Tris/HCl/boric acid/EDTA buffer (89 mM Tris/HCl, pH 8.0, 89 mM boric acid, 2.5 mM EDTA) for 1 h.

DNA from PCR-negative samples was spiked with 100 pg of DNA from the H37Rv strain of M. tuberculosis and reamplified to check for amplification inhibitors giving false-negative results. Further, to confirm the absence of the M. tuberculosis genome, all negative amplicons were dot-blotted and hybridized to an oligonucleotide probe, MPT3 (5’-CCTCAACCCGGGGAGT-3’; nt 601–617), specific to the 240 bp amplified region of the mpt64 gene and endlabelled with 32P-dCTP (Bhabha Atomic Research Centre) by the method described by Sambrook et al. (1989). For PCR, precautions against cross- and carry-over-contamination were followed as previously reported (Seth et al., 1996). While running the PCR, technicians were blinded to the clinical impression of TB.

Analysis of results. After the results of microscopy, culture and PCR were available, the clinical codes were disclosed by the collaborating gynaecologist and the clinical category of the patients revealed. The patients were classified according to the following laparoscopic findings (Rattan et al., 1993): group A, suggestive diagnosis of TB (presence of caseation, granuloma/tubercles and/or beaded/thickened tube); group B, probable diagnosis of TB (hydrosalpinx, peritubal and/or peri ovarian adhesions, tubo-ovarian mass but without frank tubercles/caseation); group C, incidental findings (pelvic pathology other than pelvic inflammatory disease including fibroid uterus, endometriosis, polycystic ovaries, etc.); group D, normal findings. The number of patients in each category and the nature of the samples used are given in Table 1.

RESULTS AND DISCUSSION

The present study assesses the role of PCR in the diagnosis of GTB, using EB, EA and POD samples, in comparison to laparoscopic findings. None of the biopsy specimens had histopathological indications of TB. One EB sample, from group A, revealed AFB on smear examination after ZN staining (1.6%). This sample as well as a smear-negative EA, also from group A, grew isolates of M. tuberculosis in culture (3-2%). Poor rates of detection, as low as 0-4% (Agarwal & Gupta, 1993) and 1-23% (Misra et al., 1996) for smear, and 3-3-10-6% positivity by culture (Manjunath et al., 1991; Roy et al., 1993; Srivastava et al., 1997), have been reported elsewhere.

Sampling plays an important role in the accuracy of detection of GTB. Since the involvement of the genital tract in TB could be generalized or localized, and the technique of sample collection is blind, there is a possibility of missing the infected area. Similarly, though the peritoneum might appear to be normal in laparoscopy, there might be an outpouring of bacilli from the uterine cavity to the peritoneum through the cornua and fallopian tubes. While laparoscopy generally detects macroscopic changes such as peritubal adhesions, tubercles on the tubes and small tubo-ovarian masses that commonly are seen in chronic cases, GTB presents unique diagnostic challenges including subtle clinical manifestations that may be overlooked in laparoscopy during early stages of infection.

The detection sensitivity of the PCR assay was evaluated and it was found that as little as 5 fg of M. tuberculosis DNA from H37Rv (corresponding to one organism) spiked in water and 25 fg (five organisms) spiked in EB, EA and POD could be detected. Although higher sensitivities, such as detection of as little as 0-23-0-023 c.f.u. ml-1 of sample by PCR of IS6110, have been reported (Forbes & Hicks, 1993), others have found that only about 52% of samples harbouring <50 M. tuberculosis c.f.u. ml-1 yield positive amplification results (Clarridges et al., 1993).

In this study, among 15 EBs, 21 EAs and 25 POD fluid samples from 25 women, eight (53.3%), ten (47.6%) and
four (16%) samples, respectively, were PCR positive. Our results are comparable with those from Ethiopia reporting 48% PCR positivity in biopsies and curettage samples from 25 women with complaints of infertility (Abebe et al., 2004).

Comparing PCR with laparoscopic findings, all patients (100%) in group A, nine out of 15 (60%) in group B and one out of three (33.3%) in group C were PCR positive. However, one out of four patients (25%) in group D was also found to be PCR positive.

It is probable that the PCR-positive patient in group D had early endometrial involvement and PCR detected the low number of tubercle bacilli and possibly early disease. In light of our earlier study demonstrating \( mpt64 \) PCR to be both sensitive and specific (Seth et al., 1996) as well as the fact that concurrent negative controls (sterile water) in this series were PCR negative, it seems unlikely that this result is a case of false-positivity. Furthermore, the high endemicity of TB in India raises the possibility of this patient harbouring a latent
infection. Regarding pulmonary TB, positive PCR signals for the IS6110 insertion sequence as an indicator of *M. tuberculosis* DNA were found in about 30% of Ethiopians and Mexicans with latent *M. tuberculosis* infections (Hernandez-Pando et al., 2000). Further, to rule out the possibility of the PCR-positive sample in group D being false-positive, we checked the specificity of our PCR assay in a second series of samples consisting of five EB, seven EA and 12 POD samples collected from patients with normal laparoscopy and diagnoses other than TB, all of which were found to be PCR negative.

In this study, among 14 patients with all three sample types available, two patients were PCR positive in the EB only, while another two patients were PCR positive in the EA only. In 10 patients where an EB was not available, the EA was positive in three patients and the POD in one (Table 1). The sensitivity of the PCR was reasonably good (76.9%) when both the EB and EA samples were considered. This study supporting the use of EB and EA samples therefore endorses the study by Halbrecht (1947) on the importance of multiple sampling in aiding the diagnosis of GTB.

In the present series, in spite of the fairly optimal positivity in different clinical groups based on laparoscopy, the majority of samples (63.9%) were negative for the *mpt64* gene. Since multiple sampling from the same sites could not be performed, we excluded the possibility of amplification inhibition by spiking all PCR-negative samples with 100 pg H37Ra DNA and reamplifying the samples. Inhibition was indicated, by the presence of a 240 bp product on reamplification, in three EBs and one EA. In addition, 37 PCR products that were negative by gel electrophoresis were hybridized to 32P-P-MPT3. Only one gel-negative amplification product (2.7%), from an EB, hybridized to the radio-labelled probe, indicating the presence of *M. tuberculosis* DNA. This could be explained by the superiority of hybridization vis-à-vis PCR, in terms of sensitivity. However, PCR and hybridization were concordant in 97.3% of the negative results. Besides, the requirement of radioactive label and the tedious procedure lasting for 2–3 days offsets the benefits obtained by inclusion of hybridization in routine laboratory use. Further, the patient with hybridization-positivity had an EA sample positive for *M. tuberculosis* by PCR, so the diagnosis in this patient was achievable without the hybridization results.

Finally, PCR identified 14 patients (56%) as harbouring *M. tuberculosis* DNA, including the two patients whose samples had grown *M. tuberculosis* on culture, affording a sevenfold increase in the sensitivity of detection compared to the former technique. Compared to smear, PCR was 14-fold more sensitive and may be a useful diagnostic adjunct for a relatively asymptomatic disease, which is one of the important causes of infertility among women. The fallopian tube is the organ most commonly involved, and the use of multiple samples collected from either end of the tube clearly shows promise in improving the diagnostic yield. Proper sampling, multiple sampling and repeat sampling from the patient will enhance the sensitivity of PCR as a diagnostic tool for GTB.

**REFERENCES**


