INTRODUCTION
Introduction

*Mycobacterium tuberculosis*, the etiological agent for tuberculosis, has been extensively studied for over a century now. But the disease still remains a major public health concern today in the 21st century. Despite the availability of anti-tubercular chemotherapy tuberculosis still remains a major health problem and is the leading cause of morbidity and mortality in many developing/ resource-poor countries. Despite the efforts that are being made to control tuberculosis worldwide, countless numbers of people die with every passing year (1).

More than a decade ago, tuberculosis was identified as a global health emergency. According to the WHO report on global tuberculosis control 2011, India and China together account for 40% of the world’s TB burden (1). In 2010, there were 8.8 million (range, 8.5–9.2 million) incident cases of tuberculosis, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB (1).

Control of the disease is complicated by the fact that one-third of the world’s population is latently infected with tuberculosis. An estimated 5-10% of the latently infected population develops active disease during its lifetime while the rest act as reservoir of pathogen thus making the disease control a significant challenge (2). The only vaccine, Bacillus Calmette-Guerin vaccine, offers some protection against several forms of tuberculosis most often contracted by children, but is not effective against adult pulmonary TB. Tuberculosis can be triggered by anything that reduces a person’s immunity, such as HIV-infection, diabetes, kidneys failure, or cancer treatment. Children are also more at risk of developing active TB than adults, because their immune systems are not fully developed. Efforts in averting the disease have further been impeded by the synergistic relationship between tuberculosis and HIV, as tuberculosis, being an opportunistic infection worsens the immunological suppression in HIV patients. HIV-infected people are anywhere from 50 to 400 times more likely than their HIV-uninfected counterparts to develop active TB disease. In addition, the
difficulty in co-administration of the anti-TB and anti-HIV drugs as a result of drug-drug interactions is well established. In addition, tuberculosis is difficult to diagnose in HIV-positive subjects as smear microscopy, a test widely used in developing countries fails to detect TB in 80% HIV-positive cases. Spread of drug resistant strains poses new challenges for prevention and control of this deadly disease as the present therapy fails to work on the drug resistant strains. Unless we act promptly and aggressively, this emerging global health threat will spiral, threatening to return to the pre-antibiotic era. Failure to develop programs to diagnose and treat such patients now will be more costly in the future, leading to increased incidence, greater residence, and more deaths. Non-availability of domestic funding in developing countries is again a fall back in the disease control. Early, accurate diagnosis and immediate curative treatment, under proper supervision to ensure that drugs are taken for the appropriate duration, is the key to disease control.

Among various clinical presentations of tuberculosis, female genital tuberculosis poses serious concern throughout the world because of various associated complications like oligomenorrhea, amenorrhea, primary or secondary infertility, chronic pelvic pain, pelvic mass and significant mortality (3-7). The disease is being increasingly recognised as a notable cause of infertility in recent years. Infertility is otherwise a common problem but only the couples afflicted with it can understand the social stigma, psychological stress and trauma behind it. The true incidence of the disease remains unknown as the disease poses diagnostic difficulties mainly because the primary symptoms are usually non-characteristic (8, 9). Infertility is a well-known sequela (10). Early diagnosis invariably helps to speed up the decision-making process and markedly reduces the time lag in starting anti-tubercular therapy. Although the reported incidence of genital tuberculosis in Asian and western countries varies between 0.69% in Australia and 17.4% in India, the actual incidence may be higher because a large proportion of cases go unreported due to lack of sensitive and specific investigations (11,12). Factors such as poverty, homelessness, a poorly functioning national tuberculosis program and dismantling of public health infrastructure have significantly contributed to the worsening situation (13). Prevalence of female genital
tuberculosis varies widely from 0.69% in Australia to 19% in India in women of reproductive age group, mostly as a secondary complication to the primary focus elsewhere in the body (14). The most commonly affected organs are the fallopian tubes and endometrium, followed by the ovaries, cervix, vagina and vulva, and it is often associated with tubal blockage and pelvic, peritubal and perihepatic adhesions (Fitz–Hugh–Curtis syndrome) (5, 14-16). Female genital tuberculosis commonly causes caseation and ulceration of the endometrium, resulting in destruction and partial or total obliteration of uterine cavity leading to Asherman’s syndrome (8, 17). In India, 5–16% infertility is reportedly caused by tuberculosis, but the actual incidence rate may be underreported due to asymptomatic presentation of the disease and paucity of investigations (17). Effective management involves rapid, accurate diagnosis and early anti-tubercular treatment.

The diagnosis of tuberculosis still relies on acid fast bacilli (AFB) microscopy and culture, despite the fact that both techniques suffer several diagnostic lacunae, as reported earlier (18). In samples derived from extrapulmonary sites that are often paucibacillary in nature, smear microscopy offers low sensitivity, requiring $10^4$ bacilli/ml for detection. Culture, although being the gold standard, requires a minimum of 10–100 bacilli/ml and a long incubation period (by rapid test the earliest that mycobacteria can be detected is within twelve days) causing delay in diagnosis and treatment. Histopathological examination by haematoxylin and eosin for granulomatous tissue reactions compatible with tuberculosis infection is usually inconclusive, as reported earlier (19). Over the last decade, polymerase chain reaction (PCR) has emerged as a rapid, sensitive and specific molecular method for detection of mycobacterial DNA by amplifying 65 kDa protein-encoding gene, 38 kDa antigen coding gene, the IS6110 and mpt64 gene in both pulmonary and extrapulmonary samples, as reported by various authors (14,15,20,21). Subclinical disease or latent tuberculosis infection might give a positive result in PCR, but this is considered insignificant as prompt diagnosis is essential for averting permanent damage to genital organs and consequent infertility. Endoscopic procedures like laparoscopy and hysteroscopy are widely used for investigation of infertile women (22-24). Their role in
the diagnosis of genital tuberculosis is well established. However, subclinical, latent or early stage infection may be overlooked during the procedure. A positive laboratory test may thus be helpful in timely diagnosis before extensive damage occurs.

PCRs for detection and identification of mycobacteria in clinical specimens have been developed and evaluated (23,24), but mRNA-based assay offers great promise in differentiating between live and dead bacilli as the average half-life of bacterial mRNA is 3 min (25). Thus as mRNA is more easily destroyed than DNA, it can distinguish viable from non viable organism.

Table 1: Comparison of various diagnostic modalities for diagnosis of genital tuberculosis

<table>
<thead>
<tr>
<th>Method</th>
<th>No of Organisms</th>
<th>Time Req'd</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB smear/Microscopy</td>
<td>10,000 org/ml</td>
<td>1 day</td>
<td>Cost effective; Takes less time</td>
<td>Low sensitivity; requires high bacterial count</td>
</tr>
<tr>
<td>Culture LJ</td>
<td>10-100 org/ml</td>
<td>6 wks</td>
<td>Old-time; Gold Standard; Very Specific</td>
<td>Long time required; Lacks sensitivity</td>
</tr>
<tr>
<td>Culture BACTEC 460 TB system</td>
<td>10-100 org/ml</td>
<td>2 wks</td>
<td>Higher sensitivity &amp; Specificity</td>
<td>High cost and use of radioactives; Lacks sensitivity</td>
</tr>
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MOLECULAR METHODS
(DNA fingerprinting-based diagnostics)

i) DNA-PCR
   No of Organism: 1-10 org/ml
   Time Req'd: ~6-8 hrs
   Advantages: Highly Sen/Specific
   Disadvantages: Highly skilled personnel required

ii) mRNA-RT PCR
    No of Organism: 1-10 org/ml
    Time Req'd: ~1 day
    Advantages: Unique for viability
    Disadvantages: Highly skilled personnel required

iii) Gene Seq for Mutation analysis
     No of Organism: 1-10 org/ml
     Time Req'd: ~2 day
     Advantages: Ultimate method for confirmation/Drug-resistance gene
     Disadvantages: Expensive

The emergence of resistance to anti-tuberculosis drugs is a matter of serious concern worldwide and has been reported by several studies (26-28). The situation is aggravated by the increasing incidence of multi-drug resistant (MDR) *Mycobacterium tuberculosis* strains that are defined as being resistant to at least Rifampicin (RMP) and Isoniazid (INH), the drugs which comprise the backbone of anti-tuberculosis chemotherapy. Rifampicin, which is a semi-synthetic derivative of Rifamycin, is one of the most potent anti-tuberculosis drugs and has a highly effective bactericidal activity against *Mycobacterium tuberculosis* which has made it the principle drug in first line of TB medication. Rifampicin binds to the \( \beta \) subunit of ribonucleic acid (RNA) polymerase.
resulting in inhibition of transcription initiation. Presence of Rifampicin resistance increases the likelihood of MDR-TB because *Mycobacterium tuberculosis* strains resistant to rifampicin are more likely to be resistant to several other anti-TB drugs (29). Table 1 shows comparison of various diagnostic tests for genital tuberculosis.

The molecular basis of resistance to anti-TB drugs is becoming better understood with time. RMP resistance is essentially mediated by the mutation in rifampicin resistance determining region (RRDR) corresponding to codon position 507–533 of *rpo B* gene (30, 31). While on the other hand, INH resistance is apparently controlled by a more complex genetic system that involves several genes, namely *kat G*, *inh A*, *kas A* and *ahp C* (32). However, extensive studies have demonstrated that INH resistance is most frequently associated with mutation in *kat G*, a gene that encodes the catalase peroxidase enzyme in *Mycobacterium tuberculosis* and *inh A* gene responsible for inhibition of mycolic acid synthesis in the bacteria (32). Sequence-based monitoring of mutation(s) in the defined targets of drug-resistance genes may improve the treatment and decision-making process. Very limited studies have been carried-out in India in case of genital tuberculosis. Hence, understanding molecular insight is important for understanding the disease, diagnostics, therapeutics and management.

Based on the aforesaid facts, we undertook the present study to assess the utility of PCR in definitive diagnosis of tuberculosis in Indian infertility patients in conjunction with endoscopic procedures—laparoscopy and/hysteroscopy and conventional tests (smear and culture based on the radiometric BACTEC system). Utility of RT-PCR in distinguishing active cases from the past infection will also be evaluated. Also, considering the clinical importance of multi-drug resistance in genital tuberculosis we plan to investigate all genital tuberculosis positive cases for Rifampicin and Isoniazid resistance by automated DNA sequencing of *rpo B*, *kat G* and *inh A* genes.
Lacunae in current knowledge on genital tuberculosis

The actual incidence of genital TB in the general population cannot be accurately measured, as most of the patients are asymptomatic and may remain undiscovered. The diagnosis thus requires high index of suspicion. Early diagnosis of genital TB and its treatment in young patients is essential and may improve the prospects of care before the tubes are damaged beyond recovery.

The diagnosis of tuberculosis still relies on acid fast bacilli (AFB) microscopy and culture, despite the fact that both techniques suffer several diagnostic lacunae. In samples derived from extrapulmonary sites that are often paucibacillary in nature, smear microscopy offers low sensitivity, requiring $10^4$ bacilli/ml for detection. Culture, although being the gold standard, requires a minimum of 10–100 bacilli/ml and a long incubation period (by rapid test the earliest that mycobacteria can be detected is within 12 days) causing delay in diagnosis and treatment.

Histopathological examination for granulomatous tissue reactions compatible with tuberculosis infection is usually inconclusive. The commonly used ‘Laparoscopy’ (the so-called Clinical Gold Standard) may be indicative only and that too may help at later stage and hence not conclusive.

Over the last decade, polymerase chain reaction (PCR) has emerged as a rapid, sensitive and specific molecular method for detection of mycobacterial DNA by amplifying 65 kDa protein-encoding gene, 38 kDa antigen coding gene, IS6110 and mpt64 gene in both pulmonary and extra-pulmonary samples, as reported by various authors. Subclinical disease or latent tuberculosis infection might give a positive result in PCR, but this is considered insignificant as prompt diagnosis is essential for averting permanent damage to genital organs and consequent infertility.

Despite significant diagnostic promise of DNA-PCR for confirming clinical diagnosis of genital tuberculosis, this method is not useful for differentiating between active and latent or dormant cases. In some other infectious diseases, mRNA-based methods have been shown to act as an important tool for pinpointing active disease; it would be
worthwhile to evaluate mRNA-based RT-PCR or Real-time PCR for confirming the active cases of genital tuberculosis. However, only limited studies have been carried-out worldwide, particularly on the innovative diagnostics and drug-resistance components of female genital tuberculosis; Indian data on the subject is almost negligible excepting couple of recent reports on limited samples.

Molecular insight directed towards understanding an evolving innovative disease diagnostics, detection of active cases earlier than later, and keeping a vigil over the possible drug-resistance mutation(s) through gene-fingerprinting seemed very important to supplement/fill the existing gap in knowledge better management of female genital tuberculosis that may prove to be a central dogma in reducing incidence of tuberculosis-associated female infertility in India. Present study was therefore intended to characterize 65kDa antigen coding gene by PCR (DNA PCR) in diagnosis of genital tuberculosis, assess the utility of mRNA-based PCR (RT-PCR) followed by gene sequencing for differentiating between active and latent cases of genital tuberculosis; and lastly to unveil mutations in the drug resistance genes (rpo B, kat G and inh A) through DNA signature sequences associated with resistance to Rifampicin and Isoniazid.