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Laboratory Diagnosis of Genital Tuberculosis: A Narrative Review

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Abstract

Genital tuberculosis is a leading cause of infertility in high tuberculosis burden countries like Bangladesh. Diagnosing this condition can be challenging due to the diverse ways it can present, which requires a physician to have a high level of suspicion. The fallopian tubes are mainly affected, and the endometrium in 50% of cases[1]. Diagnostic modalities usually include histopathology, microscopy, solid and liquid culture tests, Polymerase Chain Reaction (PCR) tests, immunohistochemistry tests, radiology imaging and laparoscopic and hysteroscopic procedures. [*Bangladesh Journal of Infectious Diseases, December 2023;10(2):92-100*]

Keywords: Female Genital Tuberculosis; infertility; diagnostic modalities

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Introduction

Diagnostic difficulties arise because the disease is paucibacillary and *Mycobacterium tuberculosis* can exist without an investigation that is pathognomic for its diagnosis[1]. Because there is no gold standard, the diagnosis of GTB is a dilemma to the clinicians necessitating high index of suspicion with clinical correlation[2].

Specimen

Different authors have emphasized multiple and repeat sampling from different sites of the patients especially from either end of the fallopian tube for increased sensitivity in determining the tuberculous aetiology[3]. Endometrium is the most common location for biopsy because of the surgical ease of its access, without affecting the fertility of the woman. On the other hand, fallopian tube, being the most common site of involvement, is not a suitable site for biopsy as it cannot regenerate thus hampers fertility. In an Ethiopian study, biopsies from fallopian tubes gave the highest positive result for MTB[4]. Biopsy is best performed in the premenstrual period[5].

Negative endometrial biopsy does not rule out pelvic involvement with tuberculosis, since

sampling errors are common and there may be tuberculous lesions in other genital parts without an associated tuberculosis endometritis. Therefore, laparoscopic assessment and multiple biopsy from suspicious areas (tubes, ovaries and peritoneum) should be done before any conclusion. Fluid from the pouch of Douglas has been used in various studies. It is believed to be diagnostic in suspicious cases of chronic inflammation where microscopy and culture are negative[6,7,8.] Studies have evaluated menstrual fluid for microscopy and culture considering the fact that endometrium is shed monthly and the possibility of missing out the bacilli[9,10]. Examination of menstrual blood by direct smear for mycobacterium tuberculosis gives a very poor result which is equally true for PCR analysis[8]. Blood is considered having many PCR inhibitors like heme, leucocyte DNA and immunoglobulins[11].

Mantoux test

The diagnostic role of a positive MT is controversial in GTB. In an Indian study, MT was compared to laparoscopic findings such as thickened tubes, areas of caseation of 100 infertile women. The test had a specificity of 80.0% but a sensitivity of 55.0%. This can be explained by the high TB prevalence in India[12]. The tuberculin test can be positive, when there is infection with non-tuberculous mycobacteria (NTM) or when the patient has had a past infection or previous vaccination with BCG[6,7]. In one study[13] it showed that TST was positive in 42.6% patients with genital tuberculosis. While another study showed sensitivity and specificity of TST as 55.0% and 80.0% respectively, in women with laparoscopically diagnosed[12].

Bacteriological Examination

AFB Smear Microscopy: AFS of smears by Ziehl-Neelsen staining method is not often useful since 10^5 organisms /ml must be present in the specimen to give a positive result. Moreover, it cannot differentiate between typical or atypical mycobacteria. Furthermore, the biopsied tissue may not necessarily represent the infected site. Other methods include Auramine-Rhodamine staining and Fluorescine diacetate staining (FDA).

Culture: Isolating TB bacilli remains the gold standard for diagnosis of tuberculosis. But the scenario is different in the case of GTB. The endometrium is infected in only half of all cases of GTB, and so, a negative culture from a uterine sample should not necessarily exclude its diagnosis[3]. A South African study found an incidence of 6.0% of culture-positive tuberculosis in an infertile population[14]. Many studies have isolated organisms other than MTB (*Mycobacterium bovis* and other atypical mycobacteria) from the endometrial tissues which would be otherwise negative by conventional culture technique. It is now believed that these organisms are equally responsible for the pathogenesis of GTB[15]. Considering the delay in conventional culture result, several methods have been developed in recent years for a rapid outcome BACTEC 460, MGIT (Mycobacterium Growth Indicator Tube), BacT/ALERT, MODS (Microscopic Observation Drug Susceptibility), Phage based assays.

Bactec culture is a rapid radiometric culture and is based on measurement of carbon dioxide released by bacteria during growth in liquid medium. Radioactive carbon labelled substrate like palmitic acid or formic acid is used as marker for bacterial growth. It has higher sensitivity 80-90% and higher detection rate 7.0% to 10.0% and gives quicker result (5 to 10 days) and is also useful for drug susceptibility. The only disadvantage of this test is its cost. In this test homogenized sample is cultured using Bactec MGIT 960 systems. About 500 microliter of sample is inoculated in MGIT tube containing 0.8 ml PANTA antibiotics and growth supplements. MGIT tubes are incubated in Bactec 960 instruments. This system automatically identifies positive sample[16]. Identification of Mycobacteria is based on its phenotypic characteristics (such as growth rate, and pigmentation) and several biochemical tests like growth on medium containing p-nitrobenzoic acid (pnb), Nitrate reduction assay, niacin test and catalase test.

Histopathology: Histological findings in endometrial TB depend on the cycle stage when the biopsy was taken and also on the site in the uterus in which it occurs. The specimens should be taken from the cornua in the premenstrual phase[17]. The granulomatous lesions are usually best recognized on cycle day 24 to 26 or within 12 hours after the onset of menses[18]. Small tubercles usually isolated, infrequent and scattered irregularly throughout the endometrium. In most cases, the lesions are extremely scanty, and careful search through all the sections of the endometrium removed at curettage may reveal only one or two TB foci. The classic lesion in tuberculous endometritis is the non-caseating granuloma, composed of epithelial cells, Langhans giant cells, and lymphocytes. These granulomas are located throughout the endometrium but occur in greater density in the

more superficial layers. The glands may be proliferative without any stromal infiltrate. However, no characteristic endometrial pattern was found to be specific for a tuberculous lesion. A deviation from normal patterns such as proliferative mixed and hyperplastic endometrium was noted in a number of cases[19]. The lesions are frequently focal and immature because they tend to be shed monthly except in postmenopausal women or women with amenorrhoea. This can also explain the reason for absence of caseation, fibrosis and calcification in advanced stages. The endometrium is re-infected regularly from the tubes or infections of the basalis by organisms in menstrual blood after sloughing of the superficial layer[20]. Other reasons for false negative histology may be due to inadequate or non-representative samples requiring multiple or repeat sampling[4,17].

Endoscopic Procedures

Both laparoscopy and hysteroscopy reliably facilitate pelvic organ visualization and specimen collection; however, they are invasive and pose potentially significant surgical risks of excessive bleeding and infection flare-ups[21]. Endoscopic evidence of tubercles or caseous nodules is often considered diagnostic. Other findings consistent with FGTB include pelvic and intrauterine adhesions, beaded or lead-pipe appearance of the fallopian tubes, hydrosalpinx, tubo-ovarian masses, and a distorted or shrunken endometrial cavity[22]. In severe cases, extensive internal adhesions cause genital organs to become attached ("frozen pelvis")[23] .

Hysterosalpingography is used to evaluate the uterine endometrium, tubal lumen, and cervix. In the endometrium, findings of FGTB include intrauterine adhesions and a distorted or obliterated uterine cavity[24]. The fallopian tubes may have a beaded appearance caused by multiple constrictions or a rigid pipe-like appearance in which the entire tube becomes encased in dense, fibrous connective tissue[25]. Tubal obstructions, caused by granulomas, fibrotic tissue, or calcification, commonly occur at the junction between the tubal isthmus and ampulla[26]. The adnexa may show calcified lymph nodes or nodules[24]. Cervical involvement appears as a stenosed cervical canal with a ragged, irregular contour and/or diverticular outpouchings[27].

Ultrasonography is often used to evaluate for uterine, tubal, and ovarian involvement. In FGTB, the endometrium appears thickened and distorted, and the endometrium and ovaries may contain hyperechoic foci of calcification or fibrosis [28]. The fallopian tubes, if involved, appear dilated and thickened[25].

Magnetic resonance imaging, CT scan, and positron emission tomography scan help detect tubo-ovarian masses. A chest radiograph (CXR) is used to identify concurrent or past PTB infection[29]. Abnormal CXR findings healed tuberculosis lesions, hilar lymphadenopathy, fibrosis, opacities, and effusions that may be consistent with tuberculosis are found in 8.0% to 27.0% of women with FGTB[30]. One study of 37 men and women with genitourinary tuberculosis in the United Kingdom found that 13.5% cases had concurrent pulmonary tuberculosis[28].

Immunology

It can be difficult to determine if someone has been infected with MTB through immunology alone. This is because antibodies and a delayed type hypersensitivity response can persist long after the disease has subsided. When MTB infests the endometrium, it triggers an inflammatory process that may be mild, but still stimulates the production of Th1 cytokines. However, for successful implantation of the blastocyst, a Th2 bias is specifically needed. The inflammatory environment in the endometrium may inhibit the down-regulation of Th1 bias and the up-regulation of Th2 bias. This can lead to increased Th1 cell cytokines in the endometrium, making it unreceptive to the embryo and causing recurrent implantation failure[31]. In extrapulmonary tuberculosis, tumor necrosis factor-alpha (TNFα) has a more pro-inflammatory cytokine profile compared to pulmonary tuberculosis [32]. Some studies suggest that treatments for pregnancy may activate latent tuberculosis and lead to failure of implantation[37, 38]. Recurrent miscarriage may occur due to the release of inflammatory cytokines such as IL-2, IFN-γ, and TNF-α. If the host tissue fails to resist the trauma, this can also contribute to the condition[32]. Increased prevalence of IFN-γ and TNF-α in endometrial tissue and aspirate in tuberculosis-positive cases may be an important clinical indicator of endometrial hostility[33]. The Quantiferon®-TB test is used to indirectly detect an MTB infection by measuring the T cells' immune response. It detects infections caused by the MTC by measuring interferon-gamma released by the patient's T cells in vitro using the ELISA technique. A recent study in Bangladesh analyzed TNFα and IFNγ levels among patients suspected to have FGTB. The levels of the biomarker were significantly higher in tuberculosis-positive patients (p=0.01) compared to tuberculosis-negative patients. The levels of the

biomarker were significantly higher in tuberculosis-positive patients (p=0.01) compared to tuberculosis-negative patients[34].

Molecular Assay

During the last decade, major advances in understanding the genetic structure of mycobacteria have been made. Based on this newer knowledge about the specific gene sequences, several gene probes/gene amplification systems for tuberculosis have been developed[35]. These molecular tools and methods can be used for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimens and also molecular detection of drug resistance[36]. These include DNA probes, Ribosomal rRNA based probes, Gene amplification methods for, Identification followed by hybridization with species specific probes, sequencing and RFLP analysis, PCR methods for detection of MTB from clinical specimen, Isothermal amplification techniques: Strand displacement amplification (SDA) system like Amplification of *Mycobacterium tuberculosis* complex rRNA followed by detection of amplicon with acridinium ester-labelled DNA probe and QB replicase based gene amplification.

Nucleic acid amplification (NAA) tests represent a major advance in the diagnosis of tuberculosis[37]. With the use of amplification systems, nucleic acid sequences unique to MTB can be detected directly in clinical specimens, offering better accuracy than AFB smear and greater speed than culture [38-40]. Advanced molecular methods such as polymerase chain reaction (PCR), a type of NAA system, 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[41]. Various targets have been used for detecting mycobacterial DNA such as IS6110, 65KD heat shock protein, MPB 64, 38KD protein and ribosomal RNA[42]. The DNA PCR test, being highly sensitive (three to five bacilli) and specific, is often questioned for comparison with a gold standard diagnostic modality for female genital tuberculosis. In the absence of a more sensitive laboratory gold standard, clinical findings including the laparoscopic findings have been put to use for evaluation. The debate that the test can be non-specific and can give false-positives often bothers clinicians. In a quality-assured laboratory set-up, the DNA-PCR test is reliable. Detection of quiescent or early disease PCR is another of its advantages. A study concluded that latent TB should be considered young Indian patients presenting with unexplained infertility[43].

GeneXpert is a cartridge based nucleic acid amplification test (CBNAAT) for simultaneous rapid tuberculosis diagnosis and rapid antibiotic sensitivity test and require less than 2 hours for result. WHO endorsed the GeneXpert for MTB diagnosis in endemic countries in December 2010. It has a specificity of 99%. It is an automated diagnostic test that can identify MTB-DNA and resistance to Rifampicin. In this test 1 ml of homogenized sample is added to 2 ml of Gene-xpert sample reagent. The mixture is vortexed for 30 seconds. The sample is left to stand for 15 minutes at room temperature and then 2 ml of mixture sample is transferred to the test cartridge. The cartridge is loaded onto the Xpert instrument. Results will be reported as positive or negative and sensitivity by RIF resistance determining region of the rpoB gene with molecular beacons within 2 hours.4 In this study out of a total of 100 samples Gene-xpert was positive in 3 samples (3%). Out of 3 positive results containing patients 2 had laparoscopic findings suggestive of FGTB and 1 had normal laparoscopic finding[16].

The practical application of PCR in a clinical laboratory setting for routine diagnosis impeded by problems of contamination and complex procedures required for sample preparation, DNA extraction, and amplification methods. The sensitivity of PCR is largely dependent on the efficiency of the DNA extraction procedure[44].

In general, a variety of methods can be used for DNA isolation from different biological materials, from boiling the sample in distilled water, autoclaving, disruption by glass beads or sonication, to the use of different enzymes and surfactants.

Physical methods like boiling-The simplest way of DNA release from mycobacterial suspension is boiling for 10 to 15 min in distilled water. Freezing and thawing- Suspensions placed in a bath of acetone with dry ice for 1 min and then in a water bath at 85°C for 2 min and repeated for a total of eight times. Sonication- the suspensions were centrifuged and floated in a sonicator probe containing distilled water and sonicated for 30 minutes at 40W; boiled for 10 min and centrifuged. Chemical methods- In cases where a higher purity of mycobacterial DNA is needed, an isolation method using Cetyltrimethyl Ammonium Bromide (CTAB) is used. Combination of physical and chemical methods-Use of Tris-EDTA and addition of lysozyme, SDS, and proteinase K, accordingly,

followed by precipitation using ethanol after chloroform-isoamyl alcohol (24:1) extraction

Isolation of Mycobacterial DNA from tissues

With respect to the fact that these samples are more complex, it is much more difficult to obtain a pure DNA extract. Moreover, they may contain various inhibitors of PCR amplification. Some of the methods are as follows: Methods of DNA extraction from tissues [45].

Methods of Isolation	PCR sensitivity or detection limit
Proteinase K, Tween 80, SDS, CTAB, PCI extraction	0.2–2 genomes[32]
TritonX-100 plus EDTA plus DTT, Suptilisin lysozyme, pronase, SDS	10 fg DNA[16]
Zirconium beads, sonication, proteinase K, immunomagnetic seperation	93%(conventional)[46]
Lysozyme plus SDS plus proteinase K, PCI extraction	230 cells/g of spiked Tissue[45]

Primers

Various studies have been done to evaluate the effectiveness of PCR in the diagnosis of FGTB. Over the last 20 years, various gene segments have been used as PCR targets for direct M. tuberculosis detection from clinical samples with varying results including IS611, *mpt*64, ESAT6, TRC4, 65 kDa.

***mpt*64:** MPT64 is one of the predominant immunogenic proteins of 24 kDa specific for MTB and not found in BCG strains[47]. The gene of this immunogenic protein is found in the culture filtrates of MTB H37Rv This antigen detection in culture isolates is a highly specific, sensitive, and rapid method of confirming MTB isolates[46].

IS6110: It is a mycobacterium-specific DNA sequence present as repeats in the genome[48] gene has been proved to be a good target because of the presence of multiple copies of its insertion sequence (1-20) in most strains of *Mycobacterium tuberculosis* complex[49-50]. This characteristic is the reason for its increased sensitivity and versatile use.

65kDa: It is a 65-kDa protein that corresponded to *Mycobacterium tuberculosis* (MTB) heat shock protein 65 (65-kDa HSP). For any mycobacterium there is only one 65kDaAg DNA sequence available for amplification, unlike that of IS6110 resulting in its low sensitivity and specificity[47].

ESAT 6: The early secreted antigenic target 6 kDa protein (ESAT-6) is a potent T-cell protein antigen synthesized by Mycobacterium tuberculosis. It has a strong antigenic nature and is produced during active tuberculous infection. A study found 43.1% positive cases whereas found 48% positive cases using ESAT-6 as the target[32].

TRC_4: It is a conserved repetitive element with specificity for *M. tuberculosis* complex. This property makes it a very sensitive molecular target for extrapulmonary sites. In a study[6] it was found sensitivity of 46.4% using TRC_4 as the target suggested incorporation of TRC_4 in the molecular diagnostic assays for detection of MTB in paucibacillary specimens from patients, with atypical clinical symptoms and negative AFB smear[7].

Fundamentals of PCR

The PCR protocol constitutes a variety of experimental applications to produce high yields of specific DNA target sequences. Since no single set of conditions can be applied to all PCR amplifications, individual reaction component concentrations (and time and temperature parameters) must be adjusted within suggested ranges for efficient amplification of specific targets. There are several possible concentration parameters and logical titrations of interrelated reaction components:

- Sample volume and reaction tubes: Most PCR protocols are performed at the 25 µL - 50 µL scale in 0.2 ml or 0.5 ml micro-centrifuge tubes.
- Template DNA or RNA: This contains the (DNA/RNA) target region to be amplified.
- PCR primers: These are oligonucleotides, typically 15-30 bases long, hybridizing to opposite strands and flanking the region of interest in the target DNA. Two primers, complementary at the 3' (three prime) ends, are used that are not complementary to themselves or with each other.

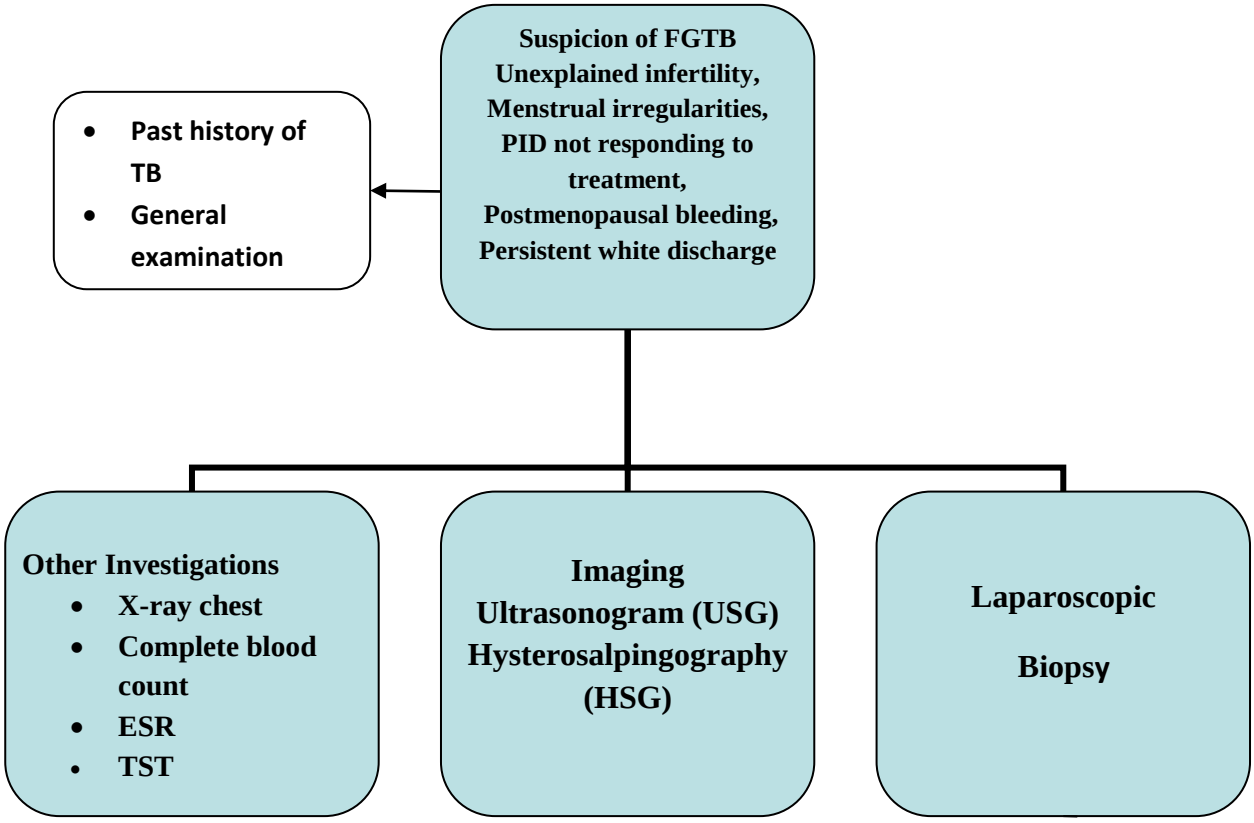

Figure I: Showing algorithm suggested for diagnosis.

Taq DNA Polymerase or another DNA polymerase- an enzyme originally isolated from the bacterium Thermus aquaticus, with a temperature optimum at around 70^0C. Deoxynucleoside Triphosphates (dNTPs)-the blocks from which the DNA polymerase synthesizes a new DNA strand. PCR Buffers provide a suitable chemical environment for optimum activity and stability of the DNA polymerase. Magnesium Ion- an essential cofactor for the DNA polymerase is Magnesium chloride. It forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes. Its concentration must be optimized for every primer-template system.

PCR Thermal Profiles

DNA Denaturation

- **Initial Denaturation:** For most amplicons from pure DNA templates, 30 seconds at 95°C is sufficient. For difficult templates such as GC-rich sequences, a longer denaturation of 2–4 minutes at 95°C is recommended for complete denaturation.
- **Denaturation:** This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes the melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing -based on the Tm (melting temperature) of the oligonucleotides chosen for PCR amplification and the range is often 37°C-55°C for 20–40 seconds. This allows the annealing of the primers to the single-stranded DNA template.

Extension-. The temperature at this step depends on the DNA polymerase used and is most effective at 72°C. Optimization is seldom needed. Typically, 25-45 cycles are required for extensive amplification (106 fold) of a specific target. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction.

Final extension of 5 minutes at 68°C is recommended.

As PCR became more widely used, scientists rapidly learned more about it and that it had its strong points and deficiencies. Very quickly, PCR demonstrated its power to amplify very small amounts (a single copy) of template nucleic acid and to amplify different nucleic acids like DNA and RNA. At the same time, laboratory personnel

learned that this biochemical reaction had a unique deficiency namely, a strong susceptibility to contamination from its own product. Early experience with the PCR soon showed that additional precautions were needed for reliable and contamination-free results[51].

Long-term sequelae FGTB often cause irreversible damage to genital organs, and women with FGTB have a poor prognosis for fertility, even after treatment. Estimates of post-treatment conception rate vary from 12.0% to 23.0% cases[41-43]. Although therapy is not always recommended based on PCR alone, there is some evidence that a positive PCR in the absence of clinical findings may indicate subclinical disease and that early therapy can prevent extensive damage to the genital organs, thereby averting permanent infertility[52-53].

Conclusion

Although FGTB remains an uncommon extrapulmonary manifestation of TB, it should remain an important consideration for the evaluation of women presenting with pelvic symptoms, including infertility, in high-TB-incidence settings or those with epidemiological risk for TB (people who have lived in high-incidence settings, or who have had a prior diagnosis of TB or contact with someone who has had TB, or with abnormal imaging findings that could be suggestive of TB) who are in low-incidence settings. FGTB presents challenges in both diagnosis and treatment due to its limited symptoms. Based on clinical presentations, a woman cannot be diagnosed with FGTB. Diagnosing the disease is difficult because it presents with a diverse range of clinical symptoms and the results of various tests, including imaging, laparoscopy, histopathology, and bacteriological and serological tests, are often inconclusive due to their limitations in diagnostic sensitivity and specificity. Since there are no standard guidelines or algorithms for diagnosing genital tuberculosis, currently available tests cannot diagnose all cases. To develop more sensitive and specific tests, larger case-control studies are required.

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None

Conflict of Interest
We declare that we have no conflict of interest.

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Contribution to authors
All the work was performed by Moureen A.

Data Availability
Any questions regarding the availability of the study's supporti ng data should be addressed to the corresponding author, who can provide it upon justifiable request.

Ethics Approval and Consent to Participate
The Institutional Review Board granted the study ethical approval. Since this was a retrospective study, not every study participant provided formal informed consent. Each method followed the appropriate rules and regulations.

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