3.1 **Tuberculosis**

Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*, which may be PTB or EPTB. TB presents with fever, cough and expectoration of more than 3 weeks, haemoptysis, weight loss etc. Extra-pulmonary tuberculosis affects many organs of the body. The prevalence of both pulmonary and extra pulmonary tuberculosis is increasing, due to the increase in number of immuno compromised patients and emergence of drug resistance in TB.

3.2 **Classification of Genus Mycobacterium**

*Mycobacterium tuberculosis* was first identified and described by Robert Koch on 24 March 1882. The classification of the genus *Mycobacterium* according to Bergey’s manual of systematic bacteriology (9th edition) (Wayne and Kubica 1986), is as follows:-

- **Kingdom** — Prokaryotes
- **Division** — Feromicutes
- **Class** — Shizomycetes
- **Order** — Actinomycetales
- **Family** — Mycobacteriaceae
- **Genus** — Mycobacterium

3.2.1 **Classification Based on Pathogenicity of Mycobacteria**

Four groups of human pathogens can be delineated on the basis of microbiologic, clinical, and epidemiologic characteristics within the genus *Mycobacterium*: *M. tuberculosis* complex, *M. leprae*, slowly growing nontuberculous Mycobacteria (NTM), and rapidly growing Mycobacteria.

3.3 **General Characteristics of Genus Mycobacterium**

1) Mycobacteria are slender rods 0.2-0.6µm wide and 1-10µm long.
2) They are obligate aerobes, non motile (sliding movement), non-spore forming, non-capsulated.
3) They have branching filamentous growth resembling fungal mycelium.
4) They are gram positive and acid fast.
5) Their G+C content of DNA ranges from 62%-72% (Goodfellow and Wayne 1982) with exceptions of *M. leprae* (58% G+C) and *M.lufu* (61% G+C content).
6) Their cell wall is rich in lipid content having complex mixture of mycolic acids and dehydrogenated menaquinones.
7) The presence of one of the three classes of catalases (Wayne and Diaz 1985) has been described as additional characteristics.

3.4 *Mycobacterium Tuberculosis*

*M. tuberculosis* causes TB in humans and has certain characteristic features such as slow growth, tendency to remain dormant, complex cell wall, and genetic homogeneity. It is an intracellular pathogen. The TB bacilli have the capability to remain dormant for long time without any symptomatic infection, thereby causing latent tuberculosis (Stewart et al., 2003).

3.4.1 Morphology

Morphologically, *MTB* is either a straight or slightly curved rod and its size ranges from 0.2-0.6µm by 1.0-10.0 µm. It occurs either singly or in pairs or clumps and is strongly acid fast with distinct beaded appearance. Acid fastness is the property of the cell wall that is related to the integrity of cell.

3.4.2 Culture Characteristics

1. Tubercle bacillus is a slow grower with a generation time *in vitro* of 14-15 hours (as compared to 15-20 minutes for *Escherichia coli*) and visible colonies usually appear within 2-8 weeks.
2. Optimum temperature is 37\(^{\circ}\) C and the growth does not occur below 25\(^{\circ}\) C or above 40\(^{\circ}\) C.
3. Optimum pH for the growth of Mycobacteria is 6.4-7.0.
4. *MTB* is an obligate aerobe but growth is stimulated by 5-10% CO\(_2\).
5. Addition of 0.5% glycerol improves the growth of human strains of TB bacilli.
6. Growth of *MTB* does not occur in media containing 500mg/L of *p*-nitrobenzoic acid.

7. Cultures of tubercle bacilli are sensitive to UV, formaldehyde and gluteraldehyde.

### 3.4.3 Resistance

Mycobacteria are not specifically heat resistant and get killed in 15-20 minutes at 60°C. Bacilli may get killed after being exposed to direct sunlight for 2 hours, but in sputum, the bacilli may remain viable for 20-30 hours. In droplet nuclei, bacilli could remain alive for 8-10 days. At room temperature, cultures remain viable for 6-8 months and may be stored for two years at -20°C. Mycobacteria are resistant to chemical disinfectants as they can survive after exposure to 5% phenol, 15% H₂SO₄ and 4% NaOH (Madigan 2012).

### 3.4.4 Antigenic Properties

Several antigens have been identified in *M. tuberculosis*. These have long been used for the diagnosis of TB through the detection of antibodies against them. However, all these antigens have been found to be useless and serologic tests have been banned by the WHO. Determination of ADA and IFN –gamma in pleural fluid may be useful in diagnosis of pleural TB. Following infection by tubercle bacilli, a delayed hypersensitivity to the protein of the bacillus (i.e. tuberculin) develops. Various antigens, e.g. the antigen 85 complex, may have protective roles and may also be useful for novel drug development (Belisle *et al.*, 1997). In a majority of TB patients, the 2 immunodominant antigens recognized by the sera are ESAT-6 and CF-10 proteins (Smith 2003).

### 3.5 Methods for Demonstration, Culture, and Identification of *MTB*

#### 3.5.1 Conventional Methods

**Demonstration of Mycobacteria in Clinical Specimens**

i) **Microscopy**

For acid-fast staining, two procedures are commonly used:

- AFB smear based on carbol fuchsin stains, ZN stain and Kinyoun procedure are examined under light microscope I oil immersion (Bishop and Neuman
1970). The acid fast bacilli appear pink in a contrasting (blue) background (Figure 1).

- AFB smear based on fluorochrome stains (auramine-rhodamine or auramine O) are examined using fluorescence microscopy (Kent and Kubica 1985). The fluorochrome stains help in rapid screening of smears and has higher sensitivity than the ZN stain.

Microscopy is an important diagnostic tool in TB control because it is a quick to perform, simple and inexpensive method of MTB detection. However, microscopy cannot discriminate between MTB and other Mycobacteria or nocardia. It lacks sensitivity, as >10⁴ bacilli per/ml are needed to get positive results (Katoch 2006).

![Figure 1: Mycobacteria seen in ZN Smear](image)

i) **Concentrated Smear Microscopy**

In the setting of human immunodeficiency virus (HIV) infection, the diagnostic sensitivity of direct smear microscopy is further reduced because of a lower bacillary burden in the lungs, a finding confirmed in multiple studies from sub-Saharan Africa (Mugusi et al., 2006). Despite these limitations, smear microscopy is rapid, inexpensive, and highly specific and will likely remain the primary diagnostic test for TB for the foreseeable future. Improved smear microscopy is therefore a primary goal for global TB control. Several methods of smear microscopy that involve sputum liquefaction and concentration have been reported to increase diagnostic sensitivity (Morcello et al., 2008). The N-acetyl-L-cysteine-NaOH (NALC) method has been the most widely investigated and has
been reported to increase sensitivity modestly compared to direct smear microscopy (Steingart et al., 2006).

ii) Culture

Culture is considered the gold standard technique for diagnosing MTB. Routine culture media are egg-based (Lowenstein-Jensen, Ogawa American Trudeau Society, Petragnani), agar-based (Middlebrook 7H10 (MB7H10) and 7H11), and liquid media (Dubos and Middlebrook 7H9). The incubation time is 8 weeks for all media.

Identification of *Mycobacterium tuberculosis* depending on colony characteristics, growth rate and pigmentation

i) Colony Characteristics:

Colonies are rough and buff-colored (Figure 2). Other Mycobacteria exhibit various other types of colony morphology ranging from smooth to dome shaped.

ii) Rate of Growth:

It is the conventional method to initially classify the organism as rapid or slow growers (Vestal et al., 1977). Even, the first scheme for identification and grouping of cultivable mycobacterium was based on growth rates and colony pigmentation. According to Good fellow et al., 1982, Mycobacteria have been classified into two broad categories on the basis of growth rate:

1. **Slow Growers**- Those Mycobacteria which do not produce colonies within 7 days of growth e.g.; *M.leprae* (*in vivo*), *M. tuberculosis*, *M. bovis*, *M. ulcerans* etc.

2. **Rapid Growers**- Those Mycobacteria which produce colonies within 7 days. e.g. *M. fortuitum*, *M. phelei*, *M. chelonae*, *M. smegmatis* etc.

   The optimum temperature for growth of both types of Mycobacteria ranges between 28°C-45°C.

iii) Pigmentation:

According to Runyon 1959, Mycobacteria have been divided into 4 groups on the basis of pigment production-
1. **Photochromogens (Runyon Group I)** - These strains form colonies that produce no pigment in dark, but a yellow orange pigment appears when the young culture is exposed to light for 1 hour in the presence of air, and then again incubated for 24-28 hours, e.g. *M. kansasii, M. simiae* and *M. marinum*. All species of this group are potentially pathogenic.

2. **Scotochromogens (Runyon Group II)** - Scotochromogens form pigmented colonies (yellow-orange-red) even in the dark. If culture is grown in light, the pigment turns orange in colour. e.g.; *M. scrofulaceum* and *M. szulgai*. Species of this group are both potentially pathogenic and common saprophytes.

3. **Non-photochromogens (Runyon group III)** - The non photochromogens are slow growing Non Tubercular Mycobacterium (NTM) whose colonies produce no pigment whether they are grown in the dark or light. Colonies may be similar to those of tubercle bacilli. *M. xenopi* and *M. avium* are thermophils, capable of growth at 45°C and cause chronic disease distinguishable from tuberculosis. Though usually classified as non-chromogen, some may form scotochromogenic yellow colonies.

4. **Rapid Growers (Runyon Group IV)** - This is a heterogeneous group of Mycobacteria whose colonies appear on solid media within 7 days of incubation at 37°C. e.g. *M. smegmatis, M. phlei, M. szulgai M. chelonae, M. abscessus, M. fortuitum* and *M. peregrinum*.

![Figure 2: MTB Growth on LJ medium](image-url)
Biochemical Identification

A number of biochemical tests have been used for identification of *Mycobacterium tuberculosis* (Vestal 1977) including Niacin test, Aryl sulphatase test, Catalase peroxidase test, Nitrate reduction, Tween hydrolysis test etc.

3.5.2 Non Conventional Methods

Rapid Methods for Early Detection Of Growth:

More rapid techniques that reduce the identification time for early detection of growth of Mycobacteria (Katoch and Sharma 1997) are as follows:

i) **BACTEC System:** Automated radiometric culture methods (e.g. BACTEC) are now being used increasingly for rapid growth of Mycobacteria. The method employs a liquid MB7H12 medium that contains radiometric palmitic acid labeled with radioactive C-14. Various antimicrobial agents are added to this medium in order to prevent the growth of non Mycobacteria. 14CO₂ production by the metabolizing organisms provides the growth index (GI) for the Mycobacteria. Growth of Mycobacteria is generally detected within 1-2 weeks (Figure 3).

ii) **Septicheck AFB System:** It has biphasic media which consists of a slide with solid media and an enriched selective broth (Isenberg *et al.*, 1991), one side of which is covered with the non-selective MB agar and the other side is divided into two parts: one containing NAP incorporated agar/ modified egg medium and the other with chocolate agar to indicate contamination.

iii) **Mycobacterial Growth Indicator Tubes (MGITs):** Using MGIT, the Mycobacterial growth is detected with the help of fluorescent sensors that are oxygen sensitive. The active growth of Mycobacteria depletes the dissolved oxygen of broth, allowing sensors to show fluorescence in a 364 nm UV light that can be seen with naked eyes. The growing time for Mycobacteria varies from 4-14 days. The technique is sensitive and specific for isolation and drug susceptibility testing of Mycobacteria (Tortoli *et al.*, 1999).

iv) **BacT/AlerT System:** BacT/ alert 3D system is a completely automated system for incubating and monitoring growth of Mycobacteria and has been
found to be equally good in giving a rapid test result. The system utilizes a colorimetric sensor to monitor the amount of CO$_2$ produced by microbial metabolism in the culture medium. CO$_2$ diffuses across the membrane, dissolves in water contained in the sensor, and generates free hydrogen ions, which in turn reduces the pH of LES, changing the color from gray to yellow.

![Figure 3: Automated Liquid Culture Systems for Mycobacteria](image)

3.5.3 Genetic Methods

All living organisms possess their first level of genetic information in terms of the DNA present within the genome of the cell. The basic structure of DNA and RNA is therefore a measure of identifying different taxa.

i) **DNA Base Composition/G+C Content:** DNA base composition may be predicted directly by hydrolysis of a DNA sample or indirectly by calculating the mole % of G+C from the hyperchromatic shift accompanying the thermal denaturation of the DNA. The G+C content of *M. tuberculosis* have been found to be between 63-65%.

ii) **DNA-DNA and DNA-RNA Hybridization:** Nucleic acid hybridization is an important technique for determining relationship between sequence of DNA and RNA products. In this technique, nucleic acids are denatured and then allowed to form a duplex structure. The reassociation may be measured between DNA-RNA, DNA-DNA and RNA-RNA (Figure 4). Although DNA-DNA hybridization has successfully established the cause of taxonomic structure of the genus Mycobacteria, it may not be useful to differentiate very closely related species or strains (Mc Fadden *et al.*, 1990).
Figure-4: Nucleic Acid Hybridization Techniques

iii) **PCR Restriction Fragment Length Polymorphism (PCR-RFLP) of Different Genes:** PCR-RFLP methods have provided newer approaches for eliciting the genetic differences among species, subspecies, and strains of Mycobacteria. Main regions targeted are: 16S-23S rRNA internal transcribed spacer (ITS) gene region, 1.8 kb rDNA (Katoch et al., 2007), hsp65 gene region (Telenti et al., 1993), rpoB gene region (Kim et al., 2001) including 16S rRNA (Vaneechoutte et al., 1993), 23S rRNA (Verma et al., 1994), dna J (Takewaki et al., 1994), gyr B (Kasai et al., 2000), rpo V (Comincini et al., 1998), oxy R (Sreevatsan et al., 1996), Sec A I (Zelazny et al., 2005), hup B (Prabhakar et al., 2004).

### 3.5.4 Use of Nucleic Acid Probes in Identification of Mycobacteria

Nucleic acid hybridization using molecular probes has become widely accepted in identification of Mycobacteria. Commercially available probes help in advanced identification of the *M. tuberculosis* complex. Sensitivity and specificity reach 100% when at least 100,000 microorganisms are present. Different probes have been used for identification including total DNA probes, probes based on rRNA genes, DNA probes based on sequences of protein antigens in Mycobacteria, and cloned fragment probes (Katoch et al., 2004).
3.5.5 Chemical Composition Based Methods

Species of Mycobacteria can be identified by several methods as follows:

- **Lipid profile:** These techniques have been developed for simple and rapid analysis by which isolates can be rapidly identified from liquid/solid medium (Katoch and Sharma 1997).

- **Protein Profile:** The protein Lactate Dehydrogenase (LDH) pattern of Mycobacteria is used for identification at the species level (Sharma et al., 1995).

3.5.6 Bacteriocins

Bacteriocins are proteins that are lethal for bacteria closely related to the strains producing them. Bacteriocins have been used for successful identification and characterization of Mycobacteria by many workers (Takeya and Tokiwa 1972).

3.5.7 Immunological Tests

i) **Seroagglutination:** Serological techniques can be used for identifying and characterizing various species of Mycobacteria from patient samples directly (Schaefer 1965). This test mainly depends upon the reaction of single or multiple antigenic components of bacteria with antibodies against them (Mukherji et al., 2006).

3.6 Epidemiology

Tuberculosis is one of the oldest diseases known to mankind with worldwide distribution. Currently it is estimated that one third of the world’s population is infected with the TB bacillus and 8.6 (8.3 to 9.0) million new cases occur annually, equivalent to 122 cases per 1,00,000 population. Approximately 1.3 million deaths can be attributed to the disease every year. In the 8.6 million incident cases, it was estimated that 0.5 million (6%) were children and 2.9 million (range, 2.7 – 3.1 million) (34%) occurred among women. Two-thirds of TB patients are between the age group of 15-44 years, with a male/female ratio among newly detected cases of 1.7 ranging from 1.0 to 2.1 globally (WHO, 2013). The burden of TB is highest in Asia and Africa. About 58% of cases are in the South-East Asia and Western Pacific regions.
3.7 **TB Burden In India**

38% of the world’s TB cases occur in SEAR nations, with India having the highest number of TB cases among these countries. India contributed 23% of the estimated global incidents of TB cases (WHO Annual TB Report 2015). Approximately 3.4 million newly diagnosed cases are estimated to occur each year and 4.4 lacs deaths were reported due to TB in 2013, mainly from 5 countries (WHO Annual TB Report 2015). According to WHO’s 2014 report, incidence rates were 133/lac population and prevalence rates were 174/lac population. Globally, notifications of newly diagnosed TB cases represented 63% (95% uncertainty interval, 60–66%) of estimated incident cases in 2014 (WHO Annual TB Report 2015).

3.8 **Clinical Presentation of TB**

The clinical presentation of TB depends on a number of factors and is therefore quite variable. TB can be classified in two types: **Pulmonary** and **Extra pulmonary**, based on the clinical manifestations. PTB is more common but EPTB is also a major concern.

3.8.1 **Extra-pulmonary Tuberculosis**

Extra pulmonary tuberculosis is defined as the occurrence of TB at body sites other than the lung. However, as per WHO guidelines, when an extra pulmonary focus is present in a patient with pulmonary tuberculosis, such a patient should be categorized under pulmonary tuberculosis (Sharma and Mohan 2004). Extra pulmonary TB involves areas that are highly vascular like meninges, kidney, vertebral column, bones, lymph nodes etc. The other sites which may be involved are pleura, peritoneum, pericardium, liver, gastro-intestinal tract and genito-urinary tract.

Diagnosing EPTB is tougher than PTB as it is comparatively less common (Sharma and Mohan 2004). Moreover, EPTB involves sites which are not easily approachable, are paucibacillary and even few bacilli can cause significant disease. The bacteriologic confirmation of a diagnosis becomes more difficult owing to two factors: i) small numbers of bacilli and ii) inaccessible sites, and therefore to confirm the diagnosis of EPTB, invasive methods have to be resorted to.
3.8.1.1 Female Genital TB

FGTB was first reported by Morgagni (1744) during autopsy on a 20-year-old girl, who was known to have died of tuberculosis peritonitis (Janjua et al., 2010). A century later, studies were conducted on the course and treatment of the disease. The disease presents with various manifestations and it occurs as a consequence of primary disease somewhere else in the body. A number of patients may remain without any symptoms. *Mycobacterium tuberculosis* is the causative agent in 90%–95% of female genital tuberculosis cases (Ahmadi et al., 2014); 5%–10% of cases are caused by *Mycobacterium bovis* especially when acquired from gastrointestinal tract (Swarnagowri 2013).

3.8.1.1.1 Epidemiology

Incidence of pulmonary and extra-pulmonary TB cases has decreased in developed countries over the last decade. However, in developing countries, there has been an increase in the incidence of EPTB and PTB, including drug-resistant forms, due to the emergence of HIV/AIDS cases (Arora et al., 2003).

The true prevalence and incidence of the disease in the community cannot be determined accurately because the disease is asymptomatic in a large number of patients, and is usually discovered incidentally or may remain undiscovered (Goldin et al., 1985). The reported frequency of GTB is based on postmortem examination, surgical specimens and endometrial biopsies from sterility studies. The incidence also varies according to the socioeconomic and public health conditions. Therefore, there is great variation in data published from different countries. Incidence of disease varies depending upon geographical location. In developed nations, less than 1% incidence has been reported of GTB cases but the incidence is 15-19% in some African countries and India (Thangappah et al., 2011). Schaefer reported 5-10% incidence of GTB in females complaining of infertility, worldwide. (Schaefer et al., 1976). In countries like Malaysia and Thailand, pelvic tuberculosis is an uncommon gynecological problem and is seen in only 0.03-0.05% of gynecological cases (Sivanesaratnam et al., 1986; Weerakiet et al., 1999). High incidences of genital tuberculosis in infertile women have been shown in studies from African countries (ref?). In Western Cape, prevalence of genital tuberculosis was found to be
7.98% (De Vynck et al., 1990). Incidence of genital tuberculosis has been estimated to range from 1 to 19% in various studies from India. Malkani and Rajani (1959) reported the incidence of genital tuberculosis to be 9.3% based on endometrial biopsies from infertile women. Deshmukh et al. (1987) reported a similar incidence at laparoscopy in 500 infertile women. Moreover, in less developed areas of the world, there is inadequacy of procedure for diagnosis of genital tuberculosis (Figueroa et al., 1996). The incidence of genital TB is also influenced by the lack of highly sensitive and specific tests to diagnose the condition (Bhanu et al., 2005).

80 to 90% of the patients of FGTB were reported to be in age group 20 – 40 years by Schaefer et al (1976). A changing trend in the age at diagnosis was highlighted by Sutherland (1979), in a large scale study done during 1951-1980 on 704 patients with FGTB. The mean age was found to be 28.2 years in the initial 10 years as compared to 38.9 years in the last decade of study. 29.2% cases of female genital tuberculosis were reported to be older than 40 years of age from a series from Turkey. However, 68 to 89% cases of genital tuberculosis were between 20 to 30 years of age in most large series conducted in India (Gupta et al., 1956; Devki et al., 1962; Hafeez et al., 1966).

3.8.1.1.2 Prevalence of Female Genital Tuberculosis

Genital TB is the most common causative factor for infertility in a significant number of females presenting with infertility (Muir et al., 1980). There has been an increase in global prevalence of GTB from 5% to 10%. This condition is endemic in countries like India, with a prevalence rate of 3% to 39% (Khanna and Aggarwal 2011). Genital tuberculosis is 10 to 15 times more common in developing countries as a causative factor for infertility (Parikh et al., 1997). High prevalence of genital tuberculosis has been reported by various authors from African countries also. Margolis et al (1992) have reported a 6.15% prevalence of genital tuberculosis in infertile women from South Africa. A bacteriological study of 114 infertile patients revealed a prevalence of 16.7% of genital tuberculosis in Northern Nigeria (Emembolu et al., 1993).
The prevalence of GTB was observed to be higher in cases where fallopian tubes were involved. The incidence of GTB was reported to be 3% in infertility cases and 41% in tubal factor infertility cases in a study from Cuttack, India (Tripathy et al., 2002) and similar findings were reported by Sharman (1952) and Halbrecht (1959). The prevalence of tuberculosis reported was between 25% and 44%, when the tubes were occluded.

3.8.1.1.3 High Risk Groups

Genital tract tuberculosis is more common in women having a family history of TB, an episode of past infection, adnexal lump, chronic abdomen pain and, secondary amenorrhea (Tripathy et al., 1998; Gatongi DK et al., 2005).

3.8.1.1.4 Symptomatology

GTB causes chronic diseases with none or very few symptoms. The varied symptomatology seen in patients with GTB was reported by Sutherland (1985): infertility (44%), pain in pelvic region (25%), unusual bleeding (18%), leucorrhoea (5%), bleeding after menopause (2%), and rarely ascitis and abdominal mass. Most confirmed cases had a normal clinical examination and 23.6% of the cases presented with an adnexal mass. Tripathy et al. (1998) observed primary and secondary infertility (58%), pain in pelvic region (18%), and leucorrhoea (26%). Secondary amenorrhea was the predominant menstrual symptom, noted in 43% cases, followed by menorrhagia in 17% cases and oligomenorrhea in 11% cases. The most significant signs were pelvic mass in 21% cases and an unhealthy cervix in 17% cases. In about 18% of cases, tuberculous lesions were seen elsewhere in the body (Sutherland et al., 1985; Tripathy et al., 1998).

3.8.1.1.5 Mode of Spread

GTB is mainly spread by hematogenous, lymphatic or direct spread from a contiguous focus. Hematogenous spread is mainly from the lungs while lymphatic spread occurs from a primary abdominal lesion in the intestine or kidneys. Rarely, infection may be transmitted from an infected male to female leading to infection of cervix or vulva.
3.8.1.6 Pathogenesis

Genital tract tuberculosis almost always occur secondary to tuberculosis infection in another part of the body. Though involvement of lungs is most common, other organs (e.g. kidneys, gastrointestinal tract, bones or joints) may be the primary focus of infection. Primary genital tuberculosis has been described in female partners of males who have been affected by active genitourinary tuberculosis. In these females, cervix or vulva may be the site of involvement. Although hematogenous or lymphatic route is the usual mode of spread, direct contiguous spread from other intraperitoneal organs may also occur in a minority of patients. The fallopian tube is the most common genital site to get infected first, with subsequent spread to other genital organs in 90% to 100% cases. Other affected sites include endometrium (50%-60%), ovaries (20%-30%), cervix (5%-15%) and vagina (1%) (Rozati et al., 2006).

3.8.1.7 Organs Involved

According to Tripathy et al (Tripathy et al., 2002), the fallopian tubes are affected most frequently (90 %-100%), followed by the uterus (50 to 60%), ovaries (20 to 30%), cervix (5 to 10%), vulva and vagina (1 to 2%), and myometrium (rare).

i) Tubal Involvement: Tubal pathology differs according to the mode of infection. If the spread of infection is through the lymphatics, tubercles are observed on the surface, along with adhesions. If the spread is hematogenous in acute stage, tubercles occur deep inside, appear oedematous, swollen and red and become fibrosed during chronic stage. Fallopian tubes may be blocked in 50% of the cases, with multiple blockages and apparently thickened tubes. Occasionally, due to a block at the outer end of the tube, pyosalpinx or hydrosalpinx formation occurs along with thick walls. Both fallopian tubes are involved in majority of the cases (Tripathy et al., 2002).

ii) Endometrial Involvement: The infection approaches the endometrium from the tubes. In the endometrium, the infection occurs in the basal layer, which may not be shed during menstruation. If shed, the layer gets reinfected after menstruation due to release of bacteria from tubes. Tuberculous endometritis is common, affecting 50-70% of the women presenting with genital
tuberculosis. Even in advanced pelvic tuberculosis, calcification, caseation and fibrosis may not be seen in the uterine cavity. Occasionally the endometrial cavity is obliterated by extensive adhesions. Total destruction of the endometrium can result in amenorrhea. Tuberculous pyometra can also develop in post-menopausal women with an occluded internal cervical os (Tripathy et al., 2002).

iii) Peritoneal Involvement: Tuberculous peritonitis is often associated with tuberculosis of the pelvis. Clinically, tuberculous peritonitis can be divided into two groups. In wet peritonitis, ascites are produced due to oozing of fluid into the peritoneal cavity. Tubercles are formed all over the peritoneum. The tubes, in addition to being covered with tubercles, are enlarged and distended. This pattern is usually associated with hematogenous spread of the tuberculous organism to the peritoneal surface and pelvic organs. Another type of tuberculous peritonitis encountered is the dry or adhesive type. In this condition the bowel adheres to the bowel through innumerable dense adhesions that blend with the musculature. The muscle is also invaded, to some degree, by the tuberculous process (Rock et al., 2003).

iv) Ovarian Involvement: A tuberculous infection of the ovary is seen in about 25% cases of genital tuberculosis and usually involves only the surface of the ovary and represents an extension of the infection from the peritoneal cavity and the adjacent fallopian tubes. The infection is occasionally limited to perioophoritis; extension to the ovarian parenchyma is prevented by the tunica albuginea. Mostly ovaries appear normal by the naked eye but upon histopathologic examination, lesions are observed. However, a break in the tunica caused by ovulation may cause the bacilli to gain access into the ovarian parenchyma. In such cases, tubercles, and thickening of capsule and adhesions may be observed along with caseous material in the ovary (Rock et al., 2003).

v) Cervical Involvement: The tuberculous lesion in cervix can either be ulcerative or proliferative. Edges of ulcers are clean cut, the outline is serpigenous and the base is yellow. Ulcers are frequently found around external os. Sessile or pedunculated papillary formation occurs in
proliferative lesions. Caseation occurs with destruction of cervix (Tripathy et al., 2002; Rock et al., 2003).

vi) **Vaginal or Vulval Involvement:** It is uncommon to have tuberculosis involving the vagina or the vulva. It is seen in less than 1% of the cases with genital tuberculosis. The gross appearance may be ulcerative or hypertrophic with the presence of multiple sinuses (Tripathy et al., 2002; Arora et al., 2003).

### 3.8.1.1.8 Clinical Presentation

The most frequent presenting symptoms in patients with female genital tuberculosis include infertility, menstrual disturbances, pelvic pain, vaginal discharge and poor general condition (Farrokh et al., 2015).

Infertility is the most common manifestation in patients with GTB, with incidence of about 40-80%. Chronic pain in pelvic region is the second most common symptom in patients with female genital tuberculosis. Reported incidence of chronic pelvic pain varies between 20-50% (Gandhi et al., 2006). Pain is non-characteristic and usually localized in the lower abdomen or pelvis. Pain tends to be chronic and dull aching. Episodes of acute pain, as a result of superadded bacterial infection, can occur and require administration of antibiotics. Acute episodes of pain may occur after diagnostic procedures such as endometrial biopsy, dilation and curettage or hysterosalpingography (HSG). Patients with chronic pain are more likely to have abnormal findings on pelvic examination.

### 3.8.1.1.9 Diagnosis

There are some nonspecific tests like ESR, Haemogram, Chest X-ray and Montoux test which help in the diagnosis of GTB. Laparoscopy, HSG, ultrasonography of pelvic organs, magnetic resonance imaging (MRI), and computed tomographic (CT) scan are the newer radiological methods available for the diagnosis of tuberculosis. The most definitive diagnosis is Mycobacterial isolation in tissue but as the disease is paucibacillary, it is not possible to demonstrate *Mycobacterium tuberculosis* in every case. However, in recent times, PCR has revolutionized microbiological diagnosis.
i) **Endometrial Biopsy**

Specimen of endometrium obtained by endometrial biopsy curette, or endometrial aspiration, or dilatation of cervix and curettage of the endometrium, is the most easily obtained tissue for the diagnosis of genital tuberculosis. The appropriate time to perform such a procedure is shortly before menstruation as lesions are likely to be close to the surface of endometrium during this phase of the menstrual cycle. Gross appearance of endometrium is mostly unremarkable. However, ulcerative or atrophic endometrium, or an obliterated endometrial cavity due to extensive intrauterine adhesions may be seen in advanced cases. Microscopically, diagnosis is based upon the presence of chronic inflammatory cells with or without caseation, granulomas with lymphocytes, Langhans giant cells, and epithelioid cells. Such lesions may be focal or localized. However, typical granulations may not be seen in all cases. In a series of 1000 cases of tuberculosis endometritis, Bazaz-Malik *et al.* (1983) noted discrete granulomas and caseation in only 60% cases. They suggested that the presence of dilated glands, destruction of epithelium, and inflammatory exudates in the lumen, should serve as additional criteria for the diagnosis of tuberculosis endometritis. Malkani and Rajani (1959) suggested that focal collection of chronic inflammatory cells, or the presence of a proliferative endometrium in the premenstrual week in a patient with past history of tuberculosis, would favor a diagnosis of female genital tuberculosis.

The pelvic involvement is not ruled out by a negative endometrial biopsy since sampling errors are common and the disease may have involved other pelvic organs without associated tuberculosis endometritis.

ii) **Ultrasonography**

Ultrasonography, being non-invasive with no radiation hazard, has been increasingly used in evaluating pelvic and other abdominal masses. Lee *et al.* (1983) described sonographic features of tuberculosis endometritis in a 59-year old female. Demonstration of bilateral, predominantly solid, adnexal masses containing scattered small calcifications is highly indicative of tuberculosis involvement.
iii) Laparoscopy

Laparoscopy is a well recognized procedure in the diagnostic work up of patients with infertility. Laparoscopy provides direct visualization of the pelvic organs and peritoneal surfaces and at the same time helps in establishing tubal patency. Laparoscopy has been found to be a superior method of bacteriological sampling, since the laparoscopic collection is done under direct vision (Sweet et al., 1979; Wolner Hanssen et al., 1983; Goagte et al., 1994). Three clinical forms of tuberculosis of the uterine appendages are distinguished: latent or minor inflammation, marked inflammation with tubo-ovarian lesion, and tuberculomas. Early/latent tuberculosis does not produce tubal or peritoneal changes. Evidence of acute infection by laparoscopy is: small military tubercles, T-O mass, peritoneal congestion, and swollen and reddened serosa of uterus and tubes. Chronic infection manifests as thickened tubes, terminal hydrosalpinx with retort shaped tubes, flimsy adhesions in the Pouch of Douglas (POD), and intravasation and extravasation on chromo perturbation (Nagpal et al., 2001). Confirmation of diagnosis with laparoscopy alone is insufficient (Wolfe et al., 1979). Genital tuberculosis presents unique diagnostic challenges including obvious clinical manifestations that may be overlooked at laparoscopy during early stages of infection (Bhanu et al., 2005). A number of conditions may be discovered during laparoscopy in these cases. These include endometriosis, pelvic inflammatory disease or fibroids.

iv) Tuberculin Test

Tuberculin reactivity was first described during a search for a remedy for tuberculosis by Koch in 1891. The characteristic dermal reaction induced by old tuberculin, however, was utilized by Von Pirquet (1907) in epidemiological studies as an indicator of past infection by tubercle bacillus (Turk et al., 1987). There are three main tests currently in use: the Mantoux test, the Heaf test and the Tine multiple puncture test. The Heaf test is mostly preferred to test a large group of people as it is quick, easy to perform, inexpensive, and reliable. The Mantoux test is preferred when a precise measurement of tuberculin sensitivity is required. The Tine test is not recommended as it is considered to be unreliable by some authorities. The sensitivity and specificity of Mantoux test was found to be 55% and 80% in women.
diagnosed with tuberculosis through laparoscopy in a study by Raut et al. (2001). Pelvic focal reaction was found to be absent in all groups including infertile women with positive Mantoux test. The authors concluded that Mantoux test has limited utility in diagnosis of active genital tuberculosis during child bearing age. However, laparoscopy is advocated early in infertile women with positive Mantoux test.

v) Microscopy

Mycobacteria are characterized by their acid-fastness, which depends on cell wall composition and integrity i.e. killed Mycobacteria may not be acid-fast. The AFB smear is an essential adjunct in the diagnosis of tuberculosis, although it is less sensitive than culture.

vi) Acid Fast Stains

Mycobacteria possess cell walls that contain mycolic acids, which are long chain and multiple cross-linked fatty acids. These mycolic acids probably serve to complex basic dyes, contributing to the characteristic acid fastness that distinguishes them from other bacteria. Acid-fastness is affected by the age of the colonies and the medium on which growth occurs. Rapidly growing species appear to be acid-fast variable. Robert Koch (1882) used hot alkaline methylene blue as the primary stain and vesuvin (Bismarck brown) as decolouriser and counterstain. Ehrlich (1882) used fuchsin as the primary stain, aniline as the mordant and a mineral acid as the decolourizer. Ziehl (1882) used phenol as mordant. Also in 1882, Rindfleisch heated the slide instead of putting it in hot water. Finally, Neelsen (1883) combined Ehrlich’s fuchsin stain with Ziehl’s mordant (Bishop and Neumann 1970). Thus, the Ziehl-Neelsen stain as we know today should properly be called the Ehrlich-Ziehl-Neelsen stain.

vii) Culture

Specimens submitted for culture of Mycobacteria usually contain many other organisms which grow in one or two days and, within a week, their overgrowth could cover the entire surface of the medium, digesting it before the Mycobacteria start to grow. Treatment of such material should be carried out in order to destroy these organisms while preserving the Mycobacteria. Some specimens such as
endometrial biopsy (EB) material, endometrial aspirate (EA) fluid, pouch of Douglas fluid, Cerebrospinal fluid (CSF) and certain pleural fluids are most likely to be free from contaminating bacteria and therefore should not be treated by any chemical agents before culture. They can be placed directly onto the culture media, but for specimens like EB material, sensitivity is increased if they are centrifuged and the sediment is inoculated onto the culture media. The probability of finding bacilli is higher by culture than by microscopy when specimens contain only a small number of Mycobacteria. Many different culture media have been devised for growing the tubercle bacillus.

viii) **Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA is an assay based on the measurement of enzyme labeled antigen, hapten or antibody. Anti-Mycobacterial antibodies are not present in healthy individuals. However, non-apparent or abortive infections due to Mycobacteria are more frequent than suspected. IgM antibodies are frequently observed, in particular, after a contact inherent to professional occupations or to adverse social conditions. A positive IgM test in the serum has been found to be most useful in latent pulmonary and extra pulmonary tuberculosis, primary infection, and prognosis of relapses. The production of IgA antibodies is mostly independent from the production of IgG antibodies. IgA antibodies easily form complexes with antigen and are readily detected in the serum of some apparently healthy individuals who are at risk, in the sputum sample of some patients suffering from pulmonary tuberculosis infection, and in biological fluids of those patients who are suffering from extra pulmonary tuberculosis.

ix) **Polymerase Chain Reaction**

Microscopy and culture techniques are not adequate for diagnosing EPTB in adults, or all forms of tubercular infections in children. The most rapid and sensitive technique for identifying *Mycobacterium tuberculosis* at the species level is PCR. PCR is a method for amplification of specific nucleic acid sequences by use of repeated cycles of DNA synthesis and involves a three step process including denaturation, annealing and extension. It has several advantages over the existing conventional diagnostic techniques for Mycobacterial infections. The *IS6110*
insertion sequence of *Mycobacterium tuberculosis* is a useful genetic marker that is now in wide use, serving as the basis for research in diagnostic applications of PCR, and also serving as a marker to be used in molecular epidemiological investigations using restriction fragment length polymorphism analysis (Desikan and Narayanan 2015). As the copy number of the target sequence is an important determinant of PCR sensitivity, it would be lower for those strains having only a low copy number of *IS6110* (Singh *et al.*, 2006). Therefore, it has become necessary to evaluate PCR protocols based on other genes of *Mycobacterium tuberculosis* in developing countries like ours, as India accounts for a large proportion of tuberculosis cases. In several studies, other genes like *MPB64*, *ESAT-6* and *hupB* gene have been used as a target for PCR amplification (Bhanu *et al.*, 2005; Rozati *et al.*, 2006; Kumar *et al.*, 2008). The *hupB* gene codes for a histone-like protein which allows differentiation of two closely related species, namely *M. tuberculosis* and *M. bovis* of the MTB complex (Prabhakar *et al.*, 2004).

x) **Reverse Transcription PCR (RT-PCR)**

Either DNA or RNA could be the template for a PCR reaction. Usually, DNA is chosen as a template for genomic studies, but for gene expression studies, RNA is the suitable template. For detecting structural changes in long genes, RNA can be better than genomic DNA. Reverse transcription PCR combines cDNA synthesis with PCR so as to provide a rapid and sensitive method for analyzing expression of genes. Real time PCR is used to quantitatively determine mRNA expression levels in a sample. Either total RNA or poly (A)+ selected RNA could be the template for RT-PCR and these reactions could be primed with random primers, gene-specific primers (GSP) or oligo(dT) primers using a reverse transcriptase. RT-PCR reactions can be either two-step or one-step. Reverse transcription and PCR take place sequentially in a single tube under conditions optimized for both RT and PCR in one-step RT-PCR, while cDNA synthesis is performed first in RT buffer and then PCR is carried out in the second step in a two-step RT-PCR.

xi) **Real-time PCR**

In real time PCR, detection and quantification of amplified PCR products is based upon the incorporation of a fluorescent reporter dye with the fluorescent
signal increasing in direct proportion to the amount of PCR product produced. This fluorescent signal is monitored at each cycle of the PCR. Data is collected throughout the PCR process rather than at the end of the PCR. This has completely revolutionized PCR-based DNA and RNA quantitation (Arya et al., 2005).

In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected. An early rise in fluorescence is observed when the starting copy number of the nucleic acid target is higher. In contrast, in an endpoint assay, the amount of accumulated PCR product is measured at the end of the PCR cycle.

3.9 International Studies for Comparison of the Various Methods for Microbiological Diagnosis of TB

Limited international studies have been conducted on extra pulmonary tuberculosis. These are reviewed below:

In a study by Jahromi et al. (2001), a total of 3088 TB cases were retrospectively studied. FGTB accounted for 1.32% of all tuberculous patients. 75.6% of these were infertile. TB endometritis was detected in 72.03% of the patients, tubal involvement was present in 34.03%, ovarian TB was present in 12.9%, and cervical TB in 2.4% of the patients. Abebe et al. (2004) did a comparative evaluation of different methods for the diagnosis of female genital tuberculosis. 25 clinically suspected cases of FGTB were investigated with histopathology, AFB smear microscopy, culture for TB, and PCR. Among these, only one sample was found to be AFB smear positive, 3 were found to be culture positive, 7 were histology positive and 12 were PCR positive (a total of 16 positives). A combination of PCR with the other methods had a higher sensitivity and specificity for the diagnosis of FGTB.

El-Adawy et al. (2006) studied the frequency of genital tuberculosis among 150 infertile women undergoing laparoscopic evaluation and assessed different diagnostic methods of genital TB. The incidence of genital TB as diagnosed by laparoscopy was found to be 8% among the study group; the incidence of positive
Review of Literature

patients was higher among rural patients having low socioeconomic and educational levels. Laparoscopic findings, confirmed by biopsy and BACTEC, showed high sensitivity and specificity (93-95%) as compared to endometrial biopsy (81-83%) and culture in LJ (72-76%) for diagnosis of genital TB. Moreover, BACTEC had a shorter detection time than LJ culture. The authors concluded that the new automated culture techniques have significantly reduced the detection time and antimicrobial testing.

Magana-Arachchi et al. (2008) used an in-house PCR method for diagnosis of extra-pulmonary tuberculosis in 465 clinical samples. Among 373 patients with suspected EPTB, 75 specimens were positive by PCR, 4 were positive by microscopy and 6 were found to be positive by culture. Of the 25 PCR-positive patients, 95% had a final diagnosis of TB. The DNA amplification method was found to be a rapid method having greater sensitivity as compared to culture and microscopy for a reliable final diagnosis of EPTB.

Ani et al. (2009) examined HIV-positive patients (101) and clinical specimens from children (40) using ZN smear microscopy, LJ culture and IS6110 PCR for the detection of MTB. 45/101 (44.6%) of the specimens from the HIV-positive patients were positive for Mycobacteria and comprised of 6% ZN positive, culture positive, PCR positive; 4% ZN negative, culture positive, PCR negative; 16% ZN negative, culture positive, PCR positive; and 19% ZN negative, culture negative, PCR positive. 22/40 (55%) children were positive with 0% smear microscopy; 4/40 (10%) were culture positive, PCR positive; and 18/40 (45%) were culture negative, PCR positive. The sensitivity of the PCR for the HIV-positive patients was 85% and the specificity was 74% against 23% and 100% for ZN smear microscopy. PCR targeting IS6110 was found to be a rapid and sensitive method specific for the MTB complex group and the authors suggested its use for the detection of MTB in high TB and HIV burden areas.

Khorshidi et al. (2009) evaluated the IS6110 PCR technique for the detection of MTB in sputum samples, and calculated its sensitivity and specificity. A total of 248 sputum samples from patients suspected of Mycobacterial diseases were
studied by the PCR method using a specific pair of primers designed to amplify 123bp and 245bp sequences of the insertion sequence, \textit{IS6110}, in the \textit{MTB} genome. 32 (12.9%) samples were found to be positive by culture. PCR yielded a sensitivity of 93.8\% and specificity of 99.1\% for the diagnosis of TB, when diagnosis was confirmed by culture. 2/32 (6.3\%) cases were PCR-positive among the patients with non-TB disease. \textit{IS6110} PCR assay was found to be important in the rapid diagnosis of TB in this study.

\textbf{Massoud et al. (2009)} examined 2123 specimens from 2006 to 2008. All the specimens were stained for AFB, cultured and tested using PCR. 113 patients were found to be positive for TB. Of these 113 patients, 48 had pulmonary tuberculosis and 65 had extra-pulmonary tuberculosis. PCR, culture and staining positivity rates were 41\%, 23\% and 12\% respectively in pulmonary TB cases, and 46\%, 26\% and 14\% respectively in extra-pulmonary tuberculosis. When compared to traditional methods, PCR was found to have a higher sensitivity for the diagnosis of TB.

\textbf{Khosravi et al. (2010)} evaluated the diagnostic value of nested PCR in GUTB, compared with AFB staining and culture method. 200 urine samples from suspected cases of GUTB were collected and were used for smear preparation, culture, and DNA extraction for PCR targeting the \textit{IS6110} gene fragment. The positivity rate was found to be 5.0\% using the culture method and PCR methods, and 2.5\% using the AFB staining method. 2\% of the samples showed positive results in all three methods. PCR sensitivity was estimated to be as high as culture method sensitivity: both equalled to 100\%. The sensitivity for direct smear staining was 41.6\%. Due to identical detection rate of culture and nested PCR methods, it was suggested that PCR is a rapid alternative to culture especially in confirmed cases of GUTB.

\textbf{Akram et al. (2012)} conducted a study on 43 CSF specimens from tuberculous meningitis patients. 76.7\% were found to be positive by PCR, whereas only 11.6\% were positive by acid-fast microscopy, and 55.2\% were culture positive. No positive results were found by acid-fast microscopy, culture, and PCR in the non-tuberculous group which was used as a control. PCR was found to be extremely useful for the diagnosis of tuberculous meningitis in this study.
Zakham et al. (2012) compared the utility of the IS6110 PCR to conventional methods for rapid detection of MTB in clinical specimens. 305 patients with different clinical manifestations were enrolled in this study and tested by both conventional methods and PCR techniques for detection of MTB. The in-house IS6110 PCR was found to have 92.4% sensitivity and 98.0% specificity with a PPV and NPV of 96.4% and 95.3%, respectively.

3.10 Studies Conducted in India to Compare the Various Methods for Microbiological Diagnosis of TB

Pahwa et al. (2005) analyzed 100 cases in which Fine Needle Aspiration Cytology (FNAC) was conducted. The prepared smears were stained using Giemsa, Ziehl–Neelsen’s, Kinyoun and Papanicolaou stains. Parts of the aspirated materials were assessed by fluorescent staining, culture and PCR methods. 74% and 22% of aspirates were positive by fluorescent stain and culture, respectively. PCR was done in 55 cases, out of which 22 (40%) were found to be positive. The sensitivity of PCR was found to be 89.5% and specificity was 86.1%. Of the conventional methods, fluorescent stain was the most sensitive (81.8%) but it had poor specificity (28.2%). PCR detected 80% of smear-negative but culture positive cases.

Bhanu et al. (2005) studied 61 samples consisting of endometrial aspirates, endometrial biopsies, and fluid from the pouch of Douglas from 25 infertile women. The MPB64 gene PCR was performed and results were correlated with laparoscopy findings. 56.0% of the patients were positive for MTB DNA, compared to 1.6% with AFB staining and 3.2% with culture method. The MTB DNA was detected in 53.3% of EBs, 47.6% of EAs and 16.0% of POD fluid samples. All patients with laparoscopy suggestive of TB, 60% of those with a probable diagnosis, and 33% of those with incidental findings were positive by PCR. The authors concluded that amplification of the MPB64 gene by PCR offered greater sensitivity in determining tuberculous infection in female infertility cases.

In a study by Rozati et al. (2006), biopsies or curettage samples from 65 women who were suspected to have genital tuberculosis were investigated with AFB smear microscopy, histopathology, culture, and PCR for MTB using ESAT-6 gene
primers. Among these, 8 were AFB smear positive, 12 were positive for culture, 17 were found to be positive for histology, and 28 were PCR positive. For achieving a high sensitivity and specificity, a combination of PCR with other conventional techniques was found to be the best method for the diagnosis of FGTB.

In a study by Singh et al. (2006), a total of 81 samples of lymph node biopsies from clinically suspected cases of tuberculous lymphadenitis were examined for AFB, culture on LJ medium, and simultaneous use of two PCRs targeting IS6110 and MPB64. Positivity with MTB culture and AFB was 13.6% and 28.4%, respectively. All samples that were culture positive for nontuberculous Mycobacteria were negative by both PCR systems. A higher proportion of positive results were observed using PCR targeting IS6110 (56/81 (69.1%) samples showed positive results) as compared to PCR targeting MPB64 (39 of 81 (48.2%) samples showed positive results). When combined, 63/81 (77.8%) samples were detected positive for MTB DNA. However, 7/81 (8.6 %) samples remained negative by IS6110 but positive by MPB64 method. These data suggest that the use of one additional PCR (along with the IS6110 system) can reduce false negativity of PCR results in samples harboring zero copy of the IS6110 element, a situation that is known to exist in the Indian population.

Oberoi and Aggarwal (2007) conducted a study on 230 pulmonary and EPTB samples. Samples were detected by ZN smear, LJ culture, BACTEC culture, and PCR. The sensitivities of these tests were found to be significantly different from one another. The sensitivity of PCR, ZN smear, LJ culture and BACTEC culture were 73.9%, 34.78%, 52.17% and 58.69%, respectively. However, these techniques did not differ significantly with respect to specificity values (p>0.05). In pulmonary and extra-pulmonary clinical samples, PCR sensitivity was 74.0% and 78.5%, respectively; significantly higher when compared to other tests (p<0.05). The mean detection time was 24.03 days by LJ medium culture, 12.89 days by BACTEC culture, and <1 day by PCR test for MTB.

Sekar et al. (2008) compared IS6110 PCR and conventional techniques for diagnosis of EPTB. 191 EPTB samples and 17 non-tuberculous samples (as
controls) were included. All the samples were processed for AFB smear while 143 samples were processed for *MTB* culture. All the samples were PCR-amplified with *IS6110* primers. 18% of the samples were AFB smear positive, 22% of the samples were culture positive, 27% samples were positive either by smear or culture for AFB, and 63% were PCR positive. Of the 140 samples that were negative by both conventional techniques, 74 (53%) samples were positive by PCR alone. Of the 51 samples that were positive by conventional techniques, 46 (90%) were found to be positive by PCR. Authors concluded that *IS6110* PCR was useful in the diagnosis of EPTB, where there is a clinical suspicion of the disease, especially in those cases when the conventional techniques are negative.

In a study by Kumar *et al.* (2008), 393 patients with various gynecological complaints were investigated for *MTB* infection using AFB microscopy, culture, nested PCR, and histopathology. Among the four methods, N-PCR assay was found to have the highest sensitivity (31.3%) for the detection of *MTB*. AFB smear microscopy showed 5.1% sensitivity while sensitivity for detection by the culture technique was 4.2%. The least sensitive was histopathological examination for granulomatous tissue reactions compatible with tuberculosis infection (2.4%).

Chawla *et al.* (2009) evaluated the PCR efficiency in 104 tissue samples compared to histopathology. The sensitivity and specificity of the PCR method were 74.1% and 96.1%, respectively. False positive and false negative rates were 2.88% and 6.73%, respectively. Substantially good agreement between histopathology and PCR was observed (0.737). It was concluded that PCR can be used for rapid diagnosis of TB in tissue samples so as to initiate timely anti-tubercular treatment.

Iqbal *et al.* (2010) evaluated 205 TB suspected patients by ZN smear examination, LJ culture, and PCR test. A significant difference in the sensitivity of the different tests was found. The sensitivities for PCR, LJ culture, and ZN smear were 67.32%, 27.81%, and 12.20%, respectively. PCR test sensitivity was found to be 77.15% and 61.6% in pulmonary and extra-pulmonary clinical samples, respectively. This was significantly higher when compared to the sensitivity of other tests.
In a study by Khanna et al. (2011), a total of 100 women with a history of infertility were studied from December 2006 to June 2008. Of these, 58 women had primary infertility and 42 had secondary infertility. 26/100 infertility patients were found to be positive for TB by PCR. Of these, only three patients had positive results by AFB culture, a method that is considered to be the gold standard technique for tuberculosis detection. One patient was AFB culture positive but PCR negative for TB. PCR was found to have 75% sensitivity. 88.46% of patients with genital tuberculosis would have been missed if TB PCR of the endometrial biopsy was not conducted in this study.

Rana et al. (2011) evaluated mRNA-based RT-PCR and DNA-PCR in various specimens collected from infertile patients. A total 200 infertile women were studied from 2006 to 2008. RT-PCR and culture results were found to be concordant. DNA-PCR showed high positivity when compared to AFB smear microscopy. 44.85% EA specimens, 9.57% PF/PW specimens, and 33.33% CB specimens were found to be positive by DNA-PCR. Genital TB can be picked up early by DNA-PCR, when it can be completely cured.

Jain (2011) conducted a study on cerebrospinal fluid, ascitic fluid, and lymph node fine needle aspirate samples from 300 patients with EPTB, who were suspected cases of tubercular meningitis, tubercular ascites, and tubercular lymphadenitis. hup B gene PCR was used. Clinical response to anti tubercular therapy was taken as the gold standard. hup B gene PCR was found to be positive in 147/155 anti tubercular treatment (ATT) responders, 85.71% of whom were infected with MTB, 9.52% with M. bovis alone and 4.76% with both MTB and M. bovis. The sensitivity and specificity of PCR was found to be 90.32% and 94.48%, respectively.

In a study by Shukla et al. (2011), the sensitivity and specificity of PCR was compared with smear and culture for diagnosis of pulmonary and EPTB suspected cases. 74 sputum, 38 endometrial biopsies, 16 CSF, and 12 gastric aspirate specimen were included, all of which were tested by ZN staining, LJ culture, and IS6110 PCR. 28.5% patients were AFB smear positive, 34.2% were AFB culture positive, and 74.2% were sensitive to MTB nested PCR. There was a significant difference in
PCR sensitivity for smear positive and negative cases, and also for culture positive and negative cases.

**Thangappah et al. (2011)** evaluated the efficacy of PCR, culture, and histopathological examination for the diagnosis of GTB in 72 infertile women. AFB smear, culture and HPE examination was carried out on endometrial samples. PCR was done on 49 samples using primers targeting *IS6110* and *TRC4*. In 7 patients, peritoneal fluid was taken for culture and PCR. To suspect GTB, a diagnostic criterion was derived based on the clinical profile and laparoscopic findings, and the diagnostic tests were evaluated using this diagnostic criterion. Based on this, 28/49 cases were suspected of GTB. The sensitivity of PCR, HPE, and culture were 57.1, 10.7, and 7.14% based on this diagnostic criterion. These results showed that conventional methods have low sensitivity when compared to PCR, which was found to be useful in diagnosing early disease, and for confirmation of diagnosis in clinically suspect cases.

**Maurya et al. (2012)** evaluated 756 specimens from suspected cases of EPTB. These samples were tested for Mycobacteria by ZN staining, BACTEC culture, and *IS6110*-based PCR. 9.3% samples were found to be positive for AFB by ZN staining, 30.1% were positive by BACTEC culture, and 20.7% isolates were PCR positive.

**Verma et al. (2012)** assessed 170 different clinical specimens suspected of tuberculosis (100 pulmonary and 70 extra-pulmonary) by PCR using *MPB64* primer, culture, and microscopy. All specimens were processed using USP methodology for inhibitor-free PCR. TB IgG, IgM, and IgA were evaluated using PATHOZYME MYCO kit. Response to ATT on clinical follow up was considered as the gold standard. The total number of pulmonary specimens found to be positive by any of the four tests was 87/100, while that for extra-pulmonary samples was 63/70. For pulmonary specimens, the diagnostic accuracy was 88.3% for microscopy, 88.3% for culture, 67.4% for ELISA, and 94.1% for PCR. For extra-pulmonary samples, the diagnostic accuracy was 30.1% for microscopy, 49.2% for culture, 44.4% for ELISA, and 87.3% for PCR. The authors concluded that, for extra-pulmonary
specimens, PCR can be used as an effective screening tool as conventional methods are not sensitive enough.

Malhotra et al. (2012) comparatively evaluated AFB smear examination, MB7H9 culture, and real-time PCR assay based on IS6110 insertion sequence to detect Mycobacteria in various female genital samples. PCR was found to be 9-fold more sensitive than smear examination and 3 fold more sensitive than the culture method for detection of Mycobacteria. The sensitivity of PCR was found to be 94.28%.

Goel et al. (2013) conducted a retrospective study from January 2011 to October 2011 at a tertiary care hospital in Delhi, with the aim of comparing different diagnostic modalities i.e., histopathological examination, acid-fast bacilli smears, Lowenstein-Jensen culture, BACTEC culture, and DNA-PCR for diagnosing genital tuberculosis in infertile women. A total of 546 samples were taken from females with the history of infertility; 360 women presented with primary infertility and 186 women with secondary infertility. HPE for tuberculosis was positive in 13, LJ culture in 10, and AFB smear was positive in one case. BACTEC and DNA PCR were feasible for 90 patients. DNA PCR was found to be positive in 20 and BACTEC was positive in 8 patients. Out of the 20 patients with PCR positive results, 15 were only PCR positive and were subjected to hyster-laparoscopy; five had evidence of tuberculosis. Histopathology and LJ culture still have an important role in the diagnosis of endometrial tuberculosis in government setups where BACTEC and PCR are not performed routinely due to lack of resources.

Siddiqui et al. (2013) compared the sensitivity, specificity, and turnaround time of BACTEC culture and PCR test for the diagnosis of EPTB. 5% of the extra-pulmonary samples were positive by ZN staining, 15% were positive by both LJ culture and BACTEC MGIT 960 TB culture, and 70% were positive by PCR. Samples positive by ZN smear, LJ culture, and BACTEC culture were all 100% positive by PCR. The mean detection time was 23.13 days by LJ culture, 9.86 days by BACTEC MGIT 960 TB culture, and <1day by PCR. PCR was found to be more
sensitive and rapid in diagnosis of EPTB as compared to conventional methods or BACTEC culture resulting in early initiation of anti-tubercular treatment.

**Makeshkumar et al. (2014)** evaluated the use of PCR in the diagnosis of definitive and probable EPTB patients. The performance of *IS6110* based PCR assay was evaluated in comparison to LJ culture for the diagnosis of EPTB. 178 non-repeated clinical specimens from clinically suspected EPTB patients were studied, all of which were subjected to AFB staining, LJ culture, and PCR based on the *IS6110* insertion sequence of MTB. 5.61% specimens were AFB smear positive. Out of 10 AFB smear positive cases, 3.37% specimens were LJ culture positive. 26.96% specimens were *IS6110* PCR positive for *MTB*. *IS6110* PCR was able to pick up more EPTB patients as compared to LJ culture for detection of *MTB*. A limitation of this study was false positive PCR results in three CSF samples, possibly due to latent TB infection.

In a study by **Bhanothu et al. (2014)**, a total of 302 specimens were processed. Of these, 202 women were infertile, highly suspected of having GTB on laparoscopic examination, and 100 were control women of reproductive age. 49.67% were premenstrual endometrial tissue biopsies (ETB), 31.46% were ovarian tissue biopsies (OTB), and 18.87% were pelvic aspirated fluids (PAFs). All specimens tested by conventional methods were compared with multi-gene PCR method using four primer sets for the detection of *MTB* DNA, and correlated with laparoscopic results. *MTB* DNA was present in 49.5% of ETBs, 33.17% of OTBs, and 5.44% of PAF specimens. All control women were confirmed as negative for TB infection. The conventional methods showed very high specificity with low sensitivity varying from 21.78% to 42.08%. H & E staining was found to have a sensitivity of 51.48%. Multi-gene PCR method had a sensitivity of 70.29% with *MPB64* gene, 86.63% with *19kDa* antigen gene at species and *TRC4* element at regional *MTB* complex level, and 88.12% with *32kDa* protein gene at genus level. The specificity of multi-gene PCR was 100% and it was found to be a better technique for diagnosis and differentiation of infection caused by Mycobacteria.
Shrivastava et al., 2014 carried out a study to compare the PCR technique, AFB culture, and AFB staining. A total of 227 aseptically collected endometrial tissue samples were processed. Out of 227 patients suspected of GTB, 133 were found to be positive either by AFB smear microscopy, culture, or PCR. 2/133 (1.5%) samples were found to be positive by all three methods, i.e. microscopy, culture, and PCR, 11 (4.8%) were found to be positive by both PCR and culture, whereas 126 (86%) samples were found to be positive only by PCR. The PCR failed to detect seven cases that were positive by the conventional culture method. The conventional methods of diagnosis like microscopy and culture are less sensitive when compared with PCR. PCR also helped in early diagnosis of infection. There are no gold standard methods in diagnosis of GTB with which PCR results can be compared.

Various methods have been described for the diagnosis of TB. These are smear microscopy, culture, and PCR. AFB smear microscopy has very low sensitivity, detecting $<10^4$ bacilli/ml. The culture method can detect 100 bacilli/ml, while PCR can detect 1-10 bacilli/ml. Various PCR assays vary in their sensitivity to detect TB. IS6110 is the most commonly used PCR target. However, many studies have reported low or zero copy number of these elements, which in turn can lead to false negative results. Other targets like ESAT-6 and MPB64 have also been tried with good results. Use of two PCR assays simultaneously has been reported to increase the positivity. Hence, we decided to use three PCR assays simultaneously in genital TB suspected cases to increase positivity, along with conventional AFB smear microscopy and culture. This helped in identifying the most efficient combination of tests for the detection of TB.