SUMMARY AND CONCLUSION

*Mycobacterium tuberculosis* causes TB, which is one of the oldest known diseases. It is one of the most common diseases of public health importance, has worldwide distribution, and has a high incidence in developing countries. 38% of all TB incident cases around the world are from WHO–SEAR countries. India is the largest country in the SEAR, having the highest number of TB cases, and accounting for 23% of the estimated global TB incident cases in 2013 (WHO Annual TB Report 2015). As per the 2014 WHO report, prevalence and incidence rates of all forms of TB were 174 and 133 per 1 lac population, respectively, showing a decline compared to previous years (WHO Annual TB Report 2015).

Pulmonary tuberculosis is the most common form of tuberculosis, but extra pulmonary tuberculosis (EPTB) is also a major clinical problem. Genital tuberculosis is a type of extra pulmonary tuberculosis which affects the genital organs. It represents 15-20% of all extra pulmonary tuberculosis cases, and is the second most common infection site after pulmonary tuberculosis (Arora *et al.*, 2003; Bapna *et al.*, 2005; Gatongi *et al.*, 2005).

Female genital TB (FGTB) is a type of extra-pulmonary TB which affects the female genital organs, with Fallopian tubes being the most commonly affected (90%), followed by endometrium (50%), and ovaries (10–30%) (Rana *et al.*, 2011). It is almost always secondary to a tubercular lesion found elsewhere in the body.

FGTB is the main cause of infertility in about 5–16% of infertile women in India. However, the exact incidence rate of FGTB remains unknown because the majority of cases are not diagnosed due to asymptomatic presentation and paucity of investigations (Malhotra *et al.*, 2012).

GTB has been a diagnostic dilemma for clinicians for decades due to the latency of the organism, asymptomatic and varied presentation in majority of the cases, and paucity of an accurate diagnostic modality. However, with the advent of newer diagnostic modalities, there has been an increase in the number of cases of genital tuberculosis that are being recognized.
An early diagnosis of TB still depends on the demonstration of acid-fast bacilli (AFB) using microscopy and/or their isolation by culture methods. Microscopy is less sensitive, requiring $10^4$ bacilli/ml for TB diagnosis (Lima et al., 2008). Histopathological examination is also of limited use in diagnosing TB infection, as reported in various studies (Chakrabarti et al., 1998; Rana et al., 2011). Culture methods are still the gold standard for the detection of genital TB, but slow growth of most pathogenic Mycobacteria (3 to 6 weeks) results in an unacceptable delay in diagnosis and treatment. Moreover, traditional AFB culture on LJ medium has a low detection rate. Colonies are seen only if the bacillary count is $>1000$ bacilli, and it takes a longer time to yield positive results (Jassawalla et al., 2006; Puri et al., 2009).

PCR is reported to be a rapid and sensitive method for the detection of TB, capable of detecting even low DNA copies (less than 10 bacteria/ml specimen) with high sensitivity and specificity. Also, the results are available within 1-2 days (Chagas et al., 2010). Its sensitivity is so high that it can detect even $<10$ microorganisms in clinical specimens to yield a positive test result. This is a critical feature since genital TB is paucibacillary.

*IS6110*-based PCR is the most commonly used PCR assay for the detection of *MTB*. However, this technique has certain limitations as it has been reported that *MTB* isolates from certain parts of India may have no copy or very low copy numbers of the *IS6110* element, potentially leading to false negative PCR results (Chauhan et al., 2007). Other targets like *MPB64* and *ESAT-6* have also been used with varying results (Bhanu et al., 2005; Rozati et al., 2006). In order to increase PCR sensitivity, several authors have used multiple targets simultaneously for the detection of *MTB* (Kulkarni et al., 2012).

Therefore, in the present study, we aimed to evaluate the utility, and to compare the results of the different PCR assays with direct AFB smear examination and culture, for the detection of genital TB in infertility patients attending SMS and attached group of hospitals, Jaipur. Therefore, the objectives of the present study were:
1. To study the occurrence of genital TB in female infertility patients by acid fast bacilli smear microscopy, culture on LJ and Middle Brook 7H9 broth, and three PCR assays in endometrial biopsies obtained from suspected cases.

2. To compare the efficiency of three PCR assays based on different primer sets: IS6110, MPB64 and ESAT-6, for the detection of M. tuberculosis in endometrial biopsies.

A total of 300 endometrium biopsy samples were collected from patients (age group 18-45 years) complaining of infertility and suspected of FGTB. The study was conducted at the Department of Microbiology & Immunology, SMS Medical College, Jaipur, from June 2011 to December 2013. Samples from FGTB suspects were received for routine diagnosis from Mahila and Zanana Hospitals. Known M. tuberculosis isolates (10) were taken as positive controls and Non tubercular Mycobacteria (10) were taken as negative controls.

Tissue samples (endometrial biopsies) obtained in sterile saline were centrifuged at 3000g for 15 min. Supernatant was decanted and deposit was crushed with glass beads by means of a nano-electric tissue homogenizer for 5 minutes; the lysate was subjected to digestion and decontamination by 4% NaOH-NALC method (Modified Petroff’s Method) (Vestal 1977). The samples were then subjected to the following procedures:

1. Acid fast staining by Ziehl-Neelsen method.
2. Culture on Lowenstein Jensen slants and Middle Brook 7H9 liquid media.
3. Identification of M. tuberculosis by biochemical tests from positive cultures on LJ slants.
4. Detection of M. tuberculosis by IS6110, MPB64 and ESAT-6 based PCR analysis.

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the ZN staining, MB 7H9 broth, and PCR results were evaluated against the gold standard LJ culture method by bivariate two by two tables using Medcalc software. Basic measures of location (i.e. mean, median), and measures of dispersion (Standard Deviation) were calculated using Microsoft Excel.
software. Chi-square test was done for comparison of proportions using Graphpad prism version 6. The level of significance was set at 5% in all analyses.

- Out of the 300 samples received, a total of 35.33% (106/300) were positive by either of the methods used. Overall positivity by AFB smear microscopy was 2.33% (7/300), 7.33% (22/300) by LJ solid culture, 9.67% (29/300) by MB7H9 liquid culture, 29.67% (89/300) by IS6110, 24.67% (74/300) by MPB64, and 20% (60/300) by ESAT-6 based PCR.

- All the smear-positive samples were positive either by culture or PCR; 6/22 (27.27%) LJ culture positive samples turned to be negative by PCR, and 4/22 (18.18%) isolates were identified as NTM by biochemical tests. 11/300 (3.67%) samples got contaminated, and were considered negative by culture. Out of these 11 contaminated samples, 3/11 (27.27%) were positive by either of the PCR methods used.

- ZN staining exhibited 31.82% sensitivity and 100% specificity. Overall positive and negative predictive value of ZN staining was observed as 100% and 94.88%.

- MB 7H9 broth culture showed 90.91% sensitivity and 97.76% specificity. Overall positive and negative predictive value of MB 7H9 was observed as 68.97% and 99.26%.

- IS6110 PCR exhibited 72.73% sensitivity and 73.74% specificity. Overall positive and negative predictive value of IS6110 PCR was observed as 17.98% and 97.16%.

- MPB64 PCR yielded 68.18% sensitivity and 78.78% specificity. Overall positive and negative predictive value of MPB64 PCR was observed as 20.27% and 96.90%.

- ESAT-6 PCR exhibited 59.09% sensitivity and 83.09% specificity. Overall positive and negative predictive value of ESAT-6 PCR was observed as 21.67% and 96.25%.

- When IS6110 PCR results were compared with result from other techniques, IS6110 based PCR was found to be significantly better than direct smear (p<0.0001), LJ culture (p<0.0001), and MB culture (p<0.0001). Also, IS6110
PCR was found to be significantly better than ESAT-6 based PCR (p<0.05) but found to be comparable with MPB64 PCR (p>0.05).

CONCLUSIONS

• Majority of the samples from FGTB suspects were paucibacillary (smear negative/scanty smear positive).

• Culture on LJ – the “Gold Standard” technique may not be very helpful for routine diagnosis of genital tuberculosis since the disease is paucibacillary.

• The liquid culture methods are faster and have higher sensitivity as compared to solid LJ culture. However, liquid cultures are more prone to contamination and hence may require greater expertise.

• PCR has higher positivity than conventional methods viz; ZN staining, solid, and liquid culture methods.

• IS6110 based PCR missed some MTB cases as it lacks applicability for detection of low/zero IS6110 copy number MTB strains. PCRs based on other primers like MPB64 and ESAT-6 are more useful in such cases.

• We found that a combination of IS6110 and MPB64 PCR is most sensitive in diagnosing the MTB infection as compared to other combinations. Based on the results of our study, we recommend the use of more than one assay in diagnosis of tuberculosis.

• Presence of PCR inhibitors in the specimens may lead to false negative results. Therefore, a good extraction procedure yielding highly pure DNA is crucial.

• Infertility is the most common presenting symptom in genital TB suspects. Absence of any clear presenting symptom makes diagnosis of FGTB all the more difficult.

• Combination of PCR methods based on IS6110 and MPB 64 genes were found to be best combination among PCR and more rapid and sensitive than conventional methods. However, there is need to include internal control gene to rule out PCR inhibitors and need to standardize multiplex PCR so as to increase the sensitivity and reduce time taken and cost involved.
Now with availability of real time PCR systems many improvements have been made viz decrease in time taken for detection, decreased chances of cross contamination and increased sensitivity for detection of *MTB*, multiplex real time PCR can be developed for simultaneous use of different targets and also add genes for detecting NTM too.

Moreover, sensitivity of any method will depend on correct sampling for which it’s better to collect sample from site of infection.

**FUTURE PERSPECTIVES**

- The multiplex PCR to be developed should preferably be real time PCR based having internal control to verify amplification so as to exclude presence of inhibitor in the sample.
- Inclusion of genus specific primer probe and probes specific for MTB will help identify nontubercular mycobacteria and the MTB both.
- Moreover to improve sensitivity of the assay multiple samples should be collected like peritoneal fluid and endometrial biopsy.