SUMMARY AND CONCLUSION

INTRODUCTION

Tuberculosis is an ancient disease that is still a major health problem facing mankind, particularly in developing countries. The causative organism of this disease is *Mycobacterium tuberculosis* which was discovered by Robert Koch in 1882. *M. tuberculosis* is reputed to have the highest annual global mortality among all the pathogens (Sharma & Mohan, 2004). Ineffective prophylaxis against TB, increasing prevalence of HIV infection & emerging drug resistant types of TB together pose an increasingly serious public health hazard with a high economic burden for developing nations like India. TB most commonly affects the lungs, but it can affect virtually any organ. A case with TB in any site other than pulmonary is considered an extrapulmonary TB (EPTB) case. The latest estimates suggest that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths around the world. An estimated 1.1 million (13%) of the 8.6 million people who developed TB in 2012 were HIV-positive. During the last decade the HIV-TB pandemic has led to an increase in the notification of smear negative & EPTB cases. Amongst the people who were diagnosed with TB for the first time (new cases) in 2012, 2.5 million had sputum smear-positive pulmonary TB, 1.9 million had sputum smear-negative pulmonary TB, and 0.8 million had extra-pulmonary TB. The most common site of EPTB is lymph nodes. Other sites include pleura, urogenital tract, bones and joints, meninges, central nervous system (CNS), bowel and/or peritoneum, pericardium, and skin. Paucibacillary tuberculosis poses difficulty in the treatment and control of tuberculosis. Since EPTB can affect virtually all organs, it has a wide variety of
clinical manifestations, which may cause difficulty and delay in diagnosis (Chakravorty et al., 2005; Cheng et al., 2005; Galimi, 2011). Timely diagnosis and prompt treatment of infectious cases are the key elements of the international effort to combat TB, providing cure of an individual patient and reducing the spread of TB by rendering infectious cases non-infectious.

Sputum smear microscopy is the most widely used diagnostic test for TB, despite its relatively low sensitivity (especially for those with paucibacillary TB such as people living with HIV and children). The conventional technique of direct smear examination with Ziehl-Neelsen staining (ZN) is cheap and easy to perform; its low sensitivity is a major drawback. Direct smear testing detects bacilli in samples containing at least 5,000 -10,000 bacilli per ml of sputum (Lima et al., 2008) and cannot differentiate between Mycobacterium tuberculosis and non-tuberculous mycobacteria (NTM) (Liu et al., 2007; Haldar et al., 2011; Derese et al., 2012).

Concentration procedures in which a liquefied specimen is centrifuged and the sediment is used for staining increases the sensitivity of the test. Mostly specimens are homogenized with a mucolytic agent (such as N-acetyl-l-cysteine) and decontaminant (such as a 1-2% sodium hydroxide solution) to render the bacterial contaminants nonviable (Heifets et al., 1994). A fluorochrome procedure using auramine-0 or auramine-rhodamine dyes is a further improvement of the conventional smear microscopy procedure. It has increased sensitivity & can differentiate between viable and nonviable organisms (Armstrong, 2009).

Isolation of mycobacteria from clinical specimen provides a clear and definitive diagnosis of TB. Culturing mycobacteria is tough as it is a fastidious
organism. Lowenstein-Jensen (LJ) medium widely used in tuberculosis microbiology laboratories and is considered as the gold standard for MTB culture but it presents certain difficulties (Ross et al., 1998). Though culture technique is very sensitive and specific, but it is complex and time consuming, require 4-8 weeks to yield growth of *M. tuberculosis* (Mehta et al., 2012). Liquid media offer a considerable time advantage over solid media for the detection of *M. tuberculosis* growth: 7–14 days in Middlebrook 7H9 liquid medium, compared with 18–28 days in Middlebrook 7H11 agar, or 21–42 days in LJ medium (Iseman et al., 2000). Use of rapid culture methods based on liquid media (BACTEC 460 and MGIT 960) has been found to be very accurate and rapid method for diagnosis of even smear negative pulmonary tuberculosis and is highly recommended for rapid and precise diagnosis of tuberculosis (Bohy et al., 2009).

Although the diagnosis of mycobacteriosis is still primarily based on conventional methods (staining, culture, biochemical analysis, proportional method), a series of molecular (nucleic acid based assays; NAA) assays are increasingly introduced and incorporated in the workflow of clinical mycobacteriology laboratories worldwide. These assays are rapid and offer high sensitivities and specificities. Various gene targets such as IS6110, 16S rRNA gene, 65 kDa protein gene (Rv0440), devR (Rv3133c), MPB-64/MPT-64 protein gene (Rv1980c), 38 kDa protein gene (Rv0934), TRC4 (conserved repetitive element) GCRS (guanine-cytosine-rich repetitive sequence), hupB (Rv2986c), dnaJ (Rv0352), MTP-40 protein gene (Rv2351c) and PPE gene (Rv0355) have been employed in these PCR assays (Bandyopadhyay et al., 2008; Garcia-Elorriaga et al., 2009; Haldar et al., 2011). The reason for widely used IS6110 in PCR tests is the presence of its multiple copies in *M. tuberculosis*
complex genome, which is believed to confer higher sensitivity (Rafi et al., 2007; Jin et al., 2010). However, a few studies from different geographical regions of the world have reported that some clinical isolates have either a single copy or no copy of IS6110 that leads to false-negative results (Thangappah et al., 2011). The method has 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 (Singh et al., 2002).

Disseminated tuberculosis remains a major health problem in countries where generalized HIV epidemics coincide with high tuberculosis incidence rates, often causing fatal illness in patients with immunologically advanced HIV disease. Early recognition and treatment are likely to be important to avoid death (Lewis et al., 2002). Although the incremental value of mycobacterial blood culture for the diagnosis of disseminated tuberculosis has been both recognized and debated (Monkongdee et al., 2009) there have been few evaluations of blood culture systems for the detection of M. tuberculosis (Archibald et al., 2000 & 2001). Mycobacterial blood culture methods in common use include visual inspection of processed blood inoculated on a solid medium (e.g., the Isolator 10 system) and continuous detection in liquid medium inoculated with blood (e.g., the BacT/Alert MB system or the Bactec Myco/F Lytic system). Various manual, semi automated, and fully automated culture systems may be used to recover these organisms from blood. Also, several different PCR systems that have been described for the diagnosis of tuberculosis have produced widely differing results with regard to the sensitivity of the assay with different types of clinical samples (Rys et al., 1993; Kox et al., 1994; Noordhoek et al., 1994). Peripheral blood appears to be the clinical material of choice for PCR, especially in cases of disseminated and extrapulmonary forms of the disease.
Earlier studies with blood-based PCR assays in humans suggested that PCR with peripheral blood mononuclear cells for the diagnosis of tuberculosis may be useful only in those who are substantially immune-compromised (DeBiasi et al., 2004; Achkar et al., 2011; Padmapriyadarsini et al., 2011) due either to AIDS or to conditions such as alcohol abuse, renal disorders, diabetes mellitus, etc. It appears that more data are required to determine the effectiveness of the blood-based PCR assay for the diagnosis of tuberculosis, especially in immune-competent patients. Since TB is endemic in our country many times clinicians are faced with the problem of diagnosing TB in cases of pyrexia of unknown origin (PUO) especially when there are no site specific sign and symptoms. In such cases the only choice is testing blood samples for presence of TB which can either be by PCR and or culture.

Therefore, the present study was undertaken to compare the various methods for microbiological diagnosis of TB: AFB smear microscopy, culture on solid (LJ medium), liquid media (Middle Brook 7H9 broth) and PCR targeting IS 6110 sequences for diagnosing extrapulmonary TB and in small number of cases we also compared site specific culture and PCR with blood culture and PCR for detection of TB.

AIMS AND OBJECTIVES:

i) To compare smear microscopy, culture on solid and liquid media with IS 6110 based PCR for diagnosis of TB.

ii) To improve sensitivity of AFB smear with concentration methods.
iii) To study utility of Myco/F Lytic medium in improving the diagnosis of TB in extra pulmonary and paucibacillary disease.

iv) To compare utility of blood PCR with site specific PCR for diagnosis of TB.

**MATERIALS AND METHODS**

**Study subjects & samples:** The study was conducted in the Department of Microbiology & Immunology, S.M.S. Medical College, Jaipur from Jan 2009 to 2013, on EPTB suspects whose samples were received for routine diagnosis from different wards and OPDs of SMS and allied hospitals. Clinical history of suspects which included age, gender, HIV status and symptoms suggestive of TB disease were noted down in a Performa.

**Specimen processing**

1. **CSF & other body fluids & aspirates** Samples were transferred to screw cap tubes and centrifuged at 3000g for 15 min. The supernatant was decanted and the pellet was re-suspended in 1 ml PBS. Sterile fluids like CSF were directly subjected to further examination but purulent, mucoid or non sterile samples were subjected to digestion and decontamination by 4% NALC-NaOH method as described later.

2. **Tissue samples** Tissue sample obtained in sterile saline was centrifuged at 3000g for 15 min. Supernatant was decanted and deposit was crushed by means of electric tissue homogenizer for 1 minute. The lysate was subjected to digestion and decontamination by 4% NALC- NaOH method described later.
**Digestion & decontamination (Modified Petroff’s Method)**

4 to 5 ml sample was taken in a 50 ml falcon tube. Equal volume of NaOH-NALC solution was added to the sample. The tube was vortexed for about 15 to 30 sec. Samples were incubated for 15-20 min; vortexing lightly in between. At the end of 15-20 min, phosphate buffer solution (pH 6.8) was added up to the top ring of the falcon tube (50 ml). After mixing well, the tubes were centrifuged (at 4°C) at a speed of 3000 g for 15-20 min. The supernatant was decanted and the sediment was re-suspended in 1-2 ml phosphate buffer solution (pH 6.8).

**AFB smear microscopy**

Smears were prepared from the above deposits, stained by routine Ziehl Neelson staining and were graded according to RNTCP guidelines.

**Culture for AFB**

*Lowenstein Jensen* medium were prepared according to RNTCP guidelines. Duplicate LJ medium slants per specimen were inoculated with one 5mm loopful of the digested & decontaminated deposit. These slants were incubated at 35-37°C. All cultures were examined 48-72 hours after inoculation to detect gross contaminants. Thereafter cultures were examined weekly for growth up to 8 weeks on a specified day of the week.

*Middle Brooke 7H9 broth* medium was also prepared and OADC enrichment was added. 50 µl of the above obtained deposit was inoculated in 1ml of Middle Brook 7H9 broth. Thereafter, cultures were incubated 35-37°C and examined weekly up to 6 week by routine ZN staining.
Biochemical confirmation of *Mycobacterium tuberculosis* The growth obtained on LJ slants & MB 7H9 broth was identified primarily by colony appearance and was biochemically confirmed by niacin, nitrate test & susceptibility to p-nitrobenzoic acid (RNTCP, 2009).

Molecular diagnosis of mycobacteria

1. **DNA extraction**

   DNA was extracted from decontaminated samples. Sample deposits were suspended in TE buffer and placed at 100°C for 5 minutes to kill the bacteria followed by snap chilling at -20°C for 20 minutes thereafter DNA was extracted by physiochemical method as described by Van sooling *et al* (1994).

2. **PCR amplification**

   PCR was performed targeting IS6110 sequence. The PCR’s were performed in a total volume of 25 µl. The reaction mixture contained 1 x PCR buffer, 1.5mM MgCl₂, 0.5U of *Taq* polymerase, 0.2mM deoxynucleoside triphosphate (dNTP), and 0.4 µM of both forward and reverse primer targeting IS6110 sequence. 3ul of DNA was added to the mix. A total of 40 cycles of PCR were performed by using a thermal cycler consisting of a denaturation step for 5 min at 94°C, an annealing step for 1 min at 60°C, and an extension step for 1 min at 74°C. After the final cycle there was a step of 5 min at 74°C. A positive (DNA from MTB H37Rv) and negative control (NFW) was included in each batch of experiment.

3. **Detection**

   PCR products were electrophoresed in 2% agarose gel. Bands were visualized after staining with ethidium bromide under UV light. The size of the fragment was
determined by comparing with 100 bp DNA ladder. 123 bp band was obtained for positive samples.

**Optimization of diagnosis of MTB from EDTA blood samples**

Blood samples were collected from known patients of pulmonary & extra pulmonary TB (diagnosed by PCR of site specific sample) & known HIV-TB patients. This was followed by culture on Myco/F Lytic media and a PCR targeting IS6110 sequence.

**Culture on Myco/F lytic media**

5 ml of aseptically collected blood was transferred to the Myco/F Lytic bottle. Bottles were inverted 2-3 times for proper mixing of samples and then incubated at 37°C for 2-8 weeks. Cultures were read within 5–7 days after inoculation and once a week thereafter for up to 8 weeks to confirm the presence or absence of Mycobacteria by routine ZN staining. If AFB were present, they were identified further using standard morphological and biochemical tests.

**DNA extraction from blood**

About 5 ml blood was taken and suspended in Buffer A (Erythrocyte lysis buffer) followed by Buffer B (Proteinase K buffer). DNA was extracted by SDS, Proteinase K. Extracted DNA after sedimentation with NaCl and isopropanol was washed with 70% ethanol and re-dissolved in 30-40µl Tris HCl. PCR amplification and detection of products was done as described above.

**Statistical analysis** Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the ZN staining and MB 7H9 broth & PCR
were evaluated against gold standard LJ culture method by using bivariate two by two tables (Binary classification method).

**OBSERVATIONS AND RESULTS:**

- **Study samples/subjects:** A total of 655 samples were collected. The study subjects included both males (n=158) & females (n=497), from age group 3-84 yrs. This included Ascitic fluid (n=4), Cerebrospinal fluid (n=254), Endometrium biopsies (n=303), Gastric aspirates (n=3), Knee aspirates (n=2), Lymph node aspirate (n=1), Menstrual blood (n=16), Pericardial fluids (n=2), Peritoneal fluids (n=41), Pleural fluids (n=18), Pus (n=6) & Synovial fluids (n=5).

- Out of 655 samples received, a total of 32.06% (210/655) were positive by either of the methods used. Overall positivity by AFB smear was 3.96% (26/655), 6.25% (41/655) by LJ solid culture, 10.22% (67/655) by MB 7H9 liquid culture and 28.54% (187/655) IS6110 based PCR.

- Out of total positives, 89.04% (187/210) were positive by the PCR, 31.90% (67/210) by liquid culture and 19.52% (41/210) by solid culture and 12.38% (26/210) by AFB smear.

- All the smear-positive samples were positive either by culture or PCR, but 28/210 (6.19%) culture positive samples were negative by PCR, twelve of which were identified as nontubercular mycobacteria (NTM) by biochemical tests. Contamination was observed 27/655 (4.12 %) samples which were considered negative by culture, 5 of these were found to be PCR positive.
• **ZN staining** has shown 10.34% sensitivity (with 95% confidential interval (CI) 24.1; 94) and 96.33% specificity (with 95% CI, 67.1; 80.6). Overall positive and negative predictive value of ZN staining was observed as 11.54 % (with 95% CI, 2.7; 20.8) and 95.87 % (with 95% CI, 93.9; 99.7).

• **MB 7H9 broth** culture has shown 44.83% sensitivity (with 95% confidential interval (CI) 24.1; 94) and 91.37 per cent specificity (with 95% CI, 67.1; 80.6). Overall positive and negative predictive value of MB 7H9 was observed as 19.40 per cent (with 95% CI, 2.7; 20.8) and 97.28 per cent (with 95% CI, 93.9; 99.7).

• **IS6110 PCR** has shown 86.21 per cent sensitivity (with 95% confidential interval (CI) 24.1; 94) and 74.12 per cent specificity (with 95% CI, 67.1; 80.6). Overall positive and negative predictive value of IS6110 PCR was observed as 13.37 per cent (with 95% CI, 2.7; 20.8) and 99.15 per cent (with 95% CI, 93.9; 99.7).

• Positivity by IS6110 based PCR was found to be significantly higher than AFB smear microscopy (P<0.0001), LJ culture (P<0.0001) and MB culture (P<0.0001).

• None of the blood samples was found to be positive by Myco/F Lytic media.

• Among the 28 PTB samples (sputum) positive for AFB smear & site specific PCR; 5 (17.85%) were positive by blood PCR. Of the 40 EPTB samples only 3 (7.5%) were positive by blood PCR. All 12 HIV-TB were negative for blood PCR and blood culture.
CONCLUSIONS

- Majority of EPTB suspects belonged to 20-40 yrs age group which represents that overall increase rate of infection in the young age group.

- Most of the samples from EPTB suspects were **paucibacillary** (smear negative/scanty smear positive).

- The liquid culture methods are faster & have fairly high sensitivity/specificity as compared to solid LJ culture. However, liquid cultures are more prone to contamination and hence may require greater expertise and automation.

- The PCR test is useful in the diagnosis of EPTB where conventional microbiological techniques for *M. tuberculosis* show poor performance.

- **IS6110** based PCR has higher positivity than ZN staining/solid/liquid culture methods. At present, nucleic acid amplification based assays are the most suitable choices for the identification of *Mycobacterium tuberculosis* in smear negative samples with high degree of sensitivity and specificity.

- However, the method lacks applicability to low/zero **IS6110** copy no. MTB strains. PCRs based on other primers may be found useful in this case. Presence of PCR inhibitors in the specimens may further lead to false negative results. This demand for a good extraction procedure leading to the yield of highly pure DNA. Also conventional PCRs are more prone to contamination which may lead to high rates of false positivity. Fully automated RT PCRs can be a better system of choice.
• 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. NAA based techniques are highly sensitive in diagnosis of FGTB as compared to conventional methods.

• TB of the CNS is one of the commonest forms of pediatric TB. Here also IS6110 based PCR analysis is highly useful for early confirmation of MTB in clinical specimen thereby enabling early diagnosis and treatment of TB meningitis.

• Blood may not be suitable sample for diagnosis of disseminated/EPTB. High NPV of blood based culture & PCR in EPTB patients strongly indicate that the test could not help in the diagnosis of the Mycobacterium tuberculosis.

We conclude that IS6110 PCR test for DNA specific to M. tuberculosis may be of hope for a rapid and accurate diagnostic test for EPTB and it helps where conventional diagnosis fails and provisional diagnosis of tuberculosis is made on the basis of clinical presentation and histology/cytology examination without evidence of AFB. IS 6110 PCR may be great potential to improve the clinician vision for the early diagnosis, treatment and prevention of EPTB. The most notable advantage of PCR tests is their turnaround time and reliability for an early detection of EPTB, which may have important implications for clinical management and TB control; the reliability of PCR to confirm an early diagnosis of TB meningitis and Genital tuberculosis when smear and culture test are rarely positive. That can help to start timely ATT and prevent progression to irreversible changes to avoid unnecessary mortality and transmission of disease. A negative PCR may result in missing the
diagnosis in a few cases. It indicates the need for further evaluation using other
diagnostic tests and repeat testing to confirm/exclude diagnosis. PCR can be improved
by using better DNA extraction methods, fully automated RT PCR systems and by
using more than one set of primers in the detection of extrapulmonary tuberculosis.
Our findings also illustrate that the idea of TB diagnosis by PCR on blood samples
should be discouraged. PCR has a potentially important role in strengthening the
diagnosis of TB both pulmonary and extra-pulmonary. For this specific site sampling
according to disease is very important.