MATERIAL AND METHODS

The present study included OPD and IPD patients from Government Medical College, Aurangabad and Mahatma Gandhi Mission’s Medical College (MGM), Aurangabad. The patients were from in and around Aurangabad district. Both the conventional techniques that is microscopy and culture were done in the Department of Microbiology at Government Medical College. Specimen processing (DNA extraction) for PCR was done in the institute of Biotechnology, MGM Aurangabad, whereas the PCR amplification was carried out at the Institute of Science, Aurangabad.

Clinical Specimens:

The study included 91 clinical specimens obtained from patients with a strong clinical suspicion of tuberculosis. All the suspected cases were from both sex and all age groups. Preliminary clinical diagnosis was made as the patient had fever, night sweats, nausea, and persistent cough for more than 3 weeks (incase of pulmonary tuberculosis), anorexia and weight loss. Clinical diagnosis was supported by positive radiological, histopathological and cytological findings.
The specimen included 50 pulmonary (sputa 32, pleural fluid 14, bronchoalveolar lavage (BAL) 2 and intercostals drain (ICD) 2) and 41 extrapulmonary specimens (ascitic fluid 11, pus 12, blood 5, tissue 4, aspirate 3, urine 3 and CSF 3). All the necessary clinical details of the patients, diagnostic evidence and treatment received were obtained from the referring clinicians in the prescribed proforma prepared for this particular study (Annex-I). PCR was also carried out on MOTT strains (\textit{M.chelonei, M.szulgai, M.phlei, M.avium and M.intracellulare}) obtained from Department of Microbiology, MGIM’s Sewagram, Wardha to check the specificity of the primers.

\textbf{Collection and Processing of Specimen :}

Sputum specimens were collected as per the RNTCP guidelines (3 specimens – two spot and one early morning, at least 5 ml in quantity) in sterile disposable wide mouthed container. All other specimens were collected in sterile containers. Specimens containing fibrinogen and traces of blood such as pleural fluid were collected in sterile bulbs having EDTA. Urine specimens from cases of suspected renal tuberculosis were collected as clean catch midstream urine
specimens on 3 successive days and held at 4°C on each day before pooling the 3 samples. Blood specimens (2 to 3 ml) were collected in EDTA bulbs. Tissue biopsy specimens were collected in sterile container having 2 to 3 ml sterile saline.

Treating clinicians were advised well before to collect maximum possible amount of specimen (5 to 10 ml) and to transport the specimens to the laboratory immediately. If there was an unavoidable delay in sending the sample the clinicians were advised to keep the specimen at 4°C in refrigerator with clear instructions to avoid freezing. All specimens on being received in the laboratory were processed as soon as possible and if processing was not possible immediately, were held at 4°C, but not for more than 24 hours. All specimens were divided into two sterile containers taking all aseptic precautions and the process was done in a biosafety cabinet. One portion was used for smear and culture, while the other portion was kept at 4°C for DNA extraction for PCR.

Microscopy:

Smears were prepared for all specimens and stained by Ziehl-Neelsen (ZN) staining. Sputum specimens were examined microscopically according to Revised National Tuberculosis
Control Programme (RNTCP) guidelines and if negative the smear negative sputum specimens were concentrated and smears repeated from the concentrated deposit. Smears for all other specimens were made after concentration by Petroff’s method using 4% NaOH (Annex-II) and then stained by ZN method.

**Culture:**

Of the three sputum specimens collected the specimen positive by microscopy was used for culture. If all the three specimens were positive the early morning specimen was selected for culture as it showed the highest number of bacilli. Half of the same specimen was also selected for PCR.

All the specimens except CSF, tissue and blood were subjected to digestion and decontamination using 4% NaOH. A part of the pellet was used to heavily inoculate two Lowenstein Jensen (LJ) medium slopes under aseptic conditions. Cultures were incubated at 37°C and watched daily for the 1st week for the growth of rapid growing mycobacteria and also for contamination and weekly thereafter for 6 weeks. If no growth was noticed after 6 weeks the specimen was labeled as culture negative.
In case the specimen was skin or tissue biopsy it was first triturated using sterile glass beads in 1-2 ml distilled water and the juices used for smear and culture. For specimens from skin (ulcers, punch biopsies) one additional LJ was inoculated and incubated at 32°C to isolate mycobacteria causing skin infections.

**Identification of Mycobacteria:**

All specimens showing growth were subjected to identification of species using limited biochemical tests (niacin, nitrate reduction, catalase and growth on PNB medium) *(Annexure-III).*

**PCR Assay:**

PCR Assay was carried out using GeneiTm Amplification Reagent Set MTB-25 for *M.tuberculosis*. This test is based on the principle of single-tube nested PCR method. This assay is a two-step sequential assay. In the first step, the IS region of *M.tuberculosis* complex DNA sequence, a 220 bp is amplified by specific external primers. In the second step, the nested primers are added to further amplify a 123 bp amplification product.
In this test, false positive reactions that may be caused by previous amplicon contamination are prevented by the use of uracil DNA glycolase (UDG) and dUTP instead of dTTP.

**Sample Processing for DNA Extraction:**

All manufacturers instructions were strictly followed.

1) **Sputum and bronchoalveolar lavage (BAL):** The specimen were first treated with equal volumes of 5 mg/ml solution of N-acetyl-L-cysteine (mucolytic solution) allowed to react for 10 minutes at room temperature with intermittent mixing and centrifuged at 6000 g (8000 rpm) for 15 minutes, the supernatant decanted and then pellet resuspended in 20-30 ml of 20 mM Tris buffer. Again centrifuged at 6000 g for 10 min and the pellet used for DNA extraction.

2) **CSF, Pleural fluid, pericardial fluid, joint fluids, needle aspirates and urine:** Sample was taken in centrifuge tube and centrifuged at 6000 g for 15 minutes. The pellet resuspended in 20-30 ml 20 mM Tris buffer and centrifugation repeated for 10 minutes and the pellet used for DNA extraction.
3) Tissue: Fine mincing was done using gloves and a new sharp scalpel blade, against the wall of the container and the minced tissue suspended in 1 ml of sterile distilled water in a 1.5 ml vial. Spined at 1000 rpm for 5 minutes to sediment large particles and 500 μl of the supernatant was centrifuged at 6000 g for 10 min. the pellet was taken for DNA extraction.

**DNA Extraction:**

1) Pellet obtained for the specimen after initial processing was resuspended in 250 ml of lysis buffer II and 20 μl of proteinase K was added to it, mixed by vortexing and incubated at 65°C for 30 min in a water bath with occasional mixing during the incubation.

2) Each vial was then centrifuged at 10,000 rpm for 10 min.

3) To 200 μl of the supernatant, 200 μl of lysis buffer II (containing the internal control DNA) was added, mixed by pulse vortexing for 15 seconds and incubated at 70°C for 10 min in a water bath.

4) To this was added 200 μl of 96-100 % distilled ethanol and mixed thoroughly by vortexing. Then the spin
columns were placed in 2 ml collection tube and the above mixture (sample-ethanol mixture) was added to the column without wetting the rim.

5) Then the collection tube with the spin column was centrifuged at 6000 g for 3 minutes and the flow through collected in the collection tube was discarded.

6) The spin column was placed in the new collection tube and 500 μl of wash buffer I was added. This was spined at 6000 g for 3 minutes.

7) The flow through in the collection tube was discarded and 500 μl of wash buffer II was added and then spined at 14,000 rpm for 3 minutes. The flow through was discarded.

8) Then the empty spin columns were spined at 14,000 rpm for 2 minutes to ensure complete removal of the wash buffer.

9) The spin columns were placed in a new labeled 1.5 ml tube and 100 μl of prewarmed (50 °C) Elution buffer was added and incubated at room temperature for 5 minutes. Then it was centrifuged at 10,000 rpm for 1-2 minutes to
elute the DNA. The DNA samples were stored at –20°C for amplification the next day.

**DNA Amplification**:

A] **First Amplification**:

Master Mix I was prepared, so that for each specimen the Master Mix I contained Amplification Premix I 8.2 μl, Genei Hot Start Taq DNA Polymerase 0.33 μl and uracil DNA glycolase (UDG) 0.5 μl. Master mix was prepared taking into account that it would be sufficient for the number of samples to be run plus one positive and one negative control.

The PCR tubes (0.2 ml) were properly labeled and 9 μl of first amplification Master Mix I was added to each FCR tube including positive and negative control tubes. To the labeled specimen tubes were added 3 μl of the extracted DNA and 3 μl of positive control to tube labeled positive control and the tube labeled negative control was left without adding anything (Reagent control).

The thermal cycler used in this study was MJ Research using the calculated mode as suggested by the manufacturer.
The first amplification reaction profile was as follows:

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<th>No. of cycles</th>
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<tr>
<td>68°C</td>
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<tr>
<td>72°C</td>
<td>1 min</td>
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B) **Nested or Second Amplification**:

Master Mix II was prepared taking into account again the number of specimen and additional for positive and negative control. For each specimen Master Mix II contained Amplification premix II 14.7 µl and Genei Hotstort Taq DNA polymerase 0.33 µl.
The above Master mix II thus prepared was added to the same PCR tubes used in the first amplification. The amount was 15 μl and the second Nested PCR was performed using the following amplification reaction profile.

<table>
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<th>Time</th>
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<td>4°C</td>
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**Analysis of amplified products:**

Analysis of amplified product was done using submarine electrophoresis using 2.5% agarose gel containing 10 μl of 10 mg/ml ethidium bromide dye solution for 100 ml of gel. Gel was prepared using 1X TAE buffer and the same was used as the reservoir buffer. Fifteen microlitre of the samples mixed with 3 μl gel loading dye was loaded in each slit. Five microlitre of ready to use marker dye (Molecular weight marker) was used to detect the bands. The electrophoretic run of 100 to 120 volts.
was used for the run. It was carried till the advancing dye moved to 3 to 4 cms. The gel was then removed and visualized under UV Transilluminator.

**Results and interpretation:**

An amplification product of size 123 bp was indicative of infection with *Mycobacterium tuberculosis* complex. The amplification product of internal control DNA was 340 bp.

Positive: Band at 123 bp and 340 bp/band at only 123 bp.

Negative: Band only at 340 bp.

Repeat the DNA extraction: No band.

(Absence of 340 bp band indicated that the sample contained inhibitors or the DNA extraction failed).