1.1 **Study design:** Cross-sectional hospital-based test validation study

1.2 **Study Setting**
Department of Microbiology, M.M. Institute of Medical Sciences and Research, Mullana

1.3 **Study Period**
October 2012- May 2014

1.4 **Study population**
A total of 1000 smear positive pulmonary TB patients (both out patients (OPD) and in patients (IPD) attending chest and TB department) and patients from RNTCP peripheral center (Ambala) were included. Specifically, stratified random sampling technique was used to recruit the patients.

1.4.1 **Inclusion criteria**
Both male and female sputum smear-positive patients irrespective of the age group and fall into one of the following criteria were included
Newly diagnosed TB Cases
  - Recently diagnosed tuberculosis case or,
Previously treated TB cases
  - Patients receiving anti tuberculosis treatment or,
  - Suspected TB treatment failure or,
  - Suspected TB relapse cases.

1.4.2 **Exclusion criteria**
- Smear negative cases of suspected pulmonary tuberculosis

1.4.3 **Ethical consideration**

1.4.4 Ethical clearance was taken from M.M. University ethical committee (No.IEC/MMIMSR/11/16 dated 21.01.2011)

1.4.5 **Data collection method**
- Performa - Annexure I attached.

1.5 Specimen and specimen collection procedures

1.5.1 Sputum

Two sputum samples (6-8 ml each) per patient were collected (Annexure II attached), of these; one sample was used for the preparation of smears, inoculation on LJ media, TTC assay and MODS assay and the other sample was preserved at -80°C for examining cross contamination of assays.

1.6 Transport

The clearly labeled specimen were transported to the Microbiology laboratory immediately after collection or if any delay occurs, the specimen were refrigerated at 4-8°C (18-24 hrs)

1.7 Specimen processing

1.7.1 Processing of sputum specimens for direct MODS, direct TTC assay and indirect LJ proportion method.

All the sputum specimens were digested and decontaminated using the N-acetyl-L-cysteine– sodium hydroxide method as described by Kent and Kubica26 - Annexure III attached.

1.8 Microscopy

Smear preparation, Ziehl–Neelsen staining, examination and grading of smears were performed as per RNTCP guidelines27 - Annexure IV attached.

1.9 Culture

1.9.1 Culture media preparation

1.9.1.1 Lownstien Jensen Media

LJ media was prepared using the standard guidelines as recommended by RNTCP30 - Annexure V attached

1.9.1.2 Middlebrook 7H9 Glycerol Casitone Broth

Middlebrook 7H9 GC Broth was prepared according to manufactures instructions (Himedia, Mumbai) with minor modifications: 0.94 g of 7H9 powder
was dissolved in 180 ml of distilled water and mixed well. Then, 0.1 g of casitone was added and mixed well. These solutions were warmed and subjected to autoclaving at 121\(^0\)C for 15 minutes. After autoclaving and cooling (at lukewarm temperature), 20 ml of OADC (oleic acid dextrose catalase) enrichment, one ml of sterile glycerol and PANTA mixture, was added and mixed well. Sterility check was carried out by leaving one night in the incubator at 37\(^0\)C. This medium was stored in a refrigerator at 4-8\(^0\)C.

### 1.10 Quality control organisms

- **Growth control** - H37Rv M tuberculosis strain ATCC 27294 (susceptible control)
- **Assay controls** - ATCC 35822 strain of M. tuberculosis INH resistant
- **Assay controls** – ATCC 35838 strain of M. tuberculosis Rif Resistant

### 1.11 Preparation of Antibiotic stock solution

1.11.1 For LJ proportion method, MODS and TTC medium - Annexure VI attached

### 1.12 MODS Assay procedures: Annexure VII attached

### 1.13 2, 3, 5–triphenyl tetrazolium chloride (TTC) test tube assay

1.13.1 **TTC preparation**

Commercially available (Merck, Mumbai) 2, 3, 5- Triphenyl Tetrazolium Chloride was dissolved in sterile distilled water to get a concentration of 5 µg/ ml. Then, this solution was filtered through a 0.22 µm filter and stored at -70\(^0\)C until needed. **Annexure VIII attached**

1.13.2 **TTC assay procedure**.

The TTC assay was carried out as described by Mohammadzadeh et al \(^8\) with few modifications - **Annexure XI attached**

### 1.14 Indirect LJ proportion Method

The proportion method was performed according to established procedures on LJ medium with critical concentrations of 0.2 µg ml for
INH and 40 µg ml for RIF\textsuperscript{24}. A strain was classified as susceptible to the particular drug only if the number of colonies that grow on the drug-containing medium is <1% of the number of colonies that grew on the drug free control tube and resistant if the number of colonies is >1 %\textsuperscript{25}.

**Annexure IX attached**

Identification of mycobacterial isolates were carried out by standard laboratory protocols\textsuperscript{26} - **Annexure X attached**

**2.0 Data analysis**

The performance of the MODS and the TTC assay was compared with the PM and results were interpreted in terms of sensitivity (ability to detect a true resistance) and specificity (ability to detect a true susceptibility), Positive Predictive Value, Negative Predictive Value.

**Statistical calculations**

\[
\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives} + \text{false negatives}} \times 100
\]

\[
\text{Specificity} = \frac{\text{True negatives}}{\text{False positives} + \text{True negatives}} \times 100
\]

\[
\text{PPV} = \frac{\text{True positives}}{\text{True positives} + \text{false positives}} \times 100
\]

\[
\text{NPV} = \frac{\text{True negatives}}{\text{False negatives} + \text{True negatives}} \times 100
\]

Significance (p value) of the test was calculated using Fisher’ Exact test (using Graph Pad Prism online software).