Materials and Methods
MATERIALS AND METHODS

The study was conducted at the Shree Krishna Hospital located in Karamsad, a tertiary care hospital with a capacity of 550 beds. It is one of the biggest rural based tertiary care hospitals covering a large population of the Anand and Kheda districts of Gujarat state. The hospital is well equipped for providing all kinds of Super speciality services to rural community such as, Cardiology, Oncology, Gastroenterology, Nephrology, Neurology and Intensive Care units (Medical, Surgical, Paediatric and Neonatal). The hospital also has an advanced Central Diagnostic Laboratory which is well equipped and has been accredited by National Accreditation Board of Laboratories for Testing & Calibration (NABL).

- **Ethical Clearance:**

This study is a prospective observational study. The study was conducted after the approval of the Human Research Ethics committee (HREC) of H. M. Patel Centre for Medical Care and Education, Karamsad and after obtaining the informed consent from the patients or the relatives (Annexure-1).

- **Study Design:**

In the present study, a total of 400 patients were enrolled, attending Shree Krishna Hospital (Tertiary Care, Teaching and Rural Hospital) Karamsad, Anand, Gujarat or referred to SKH from near by clinics and TB Hospitals because of symptoms and radiographic evidence suggestive of pulmonary tuberculosis.

A detailed history of these patients including age, sex, occupation, address and previous Anti-Koch's treatment were recorded. Based on the proper history, all the enrolled 400 patients were divided into untreated newly diagnosed cases (CAT I) and
previously treated cases (CAT II) which included Treatment failure, Relapse cases and Defaulters as per RNTCP Guidelines (Annexure-2).

- **Study Period:**

  All the patients were enrolled during the study period from Jan-2006 to Dec-2009.

- **Inclusion Criteria:**

  Patients having symptoms and radiographic evidence of pulmonary tuberculosis of any age and sex were included in the study.

- **Exclusion criteria:**

  Patients having extrapulmonary tuberculosis were excluded in the study.

**Methodology:**

- **Specimens:**

  Sputum specimens preferably one early morning and two spot specimens were collected in a sterile, wide mouth, 'leak-proof' container after giving proper instructions to the patients. Specimens were transported within 1 hour of collection to the Microbiology Laboratory, CDL, SKH and processed immediately. In case of delay, the specimens were kept in refrigerator at 4\(^0\) C for not more than one day. (Annexure-3&4)

- **Staining And Microscopy:**

  Smears were prepared from thick, purulent part of the sputum and stained by Ziehl-Neelsen staining method. Smears were examined microscopically for the presence of Acid fast bacilli and grading was done as per RNTCP guidelines.
Direct Smear Microscopy

Ziehl-Neelsen Staining Technique

All 400 sputum samples, after proper collection were processed for direct microscopy by ZN staining technique to detect Acid-fast bacilli.

Staining Procedure

- New clean and unscratched slide was taken and labelled with the Laboratory serial number on one end of the slide.

- A smear was prepared from the purulent part of the sputum using a wooden stick covering an area of 1x2 cm on a glass side in a Biosafety cabinet. Wooden sticks were discarded in 2% Cidex.

- Smear was allowed to air-dry for 15-30 mins.

- The smear was heat-fixed by passing three times through the flame of the Bunsen burner.

- A piece of small filter paper was placed over the smear and covered with Ziehl Neelsen Carbol Fuchsin.

- The slide was gently heated to steaming. Care was taken that the smear was not overheated and stain does not start boiling.

- The hot carbol fuchsin was allowed to keep on the smear for 5 mins without further heating.

- The smear was kept moist by adding more carbol fuchsin if necessary without additional heating.
- After 5 mins, the filter paper was removed and the slide was washed with tap water.

- The smear was covered with **25% H₂SO₄ as a decolorizing agent** and allowed to keep for **3 mins**. Decolourisation was repeated till the smear became pale yellow in colour.

- Then after, the smear was rinsed with tap water.

- The smear was flooded with **1% Loeffler’s methylene blue as counter stain** and left about 1 min.

- Slide was, then washed with tap water, drained and allowed to air-dry.

**Examination of the Smear in Microscope**

- The stained smears were then observed under the oil immersion lens (100 x) in microscope for the presence of Acid-fast bacilli.

- The upper part of the smear was first examined from one end to the end, followed by the lower part of the smear and then the middle part of the smear.

- Acid-fast bacilli appear red, straight or slight curved, occurring singly or in small groups, beaded appearance, branching filaments and small coccoid forms also seen while the background appears blue showing pus cells, epithelial cells, and non acid- fast bacteria.(Colour Plate-1).
Reporting of Smears

All the smears were observed for minimum of 100 oil immersion fields (OIF) on the slides. Smears found negative for AFB were reported only after the confirmation of at least 100 OIF. Any smear found AFB positive was graded as follows.

Table -11 Grading of AFB as per RNTCP Guidelines

<table>
<thead>
<tr>
<th>Number of bacilli Seen</th>
<th>Result</th>
<th>Grading</th>
<th>No. Of Fields Examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>More than 10 AFB / OIF</td>
<td>Positive</td>
<td>3+</td>
<td>20</td>
</tr>
<tr>
<td>1-10 AFB / OIF</td>
<td>Positive</td>
<td>2+</td>
<td>50</td>
</tr>
<tr>
<td>10-99 AFB / 100 OIF</td>
<td>Positive</td>
<td>1+</td>
<td>100</td>
</tr>
<tr>
<td>1-9 AFB / 100 OIF</td>
<td>Scanty</td>
<td>Exact No.</td>
<td>100</td>
</tr>
<tr>
<td>No AFB in 100 OIF</td>
<td>Negative</td>
<td>__</td>
<td>100</td>
</tr>
</tbody>
</table>

If only one or two AFB were seen then the further specimen from the patient was requested.
Colour Plate 1:
Direct Ziehl-Neelsen smear from sputum showing *M. tuberculosis.*
Concentrated Smear Microcopy

After the completion of smear preparation for direct microscopy as mentioned above, all 400 sputum specimens were subjected to decontamination and concentration procedure by Modified Petroff’s concentration method using 4% NaOH. The entire procedure was carried out in Class II Bio-safety Cabinet and using refrigerated centrifuge with proper aseptic and universal precautions during the entire procedure from receiving to discarding of the samples.

Decontamination & Concentration of Sputum by Modified Petroff’s Sodium Hydroxide Method

- Preparation of 4% NaOH

4 g NaOH was dissolved in 100 ml distilled water in a clean, dry glass flask and then autoclaved at 121°C for 15 minutes. It was then stored at room temperature and used only up to one week or less.

Procedure

- Sputum (max. 4ml) was transferred to a 10 ml sterile centrifuge screw cap bottle. If the sample volume found more than 4 ml then multiple bottles were used for processing and pooling of specimen was done at the end of processing.

- An equal volume of 4% NaOH was added in the sputum specimen.

- It was then mixed on the vortex mixer until it is liquefied for 15 to 30 seconds. Care was taken to avoid addition of excess 4% NaOH reagent since this could harm the viability of mycobacterial cells. Also care was taken to avoid
excessive vortex mixing. In some cases, where the sputum was very purulent, it was again divided into small quantities in sterile bottles and processed.

- The specimen was kept at room temperature for 15 minutes. Care was taken using timer not to exceed the 15 mins as NaOH is also toxic to mycobacteria. After some intervals, the specimen was agitated during this incubation for better liquefaction.

- After incubation equal volume of sterile distilled water was added as diluent to stop the further action of alkali.

- This was then centrifuged in a refrigerated centrifuge at 3600 rpm for 15 minutes (Colour Plate-2). Spinning at > 3000 rpm improves the recovery of mycobacteria but the conventional centrifuge may generate heat that will affect the viability of mycobacteria. So the use of refrigerated centrifuge is mandatory.

- The supernatant was carefully discarded in a suitable mycobactericidal disinfectant (2% Cidex)

- Sediment was mixed in vortex mixer and about 8 ml of sterile distilled water is added for second wash.

- It was again centrifuged at 3600 rpm for 10 minutes; Supernatant was discarded in 2% Cidex. Sediment was mixed in vortex mixer.

- Such concentrated sputum samples were used for further procedure as for concentrated smear microscopy, and inoculation on Lowenstein- Jensen medium.
Smears were prepared from the sediment obtained by decontamination & concentration technique on new glass slides. All were then stained using ZN staining procedure as mentioned above. All stained smears were observed under the microscope for minimum of 100 fields for the presence of Acid-fast bacilli (Colour Plate-3).

**Categorization of smears**

Smears were categorized into **four** categories

1) **True positive smear** was one in which the smear of the specimen showed acid-fast bacilli and culture of the same specimen yielded growth of mycobacteria.

2) **False positive smear** was one in which AFB were seen microscopically but not grown on culture.

3) **False negative smear** was defined as one in which no AFB were seen but culture of the sample yielded mycobacteria.

4) **True negative smear** was one in which no AFB were seen on smear or grown in culture.
Colour Plate-2

Refrigerated Centrifuge.
Colour Plate-3:
Concentrated Ziehl-Neelsen smear showing *M. tuberculosis*
Isolation And Cultivation

Preparation of Modified Lowenstein- Jensen Egg Medium¹⁰⁰

Mineral salt solution

- Potassium dihydrogen phosphate (anhydrous) – 2.4 g KH₂PO₄
- Magnesium sulfate (MgSO₄.7H₂O) – 0.24 g
- Magnesium citrate – 0.6 g
- Aspargine – 3.6 g
- Malachite Green – 0.5 g
- Glycerol – 12.0 ml
- Distilled Water – 600 ml

All the ingredients were dissolved by heating, autoclaved at 121°C for 15 mins and then cooled to room temperature.

Egg Emulsion

- Fresh eggs, not more than 1 week old were taken.
- Eggs were examined for the crack in the shell. If any egg found cracked, it was replaced.
- All eggs were then washed thoroughly with distilled water to remove the dust and dirt present on the shell.
• Thereafter all eggs were thoroughly cleaned with methanol soaked sterile cotton swab. This could help in removing the dirt specifically at all parts on shell if any.

• Then the eggs were left in the bowl containing methanol for about 30 mins. This would kill the organisms present on the shell, and make the shell sterile.

• Eggs were calculated based on assumption that one egg contains 50 ml emulsion.

• Eggs were then cracked in a measuring beaker and homogenized by using a blender.

• Entire procedure was carried out in a Bio-safety Cabinet (Colour Plate-4) under all aseptic precautions to prevent contamination.

**Complete L-J. Medium**

• Finally mineral salt solution (600 ml) and whole egg emulsion (1000 ml) was added in a sterile flask.

• Benzylpenicillin (100 Units / ml) was added in the L-J. medium to prevent bacterial contamination.

• 5 ml of this complete medium was then dispensed in Mc Cartney bottles. These bottles were then **inspissated at 85\(^{\circ}\) C for 60 min in an inspissator for three successive days.**

• After inspissation, L-J Media (Colour Plate-5) were incubated at 37\(^{\circ}\) C for 24 hr as a sterility check, and finally stored in the refrigerator with caps tightly closed to prevent evaporation for several months.
Quality control of L-J media was done by inoculation of standard strain of *M. tuberculosis* H37Rv (Colour Plate -6).

**Inoculation**

The sediment obtained after Petroff’s concentration method of all 400 sputum samples were inoculated with 4 mm disposable loop into four slants of Lowenstein-Jensen medium. Two slants were incubated at 37°C in dark in an incubator, one L-J slant at 25°C in BOD incubator and other slant at 42°C in other incubator.
Colour Plate-4:
Biosafety Cabinet Class II A
Observation for Growth

- Initially all 400 L-J medium were examined for the presence of growth every day for one week to pick up any rapidly growing strain or contamination.
- Thereafter the L-J. medium were examined at an interval of one week from 2nd week to the 8th week.
- L-J medium were followed up to 8th week for the presence of growth.
  (Colour Plate-7).

Confirmation of Growth

- Presence of growth was confirmed by preparing the smear from the colony grown on L-J. medium.
- The smear was then stained by ZN staining technique and examined for the presence of Acid-fast bacilli.
- Acid-fast bacilli seen in the form of micro-colony & cord form.
  (Colour Plate-8).

Test for the growth on p-nitrobenzoic acid (PNB) Medium

- P-nitrobenzoic acid medium is the L-J egg medium containing p-nitrobenzoic acid (500mg/l).
- P-nitrobenzoic acid was dissolved in propylene glycol at a concentration of 25 mg/ml. Two volumes of this solution were added to 100 volumes of the L-J egg medium before inspissation. The medium was poured in 5 ml quantities in Mc Cartney bottles and made as slants by inspissation at 85°C for 60 minutes.
- PNB medium and plain L-J medium were inoculated by one loopful of the test organism incubated at 37°C and observed weekly for the growth.

- **Identification Characteristics of *M.tuberculosis***

  - Slow, Eugonic Growth. Colonies on L-J medium were visible only after 3 weeks of aerobic incubation.

  - Rough, tough and buff colonies.

  - Non-pigmented.

  - Acid-fast bacilli.

  - Strictly grow at 37°C, fail to grow at 25°C and 42°C.

  - No growth on L-J medium containing p-nitrobenzoic acid (500 mg/l).
Colour Plate No-5
Plain L-J Medium

Colour Plate No-6:
Standard Strain of *M.tuberculosis* H$_{37}$R$_{v}$
Colour Plate No-7

Colonies of *M. tuberculosis* on L.-J Medium
Colour Plate No-8

Colony smear of *M. tuberculosis* showing cord formation.
Antituberculosis Drug Sensitivity Testing

Medium

Drug Sensitivity testing were performed on ready to use Lowenstien Jensen medium incorporated with individual antitubercular drugs of recommended specified strength.

(MICROEXPRESS SENSICULT-LJ Primary Drugs TULIP DIAGNOSTICS)

Contents

Table-12 Primary Drug Lowenstein- Jensen medium Panel (Colour Plate-9)

<table>
<thead>
<tr>
<th>DRUG</th>
<th>SYMBOL</th>
<th>PH</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid</td>
<td>IN</td>
<td>7.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>EB</td>
<td>7.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>RP</td>
<td>7.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>ST</td>
<td>7.0</td>
<td>0.1</td>
</tr>
<tr>
<td>L-J Control</td>
<td>L-J</td>
<td>7.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Sterile distilled water with glass beads for inoculum preparation. The standard strain *M. tuberculosis* H$_{37}$RV was tested with each batch of primary drugs by the manufacturer for quality control of the media. Sensitivity testing for pyrazinamide was not performed as pyrazinamide is active at acidic pH (pH 5.5). At acidic pH, most of the strains of mycobacteria show poor growth and there is inactivation of pyrazinamide if pH is above 5.5, so the results of sensitivity tests are often unreliable even with the proportion method. **Sensitivity testing to pyrazinamide is therefore not recommended in routine practice.**
• **Procedure**

Modified Proportion Method was used for determining drug susceptibility testing.

• **Inoculum preparation**

The sensitivity tests were set up with an inoculum prepared from the growth on primary L-J medium slant i.e. **indirect sensitivity tests** were carried out.

With a 22 SWG (Standard Wire Gauge) nichrome wire loop (diameter 0.7 mm) a representative sweep from the primary culture (5mg-10mg) was taken on the loop. The growth was then discharged into 0.4 ml of sterile distilled water in screw capped flat-bottomed bottles together with 6 glass beads 3 mm in diameter. A suspension was prepared by shaking the bottle for 1 min on a vortex mixer. Then it was allowed to stand for 20 mins to allow the larger particles to settle down.

The supernatant was decanted in another sterile bottle leaving sediment and glass beads. The turbidity of the bacterial suspension was then adjusted to that of McFarland 0.5. A loopful of the suspension was then inoculated on the surface of each slant of the sensitivity test.

• **Incubation and Reading of test**

The slants were incubated at 37°C. A reading was made at 2 week to give a preliminary indication of the presence of resistant strains, but the definitive reading was made at 4 weeks.

If growth of more than 20 colonies was observed on the drug containing L-J medium, the strain was classified as resistant. (Colour Plate-10)
Colour Plate No-9:
Primary Drug Lowenstein- Jensen medium Panel
Colour Plate No-10

Growth of M. TB on Plain L-J Medium and Drug Containing Medium
Comparative Study of Molecular Genetic Assay (Genotype MTB DR Plus) and Phenotypic drug sensitivity tests for identification of Resistance to Rifampicin and / or Isoniazid.

A total of 30 culture isolates on L-J medium whose sensitivity were already performed by phenotypic drug sensitivity Test were sent to the Intermediate Reference Laboratory (IRL) at State TB Demonstration Centre (STDC) Ahmedabad, Gujarat. The culture isolates were processed for the identification of resistance to Rifampicin and / or Isoniazid by a molecular genetic assay- Genotype MTBDR Plus (Hain, Life Science, Nehren and Germany). Procedure was performed as recommended by the manufacturer.

- **Methodology**

The whole procedure was divided into three steps

1) DNA Extraction from culture isolate (solid / liquid medium) or clinical specimens (Pulmonary, smear-positive decontaminated sputum).

2) Multiplex amplification with biotinylated primers.

3) Reverse Hybridization.

1) **DNA Extraction**

The procedure was carried out according to manufacturer’s instructions.

- Bacterial growth on L-J medium was collected with an inoculation loop in a sterile vial and suspended in 300 ml of water. (Molecular biology grade) provided in the kit.
• The bacteria was pelleted by spinning in a Standard table top micro centrifuge with an aerosol tight rotor in a Class II safety cabinet at 10,000 × g for 15 mins. The supernatant was discarded and bacteria resuspended in 300 ml of water (molecular biology grade) by vortexing.

• The bacteria was incubated at 95°C in a water bath for 20 mins in order to inactivate vegetative bacteria. Then it was incubated for 15 mins in an Ultrasonic bath.

• Thereafter it was spun down for 5 mins at full speed in microcentrifuge. 5µl of the supernatant was transferred to another sterile vial and used for PCR amplification.

2) Amplification

The amplification mix (master mix) was prepared in a DNA free room in the following manner.

• Primer Nucleotide Mix (PNM) - 35 µl

• PCR Buffer for Hot star Taq (15 mm MgCl₂) - 5 µl

• 2.5 mm MgCl₂ solution - 2 µl

• Hot Star Taq DNA Polymerase (Qiagen) - 0.2 µl (1U)

• Molecular biology grade (water) - 3 µl

The master mix was prepared by mixing well all the above reagents. (not by vortexing). 45 µl of the master mix was aliquoted in each of the prepared PCR tubes. (not considering the volume of enzyme). To 45 µl of master mix, 5 µl of extracted DNA solution was added in a separate area leading to a final volume of 50 µl.
In control sample 5 µl of molecular grade water was added instead of DNA solution. The PCR tubes were kept in a Thermal cycler.

The amplification protocol consisted of 15 min of denaturing at 95°C; 10 cycles of 30 sec at 95°C and 120 sec at 58°C, 20 additional cycles of 25 sec at 95°C, 40 sec at 53°C and 40 sec at 70°C; and a final extension at 70°C for 8 min.

PCR products were analysed in 1.5% agarose gel for the control of incomplete or marginal amplification. The biotinylated PCR products were then denatured and hybridized to a strip with specific oligonucleotide probes.

3) Hybridization

Procedure

Hybridization and detection were performed in an automated washing and shaking device (Twincubator; Hain Life Science GmbH, Nehren Germany) under strict aseptic precautions.

- **20 µl of Denaturation Solution (DEN, blue)** was dispensed in a corner of each of the trough of a plastic tray.

- **20 µl of amplified sample** was added to the denaturation solution and mixed well by pipetting up and down, and then it is incubated at room temperature for 5 mins.

- Meanwhile, membrane strips coated with specific probes were taken out of the tube using tweezers and labeling was done with a pencil underneath the colored marker.

- **1 ml of Prewarmed Hybridization Buffer (HYB Green)** was added carefully to each trough. The tray was shaken gently until the solution had a
homogenous colour. Care was taken not to spill solution into the neighboring troughs.

- The strips were placed in each trough using tweezers. Care was taken that the strips must be completely covered by the solution and the coated side must face upward. Tweezers were cleaned after each use to avoid contamination.

- The tray was then placed in shaking water bath / Twincubator and incubated for 30 mins at 45°C. The shaking frequency of the water bath was adjusted to achieve a constant and thorough mixing of the solution. The tray was dipped into the water to at least 1/3 of its height to allow adequate heat transfer.

- After 30 mins, the tray was taken out and Hybridization buffer was completely aspirated with the dropper.

- 1 ml of stringent wash solution (STR, red) was added to each strip and incubated for 15 minutes at 45°C in Twincubator.

- After 15 mins, the stringent wash solution was removed completely by the dropper and remaining fluid was removed by turning the tray upside down and gently striking it on an absorbent paper.

- Each strip was washed once with 1 ml of Rinse Solution (RIN) for 1 min on Twincubator at room temperature.

- 1 ml of diluted Conjugate (Streptavidin conjugated with alkaline phosphatase) was added to each strip and incubated for 30 mins on Twincubator at room temperature.

- Conjugate solution was then removed and each strip was washed twice with 1 ml of Rinse Solution (RIN) for 1 minute and once with 1 ml of distilled water for 1 minute on Twincubator. Solution was poured out each time. Care was taken to remove any trace of water after the last wash.
• 1 ml of diluted substrate was added to each strip and incubated at room temperature in the dark for 20 mins.

• The reaction was stopped by briefly rinsing twice with distilled water.

• Finally, the strips were removed from the tray and allowed to dry between two layers of absorbent paper.

❖ Evaluation and Interpretation of Results

The developed strips were pasted in the evaluation sheet provided with the kit in the designated fields by aligning the bands. Conjugate Control (CC) and Amplification Control (AC) with the respective lines on the sheet. Each strip had a total of 27 reaction zones.

• Conjugate Control (CC)

A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

• Amplification Control (AC)

When the test is performed correctly, a control amplicon will bind to the Amplification control zone. If this band is developed, mistakes during extraction and amplification set up and the carry-over of amplification inhibitors can be excluded.

In case of a positive test result, the signal of the Amplification control zone can be weak or even vanish totally. This might be due to competition of the single reactions during amplification. The test does not have to be repeated.
- **M.tuberculosis complex (TUB)**

This zone hybridizes with amplicons generated from all members of the *Mycobacterium tuberculosis* complex. If the TUB Zone is negative, the tested bacterium does not belong to the *M.tuberculosis* complex and cannot be evaluated by this test system.

- **Locus Control rpoB, Kat G and inh A**

The locus control zones detect a gene region specific for the respective locus and most always stain positive when the TUB Zone has documented the presence of a *M.tuberculosis* strain. If neither the Locus control probe nor the Wild type or Mutations probes of one of the three genes examined are developed, the test cannot be evaluated.

- **Wild Type Probes**

The wild type probes comprise the most important resistance areas of the respective genes. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. Hence, the strain tested is sensitive for the respective antibiotics. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probe hence indicates a resistance of the tested strain to the respective antibiotics. The strip has 8 rpoB wild type probes (rpoB WT1 to rpoB WT8), one Kat G wild type probe (Kat G WT) and two inh A wild type probe (inh A WT1 & inh A WT2).
• **Mutation Probes**

The mutation probes detect some of the most common resistance-mediating mutations. Only those bands whose intensities are about as strong as or stronger than that of the Amplification control zone (AC) are to be considered.

The banding pattern obtained with the rpoB probes indicates about a rifampicin resistance of the strain tested, the Kat G banding pattern indicates about a high level and the inh A banding pattern about a low level isoniazid resistance. (Colour Plate- 11.a & 11.b)

The strip has **four rpoB mutant probes**.

- rpoB MUT 1 – D516V
- rpoB MUT 2A-H526Y
- rpoB MUT 2B –H526D
- rpoB MUT 3 – S531L

**Two KatG mutant probes**

- KatG MUT1 – S315T1
- Kat G MUT 2 – S315T2

**Four inhA mutant probes**

- InhA MUT 1 – C15T
- InhA MUT 2 – A16G
- InhA MUT 3A –T 8 C
- InhA MUT 3B – T 8 A
• When all of the wild-type probes gave a positive signal and all of the mutant probes reacted negatively, the *M. tuberculosis* isolate was considered susceptible to RIF and INH.

• When at least one negative signal was obtained with the rpoB wild-type probes, the isolate was considered resistant to RIF and same was true for the Kat G and inh A wild type probe for isoniazid.

• When the resistance to RIF and INH was due to one of the ten most frequently observed mutations described above, a positive reaction was obtained with at least one of the mutant probes and was always accompanied by a negative reaction with the corresponding wild-type probe.
Colour Plate No. 11(a)

Interpretation of GenoType MTBDR plus Assay
Colour Plate No 11(b)
Interpretation of GenoType MTBDR plus Assay
Prevalence of HIV-TB Coinfection

A total of 284 patients of tuberculosis (both pulmonary and extra-pulmonary) were screened for HIV seropositivity from January 2008 to December 2009. Diagnosis of tuberculosis was based on detailed clinical history, general and systemic examination, X-ray chest, sputum smear for AFB by direct smear and / or culture. In case of extrapulmonary tuberculosis relevant samples were obtained for mycobacteriological, histopathological, biochemical and cytological examination.

All the patients included in study were referred from RNTCP to VCTC as per NACO policy for screening of HIV antibodies. The meaning and implication of this test were explained and a written consent was obtained prior to HIV testing. VCTC provided confidential counselling and testing. Serum samples were collected and assayed for antibodies against HIV using Comb Aids (Span, Diagnostics Ltd.) at the Microbiology Lab, Central Diagnostics Lab, Shree Krishna Hospital, Karamsad. In case of seropositive, a second sample of the patient was collected and retested using three different kits Tri-Dot (J.Mitra & Co. ltd.), HIV Immunocomb (Orgenics) and Comb Aids (Span Diagnostics). Procedure was carried out as per manufacturer’s instructions.