5. MATERIAL AND METHODS

Study Settings:

The present study was undertaken from Aug 2010 to Dec 2012 at a JSS tertiary care general hospital located in the city center of Mysore, Karnataka, India. The hospital caters to patients coming from Mysore, Chamarajanagar, Mandya, Hassan, and Coorg districts.

All HIV seropositive in-patients whose HIV status was diagnosed by ELISA as well as by Combaids-RS, HIV 1/2 Triline Card test and HIV 1/2 Tridot in Integrated Counseling and Testing Centre (ICTC) were screened for all the OI and were divided into three groups A, B and C. (Appendix 1)

**Group A:** Consists of HIV reactive patients with suspected tuberculosis.

**Group B:** Consists of HIV reactive patients with suspected candidiasis.

**Group C:** Consists of HIV reactive patients with features of opportunistic infections other than tuberculosis and candidiasis.

Only Group A and Group B were studied as per the objectives defined and in Group C different opportunistic infections other than TB and candidiasis were recorded.

Ethical approval was obtained from the Institutional Ethics Committee, and accordingly informed written consent was obtained from all the patients.
Group A: Tuberculosis

All HIV seropositive patients with symptoms suggestive of TB, attending JSS hospital constituted the study material.

Diagnosis of Pulmonary TB was based on

- Chest X-ray appearance
- AFB positivity in sputum/BAL.

Diagnosis of extra-pulmonary TB was based on clinical features and any of the following

- TB lymphadenitis – Histopathological findings suggestive of TB.
- Demonstration of AFB in pus/body fluids/tissues/ urine/any other relevant specimen
- Tuberculous serous effusions (Pleural, pericardial, cerebrospinal, peritoneal). Biochemical parameters, Cell count and cell type suggestive of TB in these serosal fluid.
- TB of any other tissue/ organ- presence of the tubercular granuloma with or without AFB demonstration.
- Chronic diarrhea is another important symptom of AIDS patients & the etiology is varied, so stool samples were screened for AFB.

A sensitive and reliable tool - culture was used to recover Mycobacteria from blood and various other clinical materials collected from the site of pathology. Mycobacteria were recovered from blood using a special technique called lysis - centrifugation method, to release the intra-cellular organisms from phagocytic cells in the blood through lysis. The blood was then subjected for centrifugation that acts as a
concentration technique for Mycobacteria. The specimen was then inoculated on mycobacterial culture medium (LJ Medium).

A detailed history of each case was recorded in the proforma. For complete haemogram and Erythrocyte sedimentation rate (ESR), 2 ml of venous sample was collected in an EDTA and citrate tube. CD4 counts of all the patients were recorded. Each patient was instructed to provide early morning sputum (if productive) and stool sample for AFB smear and culture. Also, blood and other clinical specimens depending on the site of pathology were collected by aseptic methods.

**Inclusion Criteria:**

HIV reactive patients with one or more of the following:

- History of prolonged fever
- Weight loss
- Cough for more than 2 weeks (sputum smear negative)
- Radiological evidence suggestive of TB, pleural effusion
- Diarrhea persisting for >1 month, pain abdomen/ascites/ lymphadenopathy or any other site with features suggestive of TB were included in the study.

**Exclusion Criteria:**

- HIV negative patients.
- HIV positive patients with no features of TB.
- HIV positive patients with sputum smear positive for AFB.
- Patients with diarrhea due to parasitic infestation.
Materials

Sample collection for tuberculosis:

1. Sputum samples: Two samples of sputa (one spot and one early morning) as per the new RNTCP guidelines were collected from patients with features of pulmonary TB.
2. Blood samples: About 5 ml of venous blood was aseptically collected into a sterile test tube containing 3.8% sodium citrate from all the patients.
3. Stool sample: Stool specimens were collected in a clean container from all the patients included in the study and the history taken to know if there were any features of enteropathy.
4. Fine needle aspiration cytology (FNAC) / Biopsy specimens were collected from lymph nodes.
5. Other specimens like CSF, pleural fluid (PF), ascitic fluid (AF), pus, ear discharge sample were collected depending on the site of pathology.

Antibiogram of mycobacterial isolates:

The isolates obtained in culture were subjected to DST by proportion method (HI Media) and confirmed by LPA.

Methods:

Sample processing:

1. Smear preparation and ZN staining: Smears were prepared from all the specimens collected as per standard protocol and stained with ZN technique. The smears were studied for the presence of AFB and the results were noted.
Two smears were prepared directly from the stool sample. One stained by regular ZN staining technique and other by modified ZN method (using 1% \(H_2SO_4\) for decolorization) and looked for acid fast bacilli and parasites like Cryptosporidium, Cyclospora and Isospora. Saline and iodine mount were prepared and looked for other pathogens like Entamoeba, Giardia and helminthic forms.

2. Culture:

Sputum: Digestion and decontamination by NaOH procedure (Petroff’s method)

- Equal volumes of sputum and 4% NaOH (40 g/l- previously sterilized by autoclaving) are mixed in a sterile, leak-proof, 50-ml sterile plastic conical centrifuge tube.

- The mixture was homogenized in the vortex mixer for 15 to 20 seconds at room temperature (25–30°C) and incubated at 37°C for 30 min with intermittent shaking till it gets liquefied.

- The specimen is then concentrated by centrifugation at 3000 rpm for 30 min. Supernatant is discarded carefully into a splash-proof container filled with a disinfectant (phenol- or glutaraldehyde-based). The sediment is neutralized drop by drop with a 2-mol/l HCl solution.

- To the neutralized deposit, 1–2 ml of sterile distilled water was added and re-centrifuged for 10 min. The sediment was then inoculated onto LJ medium.

Blood: - Lysis centrifugation technique was followed.

Sodium citrate as anticoagulant: For 5 ml of blood 3.8% of 300 µl sodium citrate was used as an anticoagulant.
**Materials and Methods**

**Saponin as lysing agent:** To 5 ml of anticoagulated blood sample, 10 µl of 0.1% saponin was added. The sample was gently inverted several times immediately after collection to enhance lysis of blood cells. To confirm the lysis of blood cells, Leishman’s * staining was carried out. The cells were intact. To concentrate white blood cells (WBC), Wintrobe’s tube was used to recover the buffy coat. Using lumbar puncture (LP) needle the buffy coat was transferred into eppendorf tube containing 50µl (trial & error) saponin to lyse WBC. The above mixture was again smeared and Leishman’s staining was carried out for lysis confirmation. At this stage, the WBC appeared to be swollen but not lysed. To enhance lysis, coarse glass beads were added to the above mixture and vortexed for 10-12 minutes (trial & error). The smears prepared and stained in this step confirmed the cell lysis (5ml blood + 300 µl of 3.8% anticoagulant + 50 µl of 0.1% saponin + glass beads - vortexed for 10 to 12 minutes).

The processed blood samples were smeared and stained by ZN method and were examined for release of AFB. The samples were then inoculated onto LJ medium.

**Leishman’s staining:**

Procedure: 0.15 gms of Leishman’s powder was dissolved in 100 ml of methanol by grounding powder in a mortar.

- The buffy coat was smeared on a clean glass slide and flooded with Leishman’s stain and left for a min.
- It was then diluted with distilled water and left for about 12 min.
- The smear was then flooded with water until the film appeared bright pink for about 30 sec.
- The excess of water was drained, blotted and air dried.
Photo 1: Swollen WBC (On addition of Saponin)

Photo 2: Lysed WBC cells (saponin+glassbeads+vortex)
**Stool specimen:**

Preparation of Middlebrook 7H9 broth: About 2.35 gms of Middlebrook 7H9 powder was suspended in 450 ml of distilled water and 2 ml of glycerol was added. The contents were heated to dissolve completely, and autoclaved at 121°C for 10 min.

About 1 gm of stool was suspended in 5 ml of Middlebrook 7H9 broth and subjected for NaOH digestion / decontamination. The homogenized sample was incubated at 37°C for 30 min with intermittent shaking. It was then neutralized with 1N HCl and centrifuged at 3000 rpm for 30 min. The supernatant was discarded and sediment inoculated onto sterile LJ slant.

FNAC/biopsy specimens were also processed for cytology / histopathological examination and to demonstrate the AFB.

Other sterile specimens like CSF and body fluids were centrifuged for 30 min at 3600 rpm for 30 min to concentrate the bacteria. The supernatant was decanted, and the sediment was vortexed and inoculated onto LJ media.

The development of characteristic colony morphology showing luxuriant growth using a small amount of inoculum at a lower rate of contamination and the low cost made us choose the LJ medium for our study.

All the inoculated LJ slants were incubated for 2–3 days at 35–37°C in a horizontal position, with the caps loosened half a turn for 6 to 8 weeks and inspected for growth of any colonies of bacteria on the surface at weekly intervals. When growth appears, the rate of growth, pigmentation and colony morphology were recorded. Smears were prepared, stained by ZN procedure and examined for AFB.
Mycobacterial speciation: On confirmation of AFB the isolates were subjected to Para - nitrobenzoic acid (PNB) test to differentiate Mycobacteria into typical or atypical Mycobacteria.

Procedure: The mycobacterial suspension was inoculated on two slopes of LJ medium, one without drug (control) and one with PNB (Hi-media SL021) at a conc. of 500 µg/ml and incubates at 37°C. Read after 28 days.

Results and interpretation:

- MTB does not grow on PNB medium. All other Mycobacteria are resistant.
- Growth on medium containing PNB – NTM.
- Abundant growth on control tube and little or no growth on PNB medium - MTB complex strain.
- No growth on either slope: non-interpretable test, to be repeated.

Drug susceptibility:

Proportion method: DST was carried out by using Himedia first line kit which consisted of 2 controls and 4 first line drugs (Table 12). LJ slants with different antitubercular drugs were inoculated as per the manufacturer’s instruction mentioned below.
Table 12: First line test kit used for Antimycobacterial susceptibility test

<table>
<thead>
<tr>
<th>No.</th>
<th>Drug</th>
<th>pH</th>
<th>Concentration (mcg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>6.8±0.2</td>
<td>-----</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>5.5±0.1</td>
<td>-----</td>
</tr>
<tr>
<td>3</td>
<td>Isoniazid</td>
<td>6.8±0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>Ethambutol</td>
<td>6.8±0.2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Pyrazinamide</td>
<td>5.5±0.1</td>
<td>200</td>
</tr>
<tr>
<td>6</td>
<td>Rifampicin</td>
<td>6.8±0.2</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>Streptomycin</td>
<td>6.8±0.2</td>
<td>4</td>
</tr>
</tbody>
</table>

Preparation of inoculum & inoculation:

- A loop full of *M. tuberculosis* growth, primarily isolated from LJ medium slant was suspended in 1.0ml of sterile distilled water in a screw capped bottle containing glass beads of 3.0mm diameter for better homogenization and declumping of cells.

- The mixture was homogenized by vortexing upto 10 min and kept at room temperature for 10 min.

- Opacity of suspension was adjusted to match McFarland 0.5 standard with saline giving approximately $1.5 \times 10^8$ CFU/ml.

- The suspension was diluted to 1:10000 and 100µl of this inoculum was seeded on LJ medium slants. The slants were then incubated at 35-37°C for 6 to 8 weeks.
Interpretation of results:

The isolates were termed either resistant /sensitive/ intermediate - depending upon the duration of growth as compared to standard strain.

Evaluation was done as follows:

- The colony characteristics and colony count on the control medium were recorded.
- Results obtained for respective dilutions were calculated as the % of resistance.

Formula to calculate the percentage of resistant cells:

\[
\frac{\text{No of colonies on the drug medium}}{\text{No of colonies on the control medium}} \times 100 = \% \text{ of resistance}
\]

Accordingly, the results were interpreted as:

- Resistant : when > 1%
- Sensitive : when < 1%
- Intermediate : when = 1%.

The results were confirmed by LPA i.e. Genotype MTBDR plus assay (Hain Life Science) method for the detection of the most common mutations in the *M. tuberculosis*.

**Line Probe Assay:** The assay was performed to detect MTB and drug susceptibility as per manufacturer’s instructions (Hain Lifescience GmbH).

- A loop full of Mycobacteria was suspended in 300µl of molecular grade water and vortexed.
• 100µl of genolyse was added and kept for 5 min at 95°C. 100µl of NA (neutralizing) buffer was added after 5 min.

• Above mixture was spun down for 5 min at full speed and 5µl of supernatant was used for polymerase chain reaction (PCR).

• Master mix was prepared by adding Primer/Nucleotide Mix (PNM) + buffer + MgCl₂ + Taq polymerase as per master chart.

• To the 45µl master mix in a 200µl PCR tube, 5µl of supernatant was added and the tube was loaded in thermal cycler and PCR program was run.

• After PCR run, the amplicon was subjected to reverse hybridization.

• 20µl of denaturation solution was added to the wells provided in the kit. To this 20µl amplicon (PCR product) was added. It was mixed up and down and incubated for 5 min.

• Pre warmed 1 ml of hybridization buffer was added to the well and mixed to get homogenous color.

• The labeled strip was put in the respective wells.

• The tray was placed in twincubator and the program S1 started i.e. incubation for 30 min at 45°C.

• The hybridization buffer was aspirated completely with pipette and the tray was tapped on tissue paper.

• 1 ml of stringent solution was added and the program S2 was run in twincubator i.e. incubation for 15 min at 45°C.

• The stringent solution was aspirated completely with pipette and the tray was tapped on tissue paper.

• 1 ml rinse solution was added and S3 program was run i.e. 1 min shaking in twincubator.
• The rinse solution was completely removed and the tray was tapped on tissue paper.

• 1 ml of conjugate was added and program S4 was run i.e. 30 min shaking in twincubator.

• The conjugate was removed completely, and the tray was tapped on tissue paper. 1 ml of rinse solution added again and program S5 was run i.e. 1 min shaking in twincubator.

• The rinse solution was removed completely and the tray tapped on tissue paper following the same rinsing step i.e. program S6.

• The rinse solution was completely removed, 1 ml of distilled water was added and program S7 was made to run i.e. 1 min shaking.

• The water was completely removed, the tray was tapped on tissue paper and 1 ml of substrate was added. The tray was then placed in twincubator and was covered with aluminum foil till the substrate incubation was over, S8 i.e. 5 to 7 min stationary.

• The substrate was removed completely and the tray was tapped on tissue paper. 1 ml of distilled water was added and the program S9 was run i.e. 1 min shaking.

• The water was completely removed from tray, tapped and step was repeated, S10 – 1 min shaking.

• After tapping the tray, the strip was pasted on evaluation sheet and the result was interpreted.

**Interpretation of result:**

The strips were pasted on evaluation sheet with designated fields by aligning the bands CC and AC with the respective lines on the sheet.
A line developed in the Conjugate Control (CC) zone documents the efficiency of conjugate binding and substrate reaction.

A band in the Amplification Control (AC) zone specifies correct extraction and amplification process.

Band in the TUB zone indicates the tested bacterium is \textit{M.tuberculosis} complex and the absence indicates atypical Mycobacteria.

When all wild type probes of a gene locus stained positive and the band intensities were stronger than AC zone, there was no detectable mutation. If at least one of the wild type bands were absent it indicated a resistance. The banding pattern with rpoB probes drew a conclusion about RIF resistance, those in the katG probes as high level of INH resistance and those in the inhA as low level INH resistance respectively.

Positive signals in at least one of the mutation bands (MUT) of rpoB, katG and inhA regions and their intensities stronger than AC zone indicated mutation positive and conferred resistance to RIF, high level and low level INH resistance respectively.

\textbf{B. Candidiasis}

\textbf{Inclusion Criteria:} HIV positive patients with oral, esophageal, vaginal and systemic candidiasis

\textbf{Exclusion Criteria:} HIV negative patients

Sample collection:

1. Oral candidiasis: Scrapings from mouth.
2. Esophageal candidiasis: Scrapings of upper GI during endoscopy.
4. In systemic candidiasis pus/aspirates were collected depending on the sites of lesions.
Materials and Methods

Dept. of Microbiology, JSSMC, Mysore

Photo 3: Oral candidiasis A & B
Sample processing:

**KOH Mount:** The specimens collected were subjected to KOH mount examination.

Procedure:

- The samples collected from rayon applicator swabs were placed on a clean glass slide.
- A drop of 10% KOH was added on the specimen and a coverslip was placed over it.
- The slide was gently heated and observed for yeast cells under the microscope.

**Gram staining:**

- The specimen was spread over a clean glass slide and heat fixed.
- The smear was flooded with crystal violet and left for 2 min.
- Rinsed with water and Gram’s iodine was added onto the smear and left for 1 min.
- Rinsed and decolorized with absolute alcohol for 30 sec.
- The smear was then counter stained with safranin for 2 min.
- The smear was rinsed, dried and observed for any yeast cells under the microscope.

The collected samples were cultured on SDA medium (2 sets) and on HiCrome *Candida* Differential Agar (CHROM agar) medium. Of the two sets one was maintained at room temperature and the other at 37°C. Cultures obtained were observed for growth of yeast like colonies every day, and incubated for a maximum period of 10 – 15 days.
Materials and Methods

Procedure:

- Sabouraud Chloramphenicol Agar (M1067): About 6.5 gms of Sabouraud chloramphenicol agar was suspended in 100 ml of distilled water. The contents were boiled to dissolve the medium completely. It was then sterilized by autoclaving at 15lbs pressure (121°C) for 15 min.
- The medium was then poured into the sterile petriplate and allowed to solidify.
- The specimen was inoculated and incubated at 25°C for 48 hrs.
- A wet mount was prepared from an isolated colony to examine purity of the colony.

Growth on Candida CHROMagar:

Procedure:

- HiCrome Candida differential Agar base (M1456A) preparation: 2.102 grams of Candida differential agar was suspended in 100ml of distilled water. The contents were heated gently to dissolve the medium completely (no autoclave). It was then cooled to 50°C and rehydrated contents of 1 ml from the vial of Candida selective supplement (FD192) were added aseptically.
- The medium was mixed well, poured into sterile petriplate and solidified.
- The culture was then inoculated into the solidified plate and incubated at 25°C for 48-72 hrs.
- The colors of the colonies were recorded.

Many researchers reported the use of CHROMagar in resource constrained settings to speciate Candida. (Anaparthy Usharani et al Luz Angelo et al).
Germ Tube test:

- A freshly grown pure culture of *Candida* species was suspended in 0.5ml of fresh human serum and incubated for 2 hrs at 37°C.
- A drop of suspension was taken on a slide with a coverslip and examined under the microscope for the germ tube.
- Observation: Germ tubes were observed as long tube-like projections extending from yeast cell without constriction.

Morphology on Cornmeal agar: (Dalmau plate)

Procedure:

- Cornmeal agar preparation (M146): 1.7 gms of CMA was suspended in 100 ml distilled water. The contents were boiled to dissolve the medium completely. 1 ml of Tween 80 was added to the medium and then sterilized by autoclaving at 15lbs pressure (121°C) for 15 min.
- Sterilized CMA 1% Tween 80 was then poured onto sterile petri plates and solidified.
- The plate was divided into 4 quadrants and labeled.
- Aseptically 2-3 streaks of approximately 3.5 - 4cm long and 1.2 cm apart were made on solidified media using a straight needle.
- A flame sterilized and cooled 22 mm square coverslip was placed over the streak to provide partial anaerobic environment at the margins of the coverslip.
- The plates were incubated at 25°C for 3-5 days and then observed at the edge of the cover glass.
- Morphological features like hyphae, pseudohyphae, spores/ sporangia were recorded.
**Biochemical tests:** Sugar fermentation, sugar assimilation, and urease tests were performed for further speciation.

**Sugar Fermentation:**

Liquid fermentation medium preparation: A medium containing 1% peptone, 0.5% sodium chloride and 0.005% Andrade’s indicator was sterilized by autoclaving at 121°C for 15 min at 15lbs pressure.

- Filter sterilized 2% sugar (glucose, lactose, maltose and sucrose) was added to the medium.
- Approximately 5 ml of the medium was dispensed into sterile test tubes along with Durham’s tube.
- Approximately 0.1 ml of inoculum was inoculated to the carbohydrate broth.
- The tubes were incubated at 25°C up to a week. The tubes were examined every 48 – 72 hrs of interval for the production of acid (pink color) and gas (in Durham’s tube).
- Production of acid in the tube was taken as fermentation positive while only acid production indicated carbohydrate assimilation.

**Sugar assimilation (Auxanographic technique):**

The Disc impregnation - Pour plate Auxanographic method of Wickerham and colleagues was carried out for sugar assimilation test. Sugars (12 types of sugar discs) tested include glucose, maltose, sucrose, lactose, galactose, melibiose, cellobiose, inositol, xylose, raffinose, trehalose and dulcitol.

Procedure:

- Preparation of Yeast nitrogen base (YNB) and agar: About 6.7 gms of YNB was dissolved in 100 ml of distilled water and sterilized by filtration. 20 gms
of agar was dissolved in 980 ml of distilled water separately. It was then dispensed in 18 ml quantity of 18X150mm screw capped tubes and then autoclaved at 121°C for 15 min.

- Yeast suspension was prepared in 2ml of (YNB) by adding heavy inoculum of 24 – 48 hrs old culture and matched with 5 McFarland Standard.
- This 2 ml of yeast suspension was then mixed with 18 ml of molten agar (cooled to 45°C) and poured onto a 90 mm petriplate.
- A total of 12 sugar discs were tested. Six types of sugar discs (HI Media) were placed aseptically into a plate and the other six in another plate of YNB agar. The plates were incubated at 37°C for 3 to 4 days.
- The presence of growth around disc was considered as positive.

**Urea Hydrolysis:**

**Procedure:**

- Christensen’s urea agar preparation: 24.01 gms of agar was suspended in 950 ml of distilled water. The contents were boiled to dissolve the medium completely. It was then sterilized by autoclaving at 15lbs pressure (121°C) for 20 min. After having cooled to 50°C, aseptically 50 ml of sterile 40% urea solution (FD048) was added, mixed well and slants were done.
- The slants were inoculated with 24-48 hrs of yeast culture and incubated at 25°C for 2 to 5 days.
- A deep pink color was considered as positive.

The results of sugar fermentation, sugar assimilation and urease test were correlated with the master chart (Table 13). Along with this data and the results of culture on CHROMagar helped in *Candida* speciation.
Table 13: Clinical and biochemical characteristics of yeasts frequently isolated from clinical specimens
Confirmation of *Candida* species and drug susceptibility were carried out by Vitek 2 automated system using the culture from Blood agar medium.

AST using Vitek 2 system was performed according to the manufacturer’s instructions. The categorical result was obtained according to the breakpoints provided by the Vitek-2 system for amphotericin B (susceptible [S], ≤1 μg/mL; intermediate, 2 μg/mL; resistant [R], ≥4 μg/mL), fluconazole (S, ≤8 μg/mL; susceptible dose dependence [SDD], 16 to 32 μg/mL; R, ≥64 μg/mL), caspofungin (S ≤0.25 μg/mL; R, ≥4 μg/mL), voriconazole (S, ≤1 μg/mL; SDD, 2 μg/mL; R, ≥4 μg/mL) and flucytosine (S, ≤4 μg/mL; intermediate, 8-16 μg/mL; R, ≥32 μg/mL).

Procedure:

- Preparation of Blood agar: To the autoclaved nutrient agar, at 50°C the sterile blood at 5% concentration was added. The molten medium was then poured to sterile petriplate and allowed to solidify.

- The plates were then inoculated with the *Candida* isolates and incubated at 37°C for 24-72 hrs and observed.

- Two tubes were taken; the first tube was labeled as ID (identification) and the second tube as AST.

- 3 ml of saline was taken in each tube. The first tube was kept at an angle of 45° and a smear was made with a loop full of inoculum (36-48 hrs) on the walls of the first tube, till a dry smear was formed, followed by mixing with the saline (to ensure uniform mixing of the inoculum).

- The O.D (optical density) was adjusted to 1.8-2.2nm. After adjusting the O.D in the turbidometer, 280 μl of the inoculum was transferred to the second tube.
The respective ID and AST cards were placed to the first and second tubes. Carefully the cassettes were loaded into the Vitek 2 system. The patient details were entered. ID and AST cards were linked in the Cassette information. The program was run. Once analyzed, the results were displayed on the computer monitor on entering the identification number of the sample. The results were recorded and subjected to statistical evaluation.