CHAPTER 3

Materials and methods

The samples of present study were collected from the suspected patients of candidiasis attended in different departments of Medical College Silchar, Assam. A total of 113 Candida species were isolated from 500 clinically suspected cases of candidiasis from the indoor and as well as outdoor patients. Each sample was first subjected to direct microscopic examination. For this potassium hydroxide mount (KOH mount), lactophenol cotton blue mount (LPCB mount) and gram stained smear were examined.

Collection of samples:

Different samples on the basis of manifestations were collected from patients. Samples were collected in a sterile, leak proof container and brought to the laboratory as soon as possible.

Blood samples were collected in BacT/Alert blood culture bottles which contained brain heart infusion broth (BHI broth). 5ml to 10 ml of blood was collected from adult and 3ml to 5ml of blood was collected from neonates. Paired blood samples were collected from the patients with suspected case of central venous catheter related blood stream infection (CRBSI). As the blood sample was collected it was then gently mixed with BHI broth and incubated at 37°C for 5
days. In case positive subculture was done in Sabouraud’s Dextrose Agar (SDA) and blood culture plates.

In case of pus, high vaginal swab (HVS), samples were collected with sterile swab and plated in SDA and blood culture plates.

For the sputum samples patients were first instructed, early morning sputum was collected in sterile wide mouth container. Smear was prepared, heat fixed and stained (Chander, 2009). It was then examined under high power and oil immersion under compound microscope for presumptive laboratory diagnosis.

Early morning midstream urine was collected in sterile wide mouth container. In case of catheterized patients samples were collected from catheter tube. Urine wet mount was observed by placing a drop of urine in to a slide and covering with cover slip. Samples were cultured on Sabouraud’s Dextrose Agar (SDA) medium with antibiotics (Chander, 2009).

Following steps were carried out to confirm the Candida species (Chander, 2009):

1) Initially KOH mount and LPCB mount were observed under microscope.
2) Grams stain of culturable samples.
3) Culture was done on SDA with antibiotics.
4) Dalmu plate culture for chlamydospore of candida was done.
5) Undisturbed morphology was also studied in Dalmu plate for confirmation.
6) Germ tube test put for all isolates from culture plates.
7) Biochemical test
   a. Sugar assimilation
   b. Sugar fermentation

8) Antifungal susceptibility test was carried out

**KOH wet mount:**

**Procedure:**

1) A drop of sample was placed on glass slide and
2) 1 to 2 drops of 10% KOH was added.
3) A cover slip was then placed over the mixture.
4) The slide was carefully examined under low power (10x) and then
   high power (40x) objective lens to detect budding yeasts cells and
   pseudohyphae.

**Lactophenol cotton blue mount (LPCB mount):**

The LPCB mount was done by preparing of each samples and staining it
with lactophenol cotton blue stain. The LPCB solution was prepared as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Phenol crystals</td>
<td>20g</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>20ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>40ml</td>
</tr>
<tr>
<td>Cotton blue</td>
<td>0.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>20ml</td>
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Wet mount preparation:

a) A small drop of sample was placed on a clean microscopic slide using a sterile loop.

b) A drop of LPCB was mixed with it.

c) Clean cover slips was put over it and observed under low power (10x) and then high power (40x) objective lens of microscope.

Staining procedure:

Lactophenol stain:

a) A small drop of sample was placed on a clean microscopic slide using a sterile loop and smeared with the help of another slide. The smear thus prepared was fixed in absolute alcohol.

b) The smear was flooded with lactophenol cotton blue solution and kept undisturbed for 1 minute.

c) The smear was washed with running tap water and observed under microscope.

Gram stain:

a) Smear was prepared in a glass slide which was then fixed.

b) The fixed smear was then covered with primary stain crystal violet for one minute.

c) Smear was then washed with water and iodine was put for one minute.

d) After washing counter stain that is saffranin was put and kept for one minute.
e) Final washing was done with water and air dried and observed under high power and oil immersion, under microscope.

**Culture and identification:**

The Sabouraud’s Dextrose Agar (SDA) medium with antibiotics was used for the primary isolation of fungus in culture from the clinical samples. Readymade as well as prepared SDA media was used for the study. The medium was prepared as follows:

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<table>
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<tbody>
<tr>
<td>Dextrose</td>
<td>40g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10g</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>

The pH of the medium was adjusted to 7.2-7.4 and autoclaved at 121°C for 15 minutes at 15lbs/in² pressure for sterilization. It was supplemented with 0.05 mg/ml of chloramphenicol after sterilization to prevent bacterial growth. For the isolation swab was streaked over the surface of SDA plates. The plate was then incubated at 28°C and observed for growth upto 7 days.

**Identification of isolated fungi:**

After 24-48 h of incubation at 27°C, the SDA plates with fungal growth were labeled as positive and plates without growth as negative and subcultured on SDA slants containing chloramphenicol.
Identification of yeast isolates: Yeasts were identified using ‘yeast Identification programme’ based on results of chlamydospore, germ tube formation and biochemical tests (Kwon Chung and Bannett, 1979 and Lodder 1970). These tests were performed with 48 h actively growing strains of yeast isolates.

**Germ tube test**

Germ tube test was carried out for presumptive identification of C. albicans. Production of germ tube represented initiation of hyphae directly from the yeast cells within two to three hours in serum and is the indicative of C. albicans and considered as rapid screening test. The germ tube formation was influenced by the medium, inoculum size and the incubation temperature.

**Procedure:**

a. A loop full twenty four hours old growth of pure culture was taken.

b. A suspension was made with 0.5 ml of pooled human serum and pure culture in a sterile test tube. $10^5 - 10^6$ cells /ml was the optimum inoculum.

c. It was incubated at $37^0$C for 2.5 to 3 hours.

d. After incubation a drop was taken on a slide and covered with a coverslip and was observed under low power magnification for the presence of germ tube.

As increased concentration of inoculum may decrease the formation of germ tube and other parameters like incubation temperature and incubation time were followed strictly according to standard procedure.
Morphological character on Corn meal agar (Dalmau plate method) (Larone, 2011):

Chlamydospore formation:

Chlamydospore formation was studied on Cornmeal Agar (CMA) Medium supplemented with Tween80. The composition of the medium was as follows:

- Cornmeal: 125g
- Agar: 50g
- Distilled water: 3000ml
- Tween80: 10ml
- Chloramphenicol: 0.05mg/ml

Corn meal agar containing 1% Tween 80 was prepared and autoclaved at 121°C for 15 minutes at 15 lbs/in² pressure. When the temperature of the medium reached around 48°C, it was then dispensed in the sterilized 90 mm petriplates and kept for solidification. Plates were kept at 4°C in refrigerator. Thereafter, these were inoculated in the following manner:

Method:

a) One plate of corn meal agar (CMA) was divided into 4 quadrants and labeled each quadrate.

b) Using a sterile needle or straight wire colony was lightly touched & 2-3 streaks of approximately 3.5-4 cm long and 1.2 cm apart was done on the CMA.
c) 22 mm square cover glass slip was flame sterilized and cooled and placed over the streak. This will provide partially anaerobic environment at the margins of coverslip, as shown below:

d) The plate was then incubated at 25°C for 3-5 days.

e) After incubation lid of the petriplate was removed and was placed in the microscope stage and the edges were observed under low power objective (10x) first and then high power objective (40x).

f) Morphological features were observed and noted.

g) With a help of forceps carefully the cover slip were removed and placed on a clean microscopic slide containing a drop of lactophenol cotton blue (LPCB). The slide was then observed under high power microscope.
Biochemical test:

Sugar assimilation medium:

Sugar assimilation test was done in Modified Wickerham Medium (Larone, 2011). This method is easier to read and reliable.

- Bromocresol purple (1.6%) - 0.2 ml
- 1/10NaOH - 1.0 ml
- Noble agar - 2.0 g
- Deionised water - 90.0 ml

Dissolved by heating.

Carbohydrate solution (stock solution)

- Carbohydrate - 1.00g
- Yeast nitrogen base - 0.67g
- Deionised water - 10.0 ml

Mixed to dissolve by gently heating.

Method of preparation:

a) The stock carbohydrate solution was mixed with agar base.

b) Mixed well.

c) pH adjusted to 7.0

d) 5ml amount of medium was poured in screw capped tubes

e) Sterilized by autoclaving

f) Allowed to solidify in slanted position

g) Stored in refrigerator at 4°C
Procedure:

Two millimeter loop full of pure culture was suspended in 9.0 ml sterile water. Each assimilation slants was inoculated with 0.1 ml of suspension. Tubes were incubated at 25°C and examined at 7th and 14th day for abundant growth and acid production (indicated by change in color to yellow).

Negative assimilation was considered when there was no difference in control without carbohydrate and the tube containing test organism with carbohydrate.

Sugar fermentation:

Peptone - 10g
Sodium chloride - 5.0g
Sugar (lactose, glucose, maltose, sucrose)- 30.0g
Andrede's indicator - 0.60g
Distilled water - 1000ml

All the constituents were mixed and pH was adjusted to 7.2, sugar was added in tubes along with Durham's tube and autoclaved at 121°C for 15 min.

Four sugars i.e., lactose, glucose, maltose, sucrose were used for the fermentation properties. With the help of sterile loop four tubes were inoculated with a portion of colony. Inoculated tubes were incubated at 37°C for 7-10 days. Culture tubes were observed every day for the production of acid and gas in inverted Durham's tube.
Anti fungal susceptibility test:

Antifungal susceptibility was done by Kirby–Bauer disc diffusion method (Kmath et al., 2009). Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg/ml methylene blue dye is used. The glucose provides a suitable growth for yeasts and the addition of methylene blue enhances the zone edge definition. The pH of the medium adjusted between 7.2 and 7.4. The inoculum is standardized to 0.5 McFarland and inoculated in the plate. Readymade antifungal disc (Amphotericin B 10U, Fluconazole 25μg, Itraconazole 15 μg) of HiMedia is placed in the media. It is then incubated at 35°C for 48 hours. Zone of inhibition was read after 24 hrs of incubation.