APPENDIX

1. **Preparation of Sabouraud's dextrose agar - Emmon's modification (NSDA)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>20 gm</td>
</tr>
<tr>
<td>Peptone</td>
<td>10 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The ingredients were added to distilled water after the medium allowed to cool to 50°C. 50 mg of chloramphenicol (Hi-Media, India) dissolved in 10 ml of ethanol (BDH Laboratory supplies, Poole, England, UK) was added to the medium. Autoclaved at 121°C for 10 min. Final pH 6.8-7.0.

2. **Preparation of Staib's agar** (Staib, 1962)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Guizotia abyssinica</em> seed</td>
<td>50 gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1 gm</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1 gm</td>
</tr>
<tr>
<td>Creatinine</td>
<td>1 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>15 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The seeds were ground finely in an electric mixer. 50 gm of pulverised seed was added to distilled water, boiled for 30 min and filtered through a
muslin cloth. The volume was made up to 1000 ml with distilled water. The other ingredients were added to the filtrate and autoclaved at 110°C for 20 min. Final pH 5.5.

i) **Supplementation of chloramphenicol**

500 mg of chloramphenicol dissolved in 10 ml of ethanol was added to the medium at 50°C.

ii) **Supplementation of 0.01% and 0.1% biphenyl**

100 mg and 1 gm of biphenyl dissolved in 20 ml of ethanol were added respectively after allowing the medium to cool to 50°C.

iii) **Supplementation of methyl violet**

2 mg of methyl violet (Hi-Media, India) was added to a litre of the medium before autoclaving.

3. **Preparation of Sunflower seed agar** (Pal and Baxter, 1985)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower seed</td>
<td>45 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The seeds were ground finely with an electric mixer. 45 gm of pulverised seed was boiled in 500 ml of distilled water for 30 min and filtered through a muslin cloth. The volume of the filtrate was made upto 1000 ml and autoclaved at 110°C for 30 min. The medium was allowed to cool to 50°C and 500 mg of chloramphenicol dissolved in 10 ml of ethanol. Final pH 5.6.
4. **Preparation of Blood culture medium**

Dehydrated BHI broth (Hi-Media, India) - 37 gm  
Agar - 17 gm  
Distilled water - 1000 ml

The ingredients were dissolved in distilled water and autoclaved at 121°C for 15 min. 30 ml of the medium was dispensed into the bottle, autoclaved and slanted. After solidification, 20 ml of BHI broth, prepared separately was added to the bottle. Final pH 5.6.

5. **Preparation of Christenson’s urea agar**

Glucose - 5 gm  
Sodium chloride - 5 gm  
Potassium dihydrogen phosphate - 2 gm  
Peptone - 1 gm  
Agar - 20 gm  
Distilled water - 1000 ml

The ingredients were dissolved in distilled water and 6 ml of phenol red (0.2% in 50% alcohol) was added. Autoclaved at 115°C for 30 min. 50 ml of urea solution (40% aqueous filter sterilised) was added, after allowing the medium to cool to 50°C. The medium was dispensed into test tubes and slanted. Final pH 5.6.
6. **Sugar fermentation test**

**Preparation of sugar fermentation broth**

Yeast extract (Hi-Media, India) - 4.5 gm

Peptone - 7.5 gm

Distilled water - 1000 ml

The ingredients were dissolved in distilled water. Enough bromothymolblue was added to give a sufficiently dense green colour. 2 ml of the broth was dispensed into test tubes containing inverted Durham’s tube. Autoclaved at 121°C for 15 min and 1 ml of 6% sugar solution (dextrose, maltose, lactose, galactose and trehalose (Hi-Media, India) sterilised by Seitz filter was added, after allowing the medium to cool to 50°C.

Two loopfuls of 72 hr old culture was transferred to 2 ml of sterile distilled water and the density of the suspension was made to McFarland No.1 standard. 0.2 ml of the suspension was added to fermentation broth tube containing 3 ml of medium, Durham’s tube and the various sugars. All the inoculated tubes were incubated at 30°C for one week. Positive fermentation was evidenced by gas bubbles being trapped in the Durham’s tube.

7. **Sugar assimilation test**

**Preparation of Basal medium**

Yeast nitrogen base (Hi-Media, India) - 6.7 gm

Agar - 20 gm

Distilled water - 1000 ml
The ingredients were dissolved in distilled water, 10 ml of medium was dispensed into tubes and autoclaved at 115°C for 15 mins. Final pH 5.6.

Prepared basal medium was cooled to 45°C and the medium was seeded with 1 ml of the test organism with the density of the suspension was equivalent to McFarland No.4 standard. The mixture was poured into sterile petriplates. After the agar yeast mixture has hardened, the disks impregnated with various sugars such as dextrose, lactose, sucrose, maltose, galactose, raffinose, xylose, rhamnose, dulcitol, inositol, melibiose, inulin and cellobiose (Hi-Media, India) were placed on the surface of the medium. The plates were incubated at 30°C for 48 hr and examined for the presence or absence of yeast growth around each disk. Growth around a carbon source indicates assimilation of the particular compound.

8. Growth at 37°C

Preparation of Yeast-malt extract agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt extract (Hi-Media, India)</td>
<td>3 gm</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3 gm</td>
</tr>
<tr>
<td>Peptone</td>
<td>5 gm</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 gm</td>
</tr>
<tr>
<td>Agar</td>
<td>20 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The ingredients were dissolved in distilled water by boiling, dispensed into tubes, autoclaved at 121°C for 15 min and slanted. Final pH 5.6.
**Procedure**

Duplicate slants of yeast-malt extract agar were inoculated with the isolates. One of each pair of slants was incubated at 37°C and the other at 26°C. Growth was compared after one week of incubation. When growth at 37°C was equal to or more than the growth at 26°C, growth at 37°C was recorded as positive.

9. **Phenoloxidase production test**

**Procedure**

The isolate was inoculated in Staib's agar slant and incubated at 26°C for one week. The formation of BCE within a week was interpreted to indicate phenoloxidase production.

10. **Nitrate reduction test** (Hopkins and Land, 1977)

**Preparation of Nitrate reduction test swab**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>-</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium nitrate</td>
<td>-</td>
<td>2 gm</td>
</tr>
<tr>
<td>Sodium di hydrogen phosphate</td>
<td>-</td>
<td>11.7 gm</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>-</td>
<td>1.14 gm</td>
</tr>
<tr>
<td>Benzalkonium chloride (17% solution)</td>
<td>-</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

The ingredients were dissolved in distilled water and pH was adjusted to 5.8. Cotton-tipped swabs were placed in the solution and allowed to saturate. The swabs were dried for 24 hr at RT and autoclaved at 121°C for 15 min.
Preparation of Nitrate reduction reagents

Solution A : 8 gm of Sulphanilic acid (Hi-Media, India) was dissolved in 1000 ml of acetic acid (E.Merck [India] Ltd., Mumbai, India), 5 mol/l.

Solution B : 5 gm of α-napthylamine (Hi-Media, India) was dissolved in 1000 ml of acetic acid 5 mol/l.

Procedure

The cotton swab containing nitrate reagent was swept across several 48 hr yeast colonies and swirled against the bottom of an empty sterile tube to assure adequate contact between the organism and substrate in the cotton. The test tube and swab were incubated at 45°C for 10 min. After incubation, the swab was transferred to a tube containing three drops each of solution A and solution B reagents. A change in colour to red indicated positive results.

11. Pathogenicity in mice

Procedure

The isolate was precultured on SDA at 26°C for 48 hr, suspended in saline with the density of McFarland No.0.5 standard and 0.2 ml of inoculum was injected into the tail veins of two swiss albino mice obtained from Tamilnadu Veterinary and Animal Sciences University, Chennai. The died and sacrificed mice, after two weeks of inoculation, post-mortum was done and the presence of lesions on the organs were observed. The organs were examined for the presence of encapsulated yeast cells by negative staining and inoculated onto SDA and Staib’s agar for the growth of the organism.
12. **Preparation of Sabouraud's dextrose agar (SDA)**

Dextrose - 40 gm  
Peptone - 10 gm  
Agar - 20 gm  
Distilled water - 1000 ml

The ingredients were dissolved in distilled water and autoclaved at 121°C for 10 min. Final pH 5.6.

13. **Preparation of test CSF and sera**

0.5 ml of CSF was inactivated by heating in a water bath at 100°C for 30 min. 0.5 ml of serum was heat inactivated at 56°C for 30 min.

14. **Co-agglutination (Co-A) reagents preparation**
a) **Production of Hyperimmune sera**

Serotype A of *C. neoformans* was grown on SDA at 27°C for 72 hr. The yeast cells were harvested with physiological saline solution and heated in a water bath at 100°C for 1 hr. The heat killed cells were used as antigen.

Male New Zealand white rabbit weighing about 2 kg obtained from Tamilnadu Veterinary and Animal Sciences University, Chennai were immunised with the prepared antigen and a concentration equivalent to a McFarland No.9 standard. The rabbit was given intravenous injection of 1.0, 2.0, 4.0, 4.0, 4.0, 4.0 and 4.0 ml of the antigen at 4 day intervals. The injected rabbit was bled from the marginal vein of the ear. The serum was tested for their titres against the immunising strain by slide agglutination test. When
the titre was 1:640 or higher, the rabbit was bled seven days after the last injection. In case, where the antibody titre did not reach the desired level, injection of 4.0 ml of antigen was continued until the desired titre was obtained. The antiserum was heat inactivated at 56°C for 30 min and stored at 4°C when in use.

b) Preparation of 10% Staphylococcus aureus Cowan-I solution (Maccani, 1981)

The strain of S. aureus Cowan-I was obtained from the Department of Microbiology, Christian Medical College and Hospital, Vellore (India) and it was inoculated into Trypticase soy broth (Hi-Media, India) and grown overnight at 35°C with constant mechanical agitation. The cells were harvested by centrifugation and then washed five times in PBS. The sedimented cells were resuspended in 0.5% formaldehyde and allowed to stand for 3 hr at RT with occasional mixing. The formaldehyde treated cells were washed three times, resuspended in PBS and then heated at 80°C for 1 hr in a constant temperature water bath.

The heat treated cells were washed three times and resuspended in PBS to a final concentration of 10% (vol/vol). The stabilised reagent staphylococci were stored at 4°C until use.

c) Co-A reagent preparation (Koshi et al., 1989)

0.1 ml of rabbit antiserum to C. neoformans was mixed with 1 ml of 10% suspension of S. aureus Cowan-I. The mixture was left at RT for 3 hr. The suspension was washed once and then resuspended in PBS to the original 1 ml
volume. For use in the Co-A test, the suspension was diluted 1:10 in PBS to
give a final concentration of 1% (vol/vol) and stored at 4°C until use. As
controls, Staphylococcal cells were sensitised similarly with rabbit pre-immune
serum (PIS).

15. **Preparation of *C. neoformans* capsular antigen** (Maccani, 1981)

Capsular antigen was prepared for the positive control used in the test.
*C. neoformans* serotype A (CDC 551) was grown on SDA at 26°C for 48 hr.
Antigen containing extracts was prepared by suspending a loopful of colony
growth into 0.5 ml of PBS with 0.5% phenol (Sigma, USA) in a screw capped
tube. The suspension was mixed for 30 seconds on a vortex mixer and the cells
were sedimented by centrifugation at 550 X g for 10 min. The supernatant
fluid was used as positive control for the Co-A test.

16. **Preparation of modified Staib’s agar**

Staib’s agar (Appendix : 2) with the creatinine concentration of 0.78
gm/1000 ml.

17. **Preparation of GCP agar**

a) **Cycloheximide solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide (Hi-Media, India)</td>
<td>0.1 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Filter sterilised</td>
<td></td>
</tr>
</tbody>
</table>
b) **Phenol solution**

Phenol red  -  0.5 gm  
Distilled water  -  100 ml  

c) **Solution A**

Glycine (Hi-Media, India)  -  10 gm  
Yeast nitrogen base  -  6.7 gm  
Cycloheximide solution  -  1.6 ml  
Distilled water  -  200 ml  

All the ingredients of solution A were added to distilled water, mixed well and sterilised by filtration.

d) **Solution B**

Agar  -  20 gm  
Phenol red solution  -  30 ml  
Distilled water  -  770 ml  

All the ingredients of solution B were added to distilled water, mixed well and autoclaved at 121°C for 20 min. Solution B was allowed to cool to about 50°C and then Solution A was added, mixed well, dispensed into tubes and slanted. Final pH 5.6.

18. **Preparation of CGB agar**

**Solution A**

Glycine  -  10 gm  
$\text{KH}_2\text{PO}_4$  -  1 gm  
MgSO$_4$  -  1 gm
Thiamine-HCl - 1 mg
L-canavanine sulphate (Sigma, USA) - 30 mg
Distilled water - 1000 ml

All the ingredients were added to distilled water and sterilised by membrane (0.45 μm) filtration. Final pH 5.6.

**Solution B**

Sodium bromothymolblue - 0.4 gm
Distilled water - 100 ml

Sodium bromothymolblue was dissolved in distilled water.

To prepare 1000 ml of the medium, 880 ml of distilled water, 20 ml of solution B and 20 gm of agar were mixed. Autoclaved at 121°C for 15 min and allowed the solution to cool to about 50°C and 100 ml of solution A was added. Dispensed into tubes to make slants, after thorough mixing.

**19. Yeast carbon base agar**

Yeast carbon base (Hi-Media, India) - 11.7 gm
Agar - 20 gm
Distilled water - 1000 ml

The ingredients were added to distilled water, pH was adjusted to 5.6 and autoclaved at 121°C for 15 min. When the medium was cooled to approximately 50°C, culture suspension of the isolate in sterile saline equivalent to McFarland No. 4 was added to the agar base and plated.
20. **Serotype specific antisera for serotyping** (Ikeda *et al.*, 1982)

**Preparation of antigens**

Reference strains of four serotypes of *C. neoformans*, A (CDC 551), B (NIH 112), C (NIH 18) and D (NIH 52) were kindly donated by Dr. Reiko Ikeda, Dept. of Microbiology, Meiji College of Pharmacy, Japan were used. Each serotype of *C. neoformans* was grown on SDA at 27°C for 72 hr. The yeast cells were harvested with physiological saline solution and heated in a water bath at 100°C for 1 hr. The heat-killed cells were used as antigens and antisera for each serotype was prepared as described in Appendix: 14 a.

21. **Preparation of serotype specific factor sera by cross adsorption experiment**

Antiserum used for final serotyping were first adsorbed with a cells of the other three heterologous serotypes. 2 ml volume of antiserum and 1 ml volume of packed wet cells of heterologous serotype were mixed. The mixture was incubated at 37°C for 2 hr and overnight at 4°C. The suspension was then centrifuged and the supernatant was tested for antibody by slide agglutination test. After adsorptions, titres against the adsorbing strains were negative at 1:2 dilution. Adsorbed antisera used for serotyping were diluted 1:4 prior to use.
22. Sporulation medium (V-8 juice agar)

44.3 gm of dehydrated V-8 juice agar medium (Hi-Media, India) was dissolved in 1000 ml distilled water and autoclaved at 121°C for 10 min. Final pH 5.7.

23. PCR reaction mix

The reaction mix was constituted in the following manner.

The reaction mix 50 µl contained

10x Taq polymerase buffer (Amersham-Pharmacia Bio-Tech Ltd., Buckinghamshire, UK) - 5 µl
MgCl₂ (25 mM) (Sigma, USA) - 12 µl
Primer CNI (250 pM) - 2 µl
Primer MYC1 (250 pM) - 2 µl
d NTPs (10 mM) (Amersham-Pharmacia, UK) - 1 µl
Taq DNA polymerase (5 U/µl) (Amersham-Pharmacia, UK) - 0.3µl
Template - 5 µl
RNAse free water (United States Biochemical, Ohio, USA) - 22.7 µl

24. Preparation of 7% Acrylamide gel

Materials

Acrylamide 30% stock solution.

Acrylamide (Sigma, USA) - 29 gm
N,N' methylene bisacrylamide (Sigma, USA) - 1 gm
Distilled water - 100 ml
Heated the solution to 37°C to dissolve the chemicals and stored at 4°C.

N,N,N',N'-tetramethylethylenediamine (TEMED)

Ammonium per sulphate (APS)) - 10% solution in water, freshly prepared before every run.

Both TEMED and APS were obtained from Amresco, Solon, Ohio, USA. Boric acid, bromophenol blue and xylene cyanol FF were obtained from Hi-Media, India.

Procedure

1. 7 ml of 30% acrylamide stock was mixed with 19.7 ml of water and 3 ml of 10x TBE.
2. 300 µl of 10% APS and 30 µl of TEMED were added, mixed and poured into vertical gel apparatus (1 mm thickness).
3. Gel was allowed to set for 1 hr at RT.

25. Silver staining

1. Fixing solution was discarded and at least 5 times the gel volumes of 30% ethanol was added. Gel was incubated for 30 min at RT with gentle shaking.
2. Step 1 was repeated.
3. Ethanol was discarded and 10 gel volumes of deionised water was added. Gel was incubated for 10 min at RT with gentle shaking.
4. Step 3 was repeated twice.
5. Water was discarded, 5 gel volumes of a 0.1% solution of AgNO₃ (E.Merck, India) freshly diluted from a 20% stock, was added and incubated for 30 min at RT with gentle shaking.

6. AgNO₃ solution was discarded and washed with 10 gel volumes of deionised water.

7. 5 gel volumes of a freshly prepared aqueous solution of 2.5% sodium carbonate (Hi-Media, India), 0.02% formaldehyde (E-Merk, India) was added and incubated at RT with gentle agitation.

8. The gel was observed for the bands to appear and incubation was continued until the desired contrast was obtained.

9. Reaction was stopped by transferring the gel into a solution containing 50% methanol (E.Merck, India) and 16% acetic acid and later the gel was washed several times in deionised water.