Materials and Methods
4. MATERIALS AND METHODS

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- ISOLATION OF C. NEOFORMANS FROM ENVIRONMENTAL SOURCES
- CHARACTERISATION OF CLINICAL AND ENVIRONMENTAL ISOLATES OF C. NEOFORMANS
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ISOLATION AND IDENTIFICATION OF C. NEOFORMANS FROM CLINICAL CASES AT CHENNAI

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4.1 SCREENING OF HOSPITALISED, SYMPTOMATIC AIDS PATIENTS FOR CRYPTOCOCCOSIS

The clinical specimens were collected from suspected AIDS patients for cryptococcosis, admitted in the AIDS wards, Government TB Hospital, Sanatorium, Chennai - 600 047 (Plate 4) and YRG CARE, Centre for AIDS Research and Education, T. Nagar, Chennai - 600 017 during the period January, 1996 to October, 1997. The available clinical data such as age, sex, symptoms, CD4 count and CSF findings were also collected from the hospital records.

4.1.1 Collection of Pulmonary Specimens

The pulmonary specimens collected include Induced sputum (n=21), expectorated sputum (n=78) and pleural fluid (n=18) from suspected cases of pulmonary cryptococcosis at the time of presentation of symptoms (Plate 5).

4.1.2 Collection of CSF

3 to 5 ml of CSF specimens were collected from patients (n=158) with suspected meningeal involvement by ward medical officer by lumbar puncture, between third and fourth lumbar vertebrae (Plate 6).

4.1.3 Collection of Other Specimens

Further, the specimens from extraneural sites such as sputum, blood and urine were collected from positive cases of cryptococcosis for culture and
Plate 4  AIDS ward, TB Sanatorium, Chennai

Plate 5  Collection of Induced sputum specimen from AIDS patient at YRG CARE, Chennai

Plate 6  Collection of CSF from AIDS patient
All the clinical specimens were collected in sterile plastic container, Clinical (Hi-Media Laboratories Pvt. Ltd., Mumbai, India).

* For sputum culture, early morning expectorated sputum was collected.

* For blood culture, 10 ml of blood was collected in Clinical containing 1 ml of balanced oxalate solution as anticoagulant.

* For detection of antigen in serum, 5 ml of blood was collected.

* For urine culture, 20 to 25 ml of early morning, voided midstream urine was collected.

The collected specimens were transported to the laboratory for microbiological investigations in appropriate conditions.

### 4.1.4 Processing of the Clinical Specimens

**CSF:** The CSF specimens were centrifuged at 250 X g for 10 min. The supernatant was used for the detection of capsular antigen of *C. neoformans* and the sediment was used for microscopic examination and culture.

**Blood:** Sera were separated by centrifugation and stored at -20°C until use.

**Urine:** The urine specimens were centrifuged at 250 X g for 15 min and the sediment was used for culture.
4.2 LABORATORY DIAGNOSIS OF CRYPTOCOCCOSIS

4.2.1 Microscopic Examination with Negative Staining

Negative staining was done with pulmonary and CSF specimens by nigrosin wet mount. A drop of nigrosin solution (Hi-Media, India) and processed specimen, were mixed on a clean glass slide and mounted with a coverslip. The wet mount was examined under 10x and then 40x objective for the presence of encapsulated yeast cells with a clear halo around them against a dark background.

4.2.2 Culture

The pulmonary and other processed specimens were inoculated on two sets of neutral Sabouraud’s dextrose agar - Emmons modification (NSDA) with antibiotic (chloramphenicol 0.05 mg/ml), Staib’s agar with biphenyl 0.01 mg/ml + chloramphenicol 0.05 mg/ml and Sunflower seed agar with chloramphenicol 0.05 mg/ml (Appendix : 1-3). Media inoculated with clinical specimens were incubated at 26°C and the another set at 37°C for an observation period of 1 to 2 weeks.

Blood culture was done with biphasic medium of Brain heart infusion (BHI) agar and BHI broth by Castaneda method (Appendix : 4). About 10 ml of blood was inoculated and incubated at 37°C for 4 weeks in an upright position. The bottle was examined daily for visual evidence of growth (turbidity and/or colonies). All yeast colonies were subcultured on SDA for further identification of C. neoformans.
4.2.3 Identification of C. neoformans

The identity of C. neoformans was determined by using a battery of morphological, biochemical and physiological tests including sugar fermentation and assimilation studies (Barnett et al., 1983).

The presumptive identification of C. neoformans was based on the formation of BCE on Staib's agar and the demonstration of the capsule in wet mount of nigrosin stain. The identification of the isolate was further confirmed by urease production, sugar fermentation, inositol assimilation, growth at 37°C, phenoloxidase production, nitrate reduction and animal pathogenicity in mice (Appendix : 5-11).

Maintenance of C. neoformans isolates

The confirmed isolates of C. neoformans were maintained as stock culture on SDA (Appendix : 12) containing chloramphenicol (0.5 mg/ml) at Room temperature (RT).

4.3 SERODIAGNOSIS - DETECTION OF ANTIGEN
4.3.1 In-House Co-Agglutination (Co-A) Test
4.3.1.1 Principle

Protein A is a cell wall component of the strain Staphylococcus aureus Cowan-I. It binds to the Fc portion of IgG antibody. The Fab portion of IgG bound to Protein A is free to combine with a specific antigen. Thus protein A with attached IgG antibody raised against capsular antigen of C. neoformans will agglutinate with freely circulating antigens in the body fluids.
4.3.1.2 Co-A Test Procedure

Equal volumes (50 µl) of heat treated specimen (Appendix: 13) and Co-A reagent (Appendix: 14 a, b and c) were mixed on a glass slide. The mixing was effected with sterile sticks and the slides were rotated manually for 2 to 5 min. The slide was read macroscopically by using indirect lighting against a dark background and the degree of agglutination was graded as 0 (smooth and milky suspension), 1+ (fine granularity and milky background), 2+ (small clumps and clear background) or 4+ (large clumps and clear background).

The prepared *C. neoformans* capsular antigen (Appendix: 15) and PBS were used as positive and negative controls respectively. Each specimen was also tested with staphylococci treated with rabbit PIS to exclude non-specific reactions.

The specimen giving positive Co-A reaction was then tested again after serial doubling dilutions with PBS. Each set of specimen dilution was tested in parallel rows with Co-A reagent and PIS coated staphylococci reagent.

4.3.2 Latex Agglutination (LA) Test

4.3.2.1 Principle

Commercially available LA test kit (Pastorex® *Cryptococcus*, Sanofi Diagnostics Pasteur, France) (Plate 7) was used for the confirmation of test positive by Co-A.
The test kit contained as follows:

i) Latex particles sensitised with an anti-glyceuronoxylomannan antibody (rat) in glycine buffer.

ii) Positive control (purified *C. neoformans* capsular polysaccharide)

iii) Glycine buffered diluent with albumin (pH 8.2)

iv) Lyophilised enzyme: Pronase (the pronase enzyme was reconstituted with 1.5 ml of distilled water and stored at -20°C until use).

v) Stopping solution (Enzymatic inhibitor)

vi) Disposable cards and

vii) Small sticks

### 4.3.2.2 Procedure

The kit was used according to the instructions provided by the manufacturer.

120 µl of the specimen (CSF or serum) was mixed with 20 µl of pronase, heated to 56°C for 30 min, added 1 drop of stopping solution and homogenised.

40 µl of the specimen was mixed with 10 µl of *Cryptococcus* latex suspension on the agglutination card. The card was placed on a rotating
platform at 160 rpm for 10 min at RT. If the latex particles agglutinate, the reaction was read as positive.

4.4 SKIN TEST

The skin test was performed with *C. neoformans* antigen (*cryptococcocin*) in culture positive AIDS cases (n=13) and other culture negative AIDS cases (n=8) as control.

4.4.1 Preparation of Cryptococcocin (Graybill and Alford, 1974)

Reference strain of *C. neoformans* serotype A (CDC 551) was grown for 14 days at 37°C in 1000 ml volume of Sabouraud's dextrose broth. At the completion of the inoculation, cryptococci were harvested, washed three times in saline by centrifugation. Cultures for fungal contamination were performed by streaking on SDA and bacterial contamination was sought by culturing on blood agar. The harvested cells were heat killed at 56°C for 1 hr, sonicated for 30 min, washed twice with PBS and resuspended in 10 ml of PBS. The suspension was mixed with 12 gm of sterile urea (Hi-Media, India) and sonicated for 1 hr in an ice bath. The suspension was incubated at 4°C for 72 hr.

The cells were centrifuged at 1000 X g for 60 min. The resulting supernatants were dialysed three times against 50 volume of sterile saline and once against Hank's buffered salt (Hi-Media, India) solution at 4°C. Dialysed *cryptococcal* extract was checked for contamination as described before,
centrifuged to remove insoluble precipitants and filter sterilised by membrane filter 0.45 μm (Sartorius, GmbH, FRG). The final product contained protein at 3 mg/ml by Lowry et al. (1951) using an egg albumin standard and polysaccharide at 0.4 mg/ml determined by the Molisch's reaction with a glucose standard (Dische, 1960).

4.4.2 Skin Test Procedure

0.1 ml of undiluted antigen was applied on forearm by the Mantoux technique and the reactions were read as millimeter induration 48 hr later.

4.5 TREATMENT AND FOLLOW-UP OF THE CULTURE POSITIVE CASES

The culture positive cases (n=8) for cryptococcosis were administered with amphotericin B intravenously in daily doses ranging from 0.4 to 0.6 mg/kg body weight for a period of 5 to 8 weeks and their outcome was observed.
COLLECTION OF CLINICAL ISOLATES OF
C. NEOFORMANS FROM OTHER STATES OF INDIA
4.6 COLLECTION OF CLINICAL ISOLATES OF *C. NEOFORMANS* FROM OTHER STATES OF INDIA

The isolates (n=144) of *C. neoformans* from clinical sources (along with clinical details) were also collected from various research institutions, major private hospitals and medical college hospitals in different states of India (Table 2). The identity of all the collected isolates were reconfirmed on the basis of morphological, biochemical and physiological tests as described earlier and, the confirmed isolates were maintained in the laboratory for further study.
Table 2: List of places from where clinical isolates of *C. neoformans* were received

<table>
<thead>
<tr>
<th>State</th>
<th>Place</th>
<th>No. of Isolate(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamilnadu</td>
<td>1. Cancer Institute, Chennai</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2. Tamilnadu Hospital, Chennai</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3. Malar Hospital, Chennai</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4. Chennai Medical College, Chennai</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5. Tietz Clinical, Diagnostic Laboratory &amp; Research Centre, Madurai</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>6. Thirunelveli Medical College, Thirunelveli</td>
<td>1</td>
</tr>
<tr>
<td>Karnataka</td>
<td>1. National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>2. Jawaharlal Nehru Medical College, Belgaum</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3. BLDEA's Medical College, Bijapur</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4. Sri Devaraj Urs Medical College, Kolar</td>
<td>2</td>
</tr>
<tr>
<td>Andhra Pradesh</td>
<td>1. Nizam's Institute of Medical Sciences (NIMS), Hyderabad</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2. Apollo Hospital, Hyderabad</td>
<td>1</td>
</tr>
<tr>
<td>Maharashtra</td>
<td>1. Krishna Institute of Medical Sciences (KIMS), Karad</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>2. LTM Medical College, Mumbai</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>3. Armed Forces Medical College (AFMC), Pune</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>4. TN Medical College, Mumbai</td>
<td>1</td>
</tr>
<tr>
<td>West Bengal</td>
<td>1. School of Tropical Medicine, Calcutta</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>144</strong></td>
</tr>
</tbody>
</table>
ISOLATION OF C. NEOFORMANS FROM ENVIRONMENTAL SOURCES

4.7.1 Specimen Collection

4.7.2 Processing of the Specimens

4.7.3 Isolation Technique
4.7 ISOLATION OF C. NEOFORMANS FROM ENVIRONMENTAL SOURCES

4.7.1 Specimen Collection

125 specimens of pigeon droppings (Plate 8) (March to October, 1996) and 86 Eucalyptus trees (E. camaldulensis and E. tereticornis) specimens includes flowers, fruits, leaves, barks and debris under the trees were collected during flowering season (November, 1996 to February, 1997) (Plate 9) from different locations in various districts of Tamilnadu and in Pondicherry (Union territory). The specimens were collected in sterile plastic bags and the information regarding its source, site and type was recorded. Then it was transported to the laboratory for processing.

4.7.2 Processing of the Specimens

1 gm of each specimen was suspended in 9 ml of sterile physiological saline and the mixture was shaken vigorously for 30 min and allowed to stand for 15 min. 1 ml of the supernatant was inoculated in 9 ml of sterile saline containing chloramphenicol (0.5 mg/ml). The solution was kept at 37°C for 1 hr.

4.7.3 Isolation Technique (Criseo et al., 1995)

0.1 ml of supernatant from the processed specimen was inoculated in duplicate onto NSDA supplemented with chloramphenicol (0.05%) and Staib's agar supplemented with biphenyl (0.01% and 0.1%). Additionally two plates of
Plate 7  Latex agglutination test kit

Plate 8  Pigeons and it's fancier

Plate 9  Eucalyptus tree in the Hospital vicinity
Staib's agar not containing biphenyl were also used. The inoculated plates were incubated in the dark at 26°C and examined daily for the growth and presence of colonies showing the BCE on Staib's agar. The plates were observed for the period of 14 days, before being discarded as negative.

Each isolate of yeast-like organism was subcultured, purified and subjected to detailed morphological, biochemical, physiological tests and pathogenicity in mice as described before.

The positive specimens were processed and diluted up to $10^4$. 0.1 ml of the supernatant was inoculated in duplicate onto Staib's agar, Staib's agar with two different concentrations of biphenyl (0.1 gm/l and 1 gm/l), Staib's agar with methyl violet (2 mg/l) and Sunflower seed agar (Appendix 2 and 3). The plates were incubated at 26°C and observed for a period of 14 days. The colonies with BCE were enumerated and the number of such colonies represented the C. neoformans count. The results were expressed as the average number of viable yeast cells per gram of droppings (CFU/gm). The efficacy of these selective media on the recovery of C. neoformans from environmental specimens was determined by positivity and colony count. The isolates were maintained in the laboratory as described before.
CHARACTERISATION OF CLINICAL AND ENVIRONMENTAL ISOLATES OF C. NEOFORMANS

4.8 ANTIFUNGAL SUSCEPTIBILITY TESTING

4.9 TYPING OF C. NEOFORMANS

4.9.1 Varietal Typing
   4.9.1.1 GCE on modified Staib's agar
   4.9.1.2 Colour change on GCP agar
   4.9.1.3 Colour change on CGB agar
   4.9.1.4 D-proline assimilation test

4.9.2 Serotyping and Virulence Study

4.9.3 Mating Typing

4.9.4 RAPD Analysis
4.8 **ANTIFUNGAL SUSCEPTIBILITY TESTING** (NCCLS, 1992)

**Antifungal agents**

The antifungal agents used were:

a) Amphotericin B (Hi-Media, India)

b) 5-Flucytosine (gifted by Hoffmann-La-Roche Inc., Nutley, New Jersey, USA).

c) Fluconazole (gifted by Pfizer Ltd., Mumbai, Maharashtra, India)

d) Ketoconazole (gifted by Torrent Pharmaceuticals Ltd., Gujarat, India).

The drugs were stored at 4°C and amphotericin B was kept in the dark to prevent degradation. Amphotericin B and ketoconazole were dissolved in dimethyl sulfoxide (DMSO) solution (Sigma Chemical Co., St. Louis, MO, USA) on the day of use and 5-flucytosine and fluconazole were dissolved in sterile distilled water. The drug solutions were sterilised by membrane (0.45 μm) filtration.

**Reference quality control strains used**

The following control strains sensitive to amphotericin B, 5-flucytosine and azoles were used in this study.

a) *Candida krusei*, ATCC 6258

b) *Candida parapsilosis*, ATCC 22019
Preparation of Inocula

The clinical isolates of *C. neoformans* (n=36) were grown on plates of SDA for 48 hr and the inoculum suspension was prepared by picking five colonies of at least 1 mm diameter and suspending the material in 5 ml of sterile physiological saline. The turbidity of the cell suspension was adjusted to McFarland No. 0.5 standard, produced a cell suspension containing $1 \times 10^6$ to $5 \times 10^6$ organisms per ml. This was further diluted with the test medium to provide a starting inoculum of $0.5 \times 10^3$ to $2.5 \times 10^3$ organisms per ml. A similar suspension was also prepared for the control organisms.

Drug dilutions and performance of the test

The test medium used was RPMI-1640 with glutamine without bicarbonate (Sigma, USA). 8.4 gm of dehydrated medium was dissolved in 1000 ml of distilled water and buffered to pH 7 with 0.165 M MOPS buffer (Hi-Media, India). Then it was sterilised by membrane (0.45 μm) filtration. Serial two fold dilutions of antifungal agents were prepared. The ranges of antifungal concentrations tested were: amphotericin B and ketoconazole 0.03 to 16 μg/ml and 5-flucytosine and fluconazole 0.125 to 6 μg/ml. 0.9 ml of the inoculum was added to the tube containing 0.1 ml of various antifungal concentrations. The tubes were incubated at 35°C for 72 hr.
The MICs for 5-flucytosine and azoles were determined as the lowest drug concentration tube that resulted in a visual turbidity ≤ 80% inhibition, when compared with that produced by the growth control (prepared by diluting 0.2 ml of drug free control growth with 0.8 ml of medium). Amphotericin B MICs were defined as the lowest drug concentration tube, in which there was absence of growth.

4.9 TYPING OF C. NEOFORMANS

Clinical and environmental isolates were subjected to the following typing procedures in order to characterise them and obtain an overall epidemiological significance.

i) Varietal typing
ii) Serotyping and Virulence study
iii) Mating typing
iv) RAPD analysis

4.9.1 Varietal Typing

The varietal status of the isolates of C. neoformans from clinical (n=164) and environmental (n=12) sources was determined by using CGB agar. The following different varietal typing methods were evaluated for their efficacy in determining the varietal status using the known strains of var neoformans (n=42) and var gattii (n=10). The reference strains of serotype A (CDC 551) and serotype B (NIH 112) were used as control.
i) *Green* colour effect (GCE) on modified Staib's agar.

ii) Colour change on glycine-cycloheximide-phenol red (GCP) agar.

iii) Colour change on L-canavanine-glycine-bromothymolblue (CGB) agar.

iv) D-proline assimilation test.

4.9.1.1 **GCE on modified Staib's agar** (Bennett *et al*., 1978)

**Principle**

When *C. neoformans var gattii* is grown on modified Staib's agar, the medium becomes green due to rapid and strong utilisation of creatinine and an accumulation of ammonia with an alkaline pH value.

**Procedure**

Slants of modified Staib's agar (Appendix : 16) was inoculated with *C. neoformans* isolates heavily and incubated at 30°C for 3 weeks to observe the green colour effect.

4.9.1.2 **Colour change on GCP agar** (Shadomy *et al*., 1987)

**Principle**

*C. neoformans var neoformans* is sensitive to cycloheximide at the concentration of 1.6 μg/ml. However, *var gattii* is resistant to the drug at the same concentration. Therefore when the *C. neoformans* is grown on GCP agar colour change is observed with *var gattii* (Appendix : 17).
Procedure

A small inoculum from culture of 48 hr old was inoculated on the centre of slant of GCP agar and incubated at 27°C. Results were interpreted in terms of growth or no growth and colour changes produced after 5 days of incubation. Scoring was done depending on the medium exhibiting 0,1+,2+,3+ and 4+ colour changes. The interpretations of the colour changes and designations of variety were determined as shown in Table 3.

4.9.1.3 Colour change on CGB agar (Shadomy et al., 1987)

Principle

*C. neoformans* var *neoformans* is sensitive to L-canavanine sulphate at the concentration of 30 µg/ml and *var guttii* is resistant to the drug at the same concentration. Therefore when the *C. neoformans* is grown on CGB agar, the colour change is observed with *var guttii* (Appendix · 18).

Procedure

The procedure and interpretation of results of colour change on CGB agar was similar to that of GCP agar

4.9.1.4 D-Proline assimilation test (Dufait et al., 1987)

Principle

*C. neoformans* var *gattii* alone can utilise D-proline as a sole source of nitrogen.
Procedure

The disk (Whatman paper No.1) impregnated with 20% of D-proline (Sigma, USA) was placed on the surface of the yeast carbon base agar medium (Appendix : 19) containing the isolate to be tested and examined for the growth around the disk after incubation at 26°C for 48 to 72 hr. The presence of growth around the disk was considered as positive.

4.9.2 Serotyping

Principle

Polyclonal antisera raised against C. neoformans in rabbit are used to establish an antigenic formula for five serotypes (A,B,C,D and AD) of C. neoformans. The antigenic formulation identifies eight distinct antigenic factors or determinants that are found on various serotypes of encapsulated cryptococci. Serotypes A and D have antigenic factors 1,2,3 and 7 and 1,2,3 and 8 respectively. Serotype AD has both antigenic factors 7 and 8, in addition to the common antigenic factors 1,2 and 3. Serotypes B and C have antigenic factors 1,2,4 and 5, and 1,4 and 6 respectively. Factor specific antisera are prepared by cross adsorption of polyclonal antibodies in anti-cryptococcal rabbit antisera. These factor specific sera are corresponded to their respective antigenic factors.
Procedure

All the isolates of *C. neoformans* from clinical and environmental sources were serotyped by slide agglutination test with the prepared serotype specific factor sera (Appendix: 20 and 21).

Equal amounts of a suspension of heat-killed cells, the density adjusted to a McFarland No. 10 standard and serotype specific factor serum were mixed on a glass slide. The slide was rotated horizontally at about 150 rpm for 5 min and then the degree of agglutination was recorded.

**VIRULENCE STUDY** (Kwon-chung *et al.*, 1992c)

i) Virulence study of serotype A alone from different states

The clinical isolates of *C. neoformans* serotype A from Tamilnadu (n=5), Andhra Pradesh (n=5), Karnataka (n=5), Maharashtra (n=5) and West Bengal (n=3) were studied for their virulence in mice. Swiss albino mice weighing 23 to 25 gm were used throughout the study. Groups of 8 mice per isolate were used. The yeast cells from 48 hr cultures on SDA were suspended in sterile saline and washed thrice. The inocula was adjusted to McFarland No. 0.5 standard and 0.2 ml of inoculum was injected in the lateral tail vein. The virulence was measured by death rate. The animals were observed for their mortality rate during the total period of 45 days. For control, 3 groups of 8 mice were injected with sterile saline.
ii) Virulence study of serotypes A, D, AD, B and C from Karnataka alone

Apart from the above isolates from various states, the clinical isolates of *C. neoformans* serotypes A, D, AD, B and C (n=1 each) from Karnataka state alone were studied for their virulence in mice as described before.

4.9.3 Mating Typing (Hironaga et al., 1983)

**Principle**

When crosses are made between two sexual compatible strains of *C. neoformans* on sporulation media, produces clamped hyphae, basidia and basidiospores.

**Reference strains used**

The reference tester strains (Table 4) kindly gifted by Dr. Takashi Mochizuki, Department of Dermatology, Shiga University of Medical Sciences, Japan and Dr. Brian Wickes, Microcide Pharmaceuticals Inc. California, USA, were used.

**Procedure**

a) Mating Experiment

Isolates of *C. neoformans* from clinical (n=30) and environmental (n=8) sources were used.
Table 3: Varietal typing by biochemical methods

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Medium</th>
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<tbody>
<tr>
<td></td>
<td><strong>GCP Agar</strong></td>
</tr>
<tr>
<td>0</td>
<td>No change</td>
</tr>
<tr>
<td>1+</td>
<td>Slight colour change on part of slant</td>
</tr>
<tr>
<td>2+</td>
<td>Orange slant/ yellow or orange butt</td>
</tr>
<tr>
<td>3+</td>
<td>Red slant/ orange butt</td>
</tr>
<tr>
<td>4+</td>
<td>Deep rose-red to bottom of tube</td>
</tr>
</tbody>
</table>

Serogroup designation

<table>
<thead>
<tr>
<th>GCP Agar</th>
<th>CGB Agar</th>
<th>Serogroup</th>
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<tbody>
<tr>
<td>0-1+</td>
<td>0-2+</td>
<td>= A/D</td>
</tr>
<tr>
<td>1-2+</td>
<td>2+</td>
<td>= Indeterminate</td>
</tr>
<tr>
<td>3+</td>
<td>3+</td>
<td>= B/C</td>
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Source: Shadomy et al. (1987)

Table 4: Reference tester strains used for mating typing

<table>
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<th>Details of the tester strain</th>
<th>Type</th>
<th>Strain Number</th>
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<tr>
<td><em>Filobasidiella neoformans</em> var neoformans</td>
<td>α</td>
<td>JEC 21</td>
</tr>
<tr>
<td><em>Filobasidiella neoformans</em> var neoformans</td>
<td>a</td>
<td>JEC 20</td>
</tr>
<tr>
<td><em>Filobasidiella neoformans</em> var bacillispora</td>
<td>α</td>
<td>NIH 444</td>
</tr>
<tr>
<td><em>Filobasidiella neoformans</em> var bacillispora</td>
<td>a</td>
<td>NIH 191</td>
</tr>
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</table>
Cultures of 72 hr old were suspended in sterile physiological saline and the suspensions were adjusted to McFarland No.9 standard.

Equal amounts of the yeast cell suspensions to be mated and reference strain were combined, stirred on a vortex mixer and centrifuged at 1500 X g for 20 min. A small amount of the mixed sediment was spread on the centre of duplicate plates of sporulation medium, V-8 Juice agar (Appendix : 22). Inoculated plates were incubated at 25°C and the cultures were monitored for the development of clamped hyphae, basidia and basidiospores for 2 to 4 weeks.

b) Self-fertility test

Each isolate was inoculated on duplicate plates of V-8 Juice agar and incubated at 25°C. Cells on the surface of the agar were monitored for the development of hyphae, clamp connection, basidia and basidiospores for 2 to 4 weeks.

4.9.4 Random amplification of polymorphic DNA analysis (RAPD) Principle

RAPD method discriminates between strains of the same organism, in which the DNA of the organism is extracted, polymorphic DNA is amplified using oligonucleotide primers by polymerase chain reaction (PCR) and the products of amplification is electrophoresed in a polyacrylamide gel. The separated bands are visualised by silver staining method.
Isolates used

Clinical (n=6) and Environmental (n=3) isolates of *C. neoformans* serotype A from same locality were used. Each isolate was subcultured on SDA and incubated at 30°C for 72 hr and the cells were harvested with sterile physiological saline.

Extraction of DNA

Materials

1. Extraction buffer (0.2 M sodium hydroxide, 0.4% N-lauryl sarcosine, 20 mM EDTA).
2. 7.5 M ammonium acetate.
3. Isopropanol
4. TE solution (10 mM Tris and 1 mM EDTA, pH 8)
5. Phenol-chloroform-isoamyl alcohol mixture (25:24:1)
6. 3 M sodium acetate, pH 5.2
7. 0.28 M sodium chloride, pH 6
8. Cetyltrimethyl ammonium bromide (CTAB)
9. Ethanol

All the chemicals were obtained from Sigma,USA except CTAB (Qualigens, India) and Ethanol (BDH, UK).
**Procedure**

For DNA extraction, the procedure of Chen *et al.* (1996) was followed. Essentially this involves the following steps.

1. The harvested cells with saline was centrifuged and the supernatant was removed.
2. The cells were disrupted by boiling for 20 min in 0.5 ml of extraction buffer in microcentrifuge tubes and the samples were centrifuged for 5 min at 14,000 X g.
3. Supernatants were transferred to a microcentrifuge tube containing 0.5 ml of 7.5 M ammonium acetate, mixed and centrifuged as before.
4. The supernatant was transferred to a another microcentrifuge tube and DNA was precipitated with 0.6 ml of isopropanol at RT.
5. Centrifuged pellets were suspended in 1 ml of TE solution and extracted twice with phenol-chloroform-isoamyl alcohol mixture.
6. DNA was precipitated using 1/10 volume of 3 M sodium acetate and 1 volume of isopropanol.
7. The centrifuged pellet was redissolved in 0.5 ml of 0.05 M TE solution and 0.28 M sodium chloride.
8. Precipitated with 0.1% CTAB, washed with 70% ethanol and resuspended in 50 μl of TE solution.
9. DNA concentrations were determined spectrophotometrically.
PCR

Primers used

The following primers gifted by Dr. Tania Sorrell, Centre for Infectious Diseases and Microbiology, Westmead Hospital, New South Wales, Australia, were used.

i) CN1 (TACCCCGCCCATATTCCAT)

ii) MYC1 (GAGGAAGGTGGGATGACGT)

Thermocycling profile (Chen et al., 1996)

The reaction mix (Appendix. 23) was subjected to the following thermocycling profile using a thermocycler (PTC-100, MJ Research, MA, USA).

Reactions were cycled 10 times at 93°C for 1 min, 35°C for 1 min and 72°C for 1 min followed by 20 cycles at 93°C for 1 min, 55°C for 1 min, 72°C for 1 min and 72°C for 5 min

Polyacrylamide gel electrophoresis (Sambrook et al., 1989)

PCR amplified product was run on non-denaturing gel containing 7% acrylamide (Appendix . 24)

Procedure

10 µl of the PCR product was mixed with 2 volumes of tracking dye (containing bromophenolblue and xylene cyanol FF) and were loaded onto the
The gel was kept in the fixative containing ethanol:acetic acid:water (30:10:60) for 2 to 16 hr and was subjected to silver staining (Appendix : 25) and the gel was examined against the light of illumination for visual bands and recorded.
STATISTICAL METHODS FOLLOWED
4.10 **STATISTICAL METHODS**

Comparison of different diagnostic tests, computation of their sensitivity, specificity, positive and negative predictive values with their 95% confidence intervals and hypothesis testing were analysed using Chi-square test, Fisher's exact test and calculation of relative risk were done using Epi-Info statistical software (Dean *et al.*, 1996)