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STUDIES ON PHENOTYPIC CHARACTERIZATION AND SELECTIVE VIRULENCE FACTORS OF CANDIDA SPP. AND CRYPTOCOCCUS NEOFORMANS AND *IN VITRO* SUSCEPTIBILITY OF THESE ORGANISMS TO THE CONVENTIONAL AMPHOTERICIN B AND FUNGISOME™

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Keywords:

Candida spp, *Cryptococcus neoformans*, Phenotypic characterization, MIC, Amphotericin B, Fungisome ™

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ABSTRACT: The human fungal infections have increased tremendously in our country, especially candidal infections. *Cryptococcus neoformans* is encapsulated yeast, causes life-threatening infections in immuno-compromised individuals such as AIDS. Meningoencephalitis is the serious consequence of C. neoformans infection. Studies are therefore, required for identification of these fungal agents and search for suitable antifungal therapy. Keeping this in view, Candida spp. and *Cryptococcus neoformans* strains were characterized. Also, the comparative *in vitro* antifungal susceptibility to conventional Amphotericin B (AMB-d) and Indian Liposomal Amphotericin B (Fungisome™) shall be determined. Candida albicans, non-Candida albicans and Cryptococcus neoformans strains originating from clinical cases have been characterized using phenotypic methods and selective virulence traits. The isolates were obtained from National Culture Collection of Pathogenic Fungi, PGIMER, Chandigarh, India. The MIC ranges of conventional Amphotericin B (AMB-d) and Indian Liposomal Amphotericin B, Fungisome™ against all the strains of Candida spp. and *Cryptococcus neoformans* were also determined. Both the fungal genera could be identified using various phenotypic characteristics and selective virulence factors. The MIC ranges of Fungisome™ ranged from 0.25-0.125 mg/l as against0.5-8.0 mg/l in case of AMB-d for Candida spp. and the MIC ranges of Fungisome™ ranged from 0.25-0.125 mg/l as against 0.125-1.0mg/l in case of AMB-d for *Cryptococcus neoformans*. The phenotypic characteristics can be used to identify isolates of Candida and Cryptococcus species. Fungisome™ proved more efficacious against both the fungal species as compared to conventional Amphotericin B (AMB-d).

INTRODUCTION: The fungal infections are on the rise as newer species are being implicated as causative agents of disease [1].

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Over the last decade, the fungal infections and the range of yeasts associated with human infections has increased, especially *Candida* [2, 3].

However, nowadays non-*Candida albicans* species are being isolated which are clinically important. *Candida* is a part of normal microflora of human body; however, it causes disease in the immune compromised host, it is thus classified as an opportunistic pathogen [4].

In India, candidal infections are on the rise with *C. albicans* being the most common isolate [5,6]. *Cryptococcus neoformans* is encapsulated yeast which causes life-threatening infections in approximately 2 to 3% of patients with AIDS in the United States [7] and in up to 40% of AIDS patients in Africa. Meningoencephalitis is the most common and serious clinical manifestation of *C. neoformans* infection [8]. This fungus is the leading cause of infection in immune compromised patients [9].

Keeping in view the involvement of *Candida albicans*, non *albicans* spp and *Cryptococcus neoformans* in various disease conditions, the rapid diagnosis is an important aspect for initiating suitable therapy. The characterization of the strains from the clinical cases is therefore, of utmost significance.

For treatment of these fungal infections, a number of drugs are available as therapeutic choices. However, differences exist in terms of antifungal spectrum of activity, bioavailability, formulation, drug interactions, and side effects of these drugs [10]. Amphotericin B (AMB) represents the oldest antifungal drug, and remains the drug of choice for many life-threatening invasive fungal infections [11]. It has a wide spectrum of action, and is useful in treating a variety of fungal infections [12]. Also, it is the only polyene drug that is prescribed for systemic infections.

Another advantage of this drug is that there are fewer reports of acquired resistance to this drug. However, despite being one of the most potent antifungal agents, the clinical use of amphotericin B is impeded owing to its poor aqueous solubility and toxicity [13]. The toxic effects of this drug frequently result in discontinuation of the therapy, high morbidity and mortality and increased treatment and hospitalization cost [12].

The lipid based formulations of amphotericin B are now in use to treat a wide range of fungal infections which have greatly augmented AMB's therapeutic utility [11]. These formulations differ with respect to their lipid composition, shape, size, stability, pharmacokinetics and toxicity, and therefore, vary in their therapeutic use [14]. One of the new formulations is L-AMB (Fungisome^{TM}) which can overcome the side effects of amphotericin-B). This formulation has been indigenously developed in India and is being manufactured and marketed by Lifecare Innovations Pvt. Ltd., Gurgaon, India [15, 16]. Fungisome^{TM} contains AMB in small unilamellar phosphatidylcholine and cholesterol in the ratio of 7:3 in liposomes [17]. This formulation is still one of the most active drugs against majority of invasive fungal infections [18].

Keeping in view, the emergence of newer strains of non *albicans* group and re-emergence of opportunistic fungal pathogen, the present study has been planned to evaluate comparative *in vitro* efficacy of amphotericin B and Fungisome^{TM}.

MATERIALS AND METHODS:

Collection of fungal strains: A total of 31 clinical isolates of yeasts (24 Candida spp. *16* Candida albicans and 8 non-Candida albicans) and 7 strains of Cryptococcus neoformans were obtained from National Culture Collection of Pathogenic Fungi (NCCPF), Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. These strains isolated from tissue biopsies, blood, pus, sputum, bronchoalveolar lavage, corneal scrapings, vitreous fluid and CSF, were used in the study.

The distribution of *Candida* isolates was as *C. albicans* (16), *C. glabrata* (3), *C. tropicalis* (2). *C. parapsilosis* (2), *C. guillermondii* (1). *Candida krusei* ATCC 6258, *Candida albicans* ATCC 90028 and *Cryptococcus neoformans* ATCC 34664 were used as quality-control strains for antifungal susceptibility testing. The study was cleared by the Institute Ethics Committee (IEC) vide letter number SUBMS/ IEC/11/53-54 dated 30.05.2011, project number SUIEC/11/05.

Maintenance of fungal strains: All the fungal strains were sub-cultured and preserved in 20 % glycerol at -70°C, according to the standard protocol of CLSI.

Phenotypic characterization: Two strains of each species were selected randomly for phenotypic characterization on different media.

Growth on Sabouraud's Dextrose Agar (SDA): Isolates or strains of the fungal species were sub-

cultures on SDA (Hi-Media) and incubated at 37 °C for 24-72h for obtaining growth and subsequently other tests such as chlamydospore production, pseudohyphae production, germ tube production, sugar assimilation, sugar fermentation, biofilm formation, etc. were performed for phenotypic characterization [19].

Hicrome *Candida* differential agar medium: Hichrome Candida differential agar medium (Hi-Media) was used for presumptive identification of different Candida spp. and for detecting mixed colonies, if any. The method is based on the differential release of chromogenic breakdown products from various substrates following differential exoenzyme activity. This media was in powdered form. The plates were repared according to the manufacturer's instructions. Using an inoculating needle, a single colony from a pure culture was seeded into this media and incubated at 35 °C for 48h. The cultures were then observed for color change [19].

Growth on Corn meal agar (CMA):

1. **Chlamydospore and Pseudohyphae production:** Corn Meal Agar (Hi-Media) was used for the identification of chlamydospore and pseudohyphae formation by yeasts. Briefly 17g of CMA containing tween 80 in final concentration of 1% was prepared according to the manufacturer's instructions. The test strains were inoculated on CMA plates by slide culture technique which involves streaking and stabbing the media with a 48 hour old yeast colony, and covering it with sterile cover slip, followed by incubation at 25°C for 72h. The plates were observed under microscope for the presence of pseudohyphae development and chlamydospore.

2. **Germ Tube Production:** Various Candida spp. used in the study were examined for their ability to produce germ tubes. The test started with a fresh growth from a pure culture of the organism. A very light suspension of the test organisms was thus, formed in 0.5ml of sterile serum (pooled from human serum or calf serum) in eppendorf tube. The optimum inoculum contained 10^5 to 10^6 cells per ml was incubated at 37˚C for 2h. One drop of the culture suspension was then placed on a slide with a cover slip and observed under microscope. Germ tube represents initiation of hypal growth arising from the yeast cell. For recording a positive result, about 30% of the cells should show germ tube production. A standard strain *Candida albicans* ATCC 90028 was kept as positive control with each test [19].

Identification of the fungal strains:

1. **Direct mount:** In order to study the microscopic morphology of yeasts, direct mount method was followed using Lactophenol Cotton Blue stain (LCB) and India ink preparation.

2. **Lactophenol Cotton Blue (LCB):** The lactophenol cotton blue (LCB) wet mount preparation is composed of: phenol, lactic acid and cotton blue. The former kills live organisms while the latter preserves the fungal structures. Cotton blue stains the chitin in the fungal cell walls. For examination LCB method, a drop of 70% alcohol was placed on a microscope slide and the specimen was immersed in it. Two drops of the lactophenol cotton blue stain were added before the alcohol fully dried out. The coverslip was then placed on the specimengently, taking care that no air bubbles was introduced. The preparation was then examined under microscope.

3. **India ink Preparation:** India ink basically consist of carbon black particles which block out all the light except the polysaccharide coating that is produced by the micro-organism. For examining the preparation, a drop of India ink was placed in the centre of a glass slide to which a loopful of the specimen was mixed well with the loop. The coverslip was placed gently on the specimen, avoiding introduction of air bubbles. The preparation was thus ready for microscopic examination under the light microscope [20].

Biochemical tests:

1. **Sugar Fermentation Test:** The liquid fermentation medium consisted of peptone (1gm), sodium chloride (0.5gm), Andrade's indicator (0.005gm).

It was sterilized by autoclaving at 15lb. pressure for 15 minutes. The filter-sterilized sugar at the concentration of 2% of the medium was then added and poured into sterile test tubes (approx. 5ml). Sterile Durham's tube was then placed into each tube. The tubes were plugged with cotton plugs. Inoculums were prepared by suspending heavy inoculums of yeast grown on Yeast Nitrogen Base (Hi-Media).

Each carbohydrate broth was inoculated with approximately 0.1 ml of inoculum. The tubes were incubated at 25˚C for up to one week and observed every 48-72 hrs interval for the production of acid (pink color) and gas (in Durham's). Production of gas in the tubes was taken as fermentation positive while acid production alone is simply indicative of carbohydrate assimilation.

2. **Sugar Assimilation test (Auxanographic technique):** Sugar Assimilation test was performed by the Disc Impregnation- Pour plate auxanographic method. Precisely, the yeast suspension was prepared from a 24-48 hrs old culture in 2ml of YNB (Yeast Nitrogen Base). The suspension was added to 18ml molten agar (cooled to 45˚C) and mixed well. The entire content was poured into 90mm petri plate; the agar was allowed to set at room temperature.

3. The sterilized discs were placed onto the surface of the agar plate. A drop of 10% sterilized sugar solution was then added to each disc. The plates were incubated at 37˚C for 3-4 days. The presence of growth around the disc was considered as positive for that particular carbohydrate. Growth around glucose disc was recorded first which served as positive control i.e. to ensure viability of the yeast.

Studies on virulence factors:

1. **Growth on Blood agar:** The Blood agar media was prepared by adding 5% human blood to nutrient agar media, which is enrichment medium used to isolate fastidious organisms and for detecting hemolytic activity of such organisms.

Hemolysis patterns of fungal strains on Blood Agar:

2. ***β-hemolysis*:** β-hemolytic activity results due to complete digestion of the contents of red blood cells surrounding the colony which is displayed by appearance of clear halos around fungus colonies.

3. ***Alpha hemolysis:*** Alpha hemolysis (α-hemolysis) results due to chemicals generated by fungus which partially break down the blood cells. This is displayed by yellowish/greenish/ brownish discoloration around the colonies of the fungus indicating incomplete hemolysis.

4. **Detection of Biofilm formation by spectrophotometric method:** All the randomly selected strains of *Candida* species (10) and *Cryptococcus neoformans* (8) were examined for biofilm formation by spectrophotometer detection test. Precisely, the test organisms were grown at 37^0C for 24 h on SDA (Hi-Media, Mumbai) plates. One ml of the broth containing active test isolates was dispensed into nine ml SDB supplemented with 8% glucose.

Yeast cell suspension in a volume of 200 µl was inoculated into each well of microtiter plates and incubated at 37^oC for 24h without agitation. The microtiter plates were washed four times in phosphate buffer saline and stained with 1% safranin, aspirated, and spectrophotometer readings were recorded at 490 nm with a microtiter plate reader.

The percent transmittance (*%T* value) for each test sample was subtracted from the %T value for the reagent blank to obtain a measure of the amount of light blocked when passing through the wells ($\%T_{block}$). The entire test was conducted in triplicate and results were expressed as negative (-) ($\%T_{block,}$ <5), weak (+) ($\%T_{block,}$ 5-20), moderate (++) ($\%T_{block,}$ 20-50), strong (+++) ($\%T_{block,} \geq 50$). A standard strain *Candida albicans* ATCC 90028 was kept as positive control with each test [21].

Antifungal susceptibility testing:

1. **Antifungal Drugs:** Fungisome™ (1mg AMB intercalated in liposomes, Lifecare Innovations Pvt. Ltd., Gurgaon, India) and Conventional amphotericin B (AMB-d) (Hi-Media.) were used in this study. The drugs were obtained as

reagent-grade powders, in liquid form and preserved according to the instructions of manufacturer. The stock solution of AMB-d was prepared according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2008) - M27-A2 (yeasts). The stock solution of Fungisome™ was prepared according to manufacturer's instructions. Serial dilutions of Fungisome™ were prepared by sonicating the drug at 15 °C for 45 min in Ultrasonicator (Fast Clean Ultrasonic Cleaner, Lifecare Innovations Pvt. Ltd., India), so as to obtain uniform, small lipid vesicles, followed by dilution making with RPMI-1640 media.

2. **Procedure of determining antifungal susceptibility testing by Micro-broth dilution test and determination of Minimum Inhibitory Concentration (MIC):** The comparative antifungal efficacy of Conventional Amphotericin B and Indian Liposomal Amphotericin B (Fungisome™) against fungi *in vitro* was performed by the micro–broth dilution method as per CLSI M27-A2 protocol [22]. This protocol describes the method for the susceptibility testing of yeast to antifungal agents that cause invasive fungal infections, including *Candida spp.*, *Cryptococcus spp*.

   According to this protocol, the dilutions of antifungal agents are made in RPMI 1640 media (with glutamine, without bicarbonate and with phenol and pH indicator) (pH 7.0±0.1) in MOPS buffer (3-CN-morphotino pyropane-sufonic acid at a final concentration of 0.165 mol/ l at pH 7.0. The inoculum was prepared by picking up five colonies, one mm in diameter, and suspending them in 5ml of sterile 0.145mol/L saline (8.5g/l NaCl) or sterile water. The suspension was vortexed for 15 sec. and cell density was adjusted with spectrophotometer by adding sterile saline or sterile water. A working solution was also made by 1:50 dilution followed by 1:20 dilution of stock suspension with RPMI 1640 broth medium which resulted in 2.5 x 10^3 to 5.0x 10^6 cells per ml. The inoculums of the test strains in media and the drug dilutions were mixed in equal volumes in a microtiter plate. The growth in the wells of the plates was observed and compared to that in the positive control tubes.

   For performing the test, sterile, disposable, multi-well micro-dilution plates (96 U-shaped wells) were used for performing micro-broth dilution test. In this test, precisely, the 2x drug concentrations of each drug was dispensed into the wells of rows 1 to 10 of the micro-dilution plates in 100µl volumes with a multichannel pipette. Row 1 contained the highest (64 or 16 µg/ml) drug concentration, respectively for Fungisome™ (water soluble antifungal agent) or Amphotericin B (water insoluble antifungal agent) and Row 10 contained the lowest drug concentration (0.125 or 0.0313 µg/ml) respectively. These trays were sealed in plastic bags and stored frozen at -70 °C until their use in the test. Each well of a micro-dilution tray was inoculated on the day of the test with 100 µl of the corresponding 2x diluted inoculum suspension, which brought the drug dilutions and inoculum densities to the final concentrations mentioned above.

   The growth control wells contained 100 µl of sterile, drug-free medium and were inoculated with 100 µl of the corresponding diluted (2x) inoculum suspensions. The QC organisms were tested in the same manner and were included each time an isolate was tested. Row 12 of the micro dilution plate was used to perform the sterility control (drug-free medium only). With the exception of *C. neoformans*, plates were incubated (without agitation) at 35 °C for 46 to 50h in ambient air.

   While testing C. neoformans, tubes were incubated for a total of 70 to 74h before determining results. The MIC is the lowest concentration of an antifungal that substantially inhibits growth of the organism as detected visually. The amounts of growth in the wells containing the agent were compared with the amount of growth in control wells (no antifungal agent). The micro dilution plates were incubated at 35°C and observed for the presence or absence of visible growth. The micro dilution wells were scored with the aid of a reading mirror; the growth in each well was compared with that of the growth control (drug-free) well. A prominent decrease in turbidity corresponded to approximately 50% inhibition in growth when determined spectro-photometrically.

RESULTS: A total of twenty four *Candida spp.* and seven *Cryptococcus neoformans* strains isolated from tissue biopsies, blood, pus, sputum, bronchoalveolar lavage, corneal scrapings, vitreous fluid and CSF were studied for their growth characteristics on different media such as Sabuoraud's Dextrose agar (SDA), Blood agar, Hicrome *Candida* differential agar, Corn meal agar (CMA). Their gross and microscopic morphology, production of germ tube, pseudohyphae production, chlamydospore production, hemolysis pattern, biofilm formation, sugar fermentation and sugar assimilation, etc. were studied as detailed below.

Phenotypic characterization:

1. **Growth on Sabuoraud's Dextrose agar (SDA):** All the *Candida spp.* and *Cryptococcus neoformans* isolates showed growth on Sabuoraud's Dextrose agar (SDA) (**Fig. 1 a-g**). The colonies of *Candida spp.* were white to cream colored, smooth, glabrous and yeast-like in appearance whereas the colonies of *Cryptococcus neoformans* were cream-colored, smooth, mucoid yeast like on the medium.

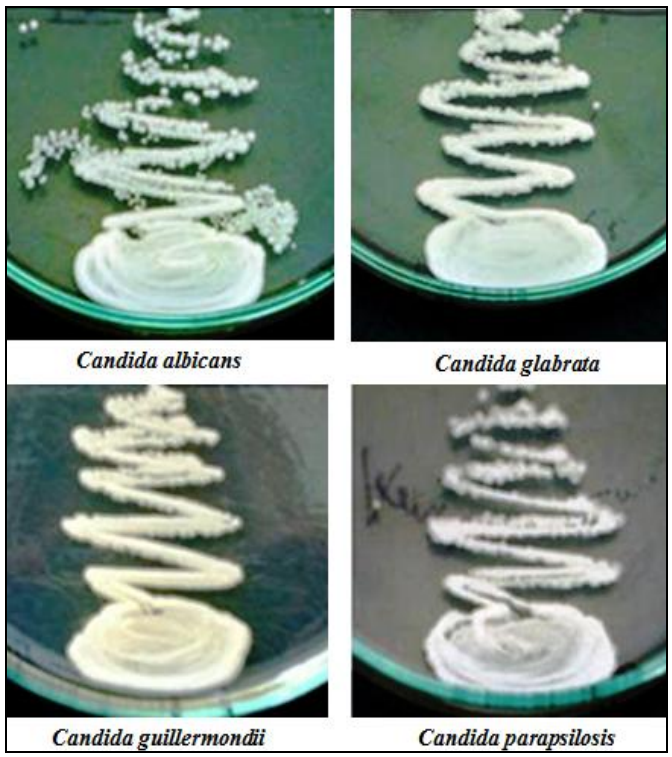


FIG. 1(A-D): COLONY MORPHOLOGY OF DIFFERENT *CANDIDA* SPP. ON SDA. A *CANDIDA ALBICANS* B. *CANDIDA GLABRATA* C. *CANDIDA PARAPSILOSIS* D. *CANDIDA GUILLERMONDII*

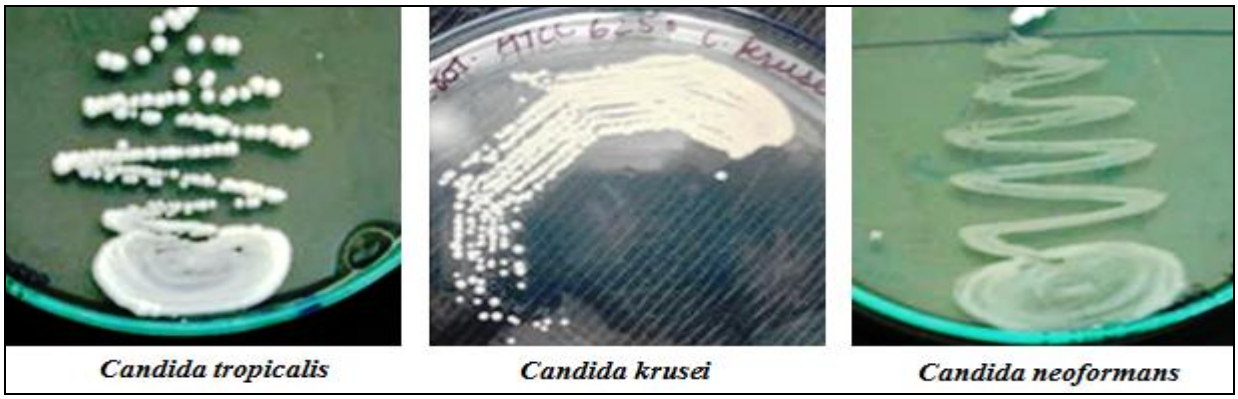


FIG. 1(E-G): COLONY MORPHOLOGY OF DIFFERENT *CANDIDA* SPP. ON SDA. E *CANDIDA TROPICALIS* F. *CANDIDA KRUSEI* G. *CANDIDA NEOFORMANS*

2. **Growth on Hicrome *Candida* differential agar™:** Different isolates of *Candida albicans* and *Candida non* albicans spp produced different shades of colors on this media (**Fig. 2a-g**). All *Candida albicans* produced green color after 48h of incubation at 35˚C while all other species produced different colors: *C. tropicalis* (dark blue), *C. parapsilosis* (pink), *C. guillermondii* (pinkish purple), *C. glabrata* (light pinkish) and *C. krusei* produced whitish pink.

3. **Growth on Corn Meal Agar (CMA):**

   a. **Chlamydospores and pseudohyphae production:** *C. albicans* produced abundant chlamydospores and pseudohyphae with clusters of spore's on Corn Meal Agar (CMA) (**Fig-3 a-g**). *C. tropicalis* formed blastoconidia singly and long pseudohyphae while *C. parapsilosis* formed blastoconidia along curved pseudohyphae and giant mycelial cells. *C. guilliemondii* formed fairly short, fine pseudohyphae and clusters of blastoconidia at septa. *C. glabrata* did not form any pseudohyphae but small, oval, single terminal budding, non-encapsulated

yeast cells formed while *C. krusei* formed pseudohyphae with cross-matchsticks or tree-like blastoconidia. *Cryptococcus neoformans* formed budding yeast cells no pseudohyphae

b. **Germ tube production:** All *C. albicans* produced germ tubes whereas all the *Candida* non *albicans* spp and Cryptococcus neoformans did not produce any germ tube (**Fig. 4**).

Microscopic morphology:

1. ***Candida* species:** Lactophenol Blue Stained (LCB) preparation of *C. albicans, C. tropicalis and C. guillermondii* showed spherical to subspherical budding yeast-like cells or blastoconidia whereas *C. glabrata* showed numerous ovoid, budding yeast-like cells or blastoconidia. *C. parapsilosis* showed predominantly small, globose to ovoid budding yeast-like cells or blastoconidia, with some larger elongated forms present whereas predominantly small, elongated to ovoid budding yeast-like cells or blastoconidia of *C. krusei* were observed (**Fig-5 a-f**).

4. ***Cryptococcus neoformans*:** Distinct, wide gelatinous capsules were observed by India ink method were suggestive of *Cryptococcus neoformans* (**Fig. 6**).

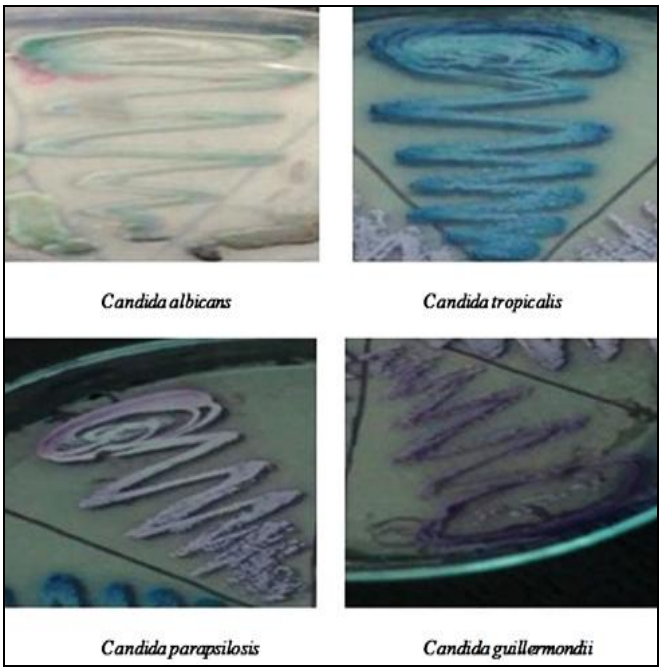


FIG. 2(A-D): GROWTH OF DIFFERENT CANDIDA SPECIES ON HICHROME *CANDIDA* DIFFERENTIAL AGAR. DIFFERENT STRAINS PRODUCED DIFFERENT COLORS. A *CANDIDA ALBICANS*-APPLE GREEN B. *CANDIDA TROPICALIS*-BLUE C. *CANDIDA PARAPSILOSIS*- PINK D. *CANDIDA GUILLERMONDII*- PINKISH PURPLE

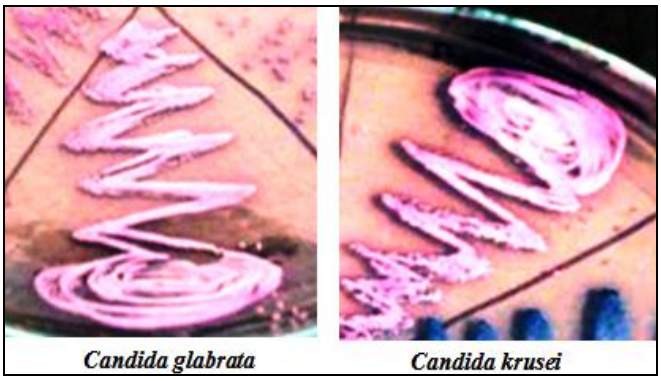


FIG. 2 (E-F): GROWTH OF DIFFERENT CANDIDA SPECIES ON HICHROME *CANDIDA* DIFFERENTIAL AGAR. DIFFERENT STRAINS PRODUCED DIFFERENT COLORS. A *CANDIDA GLABRATA*-LIGHT PINKISH B. *CANDIDA KRUSEI*-WHITISH PINK

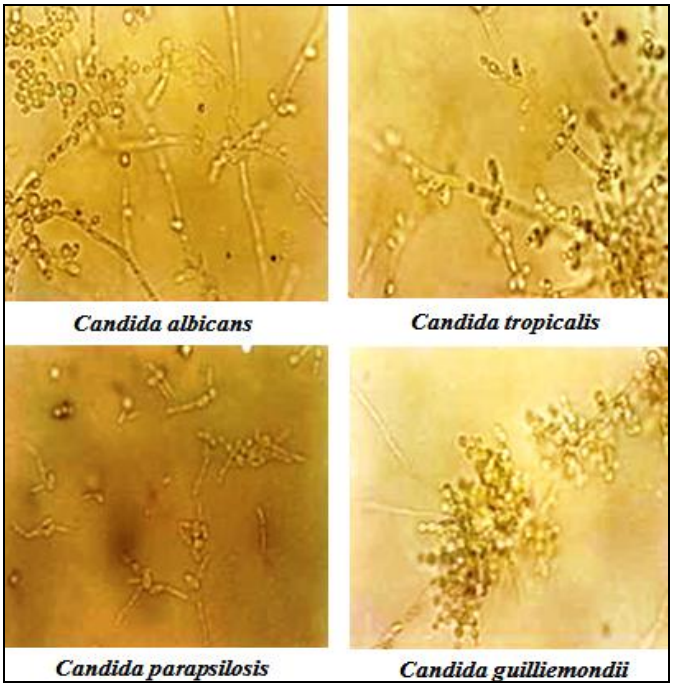


FIG. 3 (A-D): MICROSCOPIC MORPHOLOGY OF DIFFERENT *CANDIDA* SPP. ON CORN MEAL AGAR CONTAINING TWEEN 80A. A *CANDIDA ALBICANS* - ABUNDANT CHLAMYDOSPORES AND PSEUDOHYPHAE WITH CLUSTERS OF SPORES ARE SEEN B. *CANDIDA TROPICALIS*-BLASTOCONIDIA SINGLY AND LONG PSEUDOHYPHAE ARE SEEN C. *CANDIDA PARAPSILOSIS*- BLASTOCONIDIA ALONG CURVED PSEUDOHYPHAE AND GIANT MYCELIAL CELLS ARE SEEN D. *C. GUILLERMODII*- FAIRLY SHORT, FINE PSEUDOHYPHAE AND CLUSTERS OF BLASTOCONIDIA AT SEPTA ARE VISIBLE.

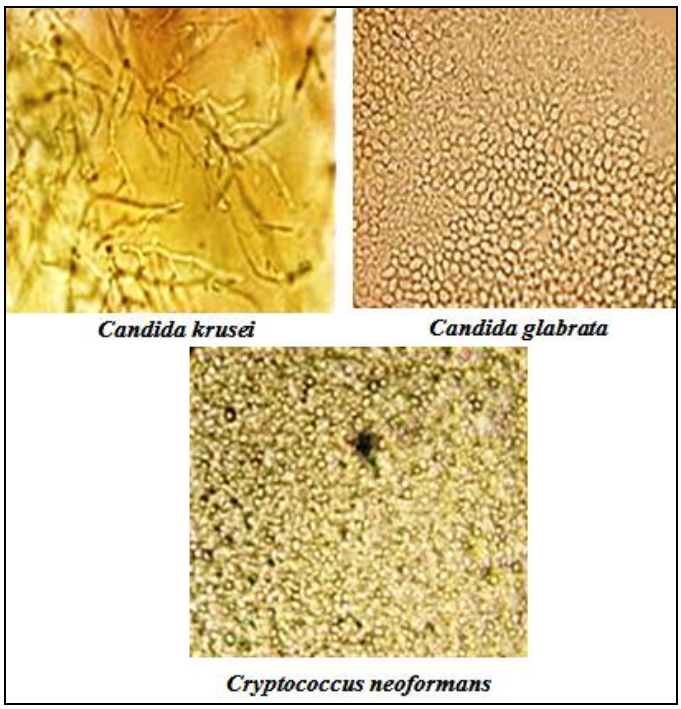


FIG. 3 (E-G): MICROSCOPIC MORPHOLOGY OF DIFFERENT *CANDIDA* SPP. ON CORN MEAL AGAR CONTAINING TWEEN 80. E *CANDIDA GLABRATA* – SMALL, OVAL, SINGLE TERMINAL BUDDING, NON ENCAPSULATED YEAST CELLS ARE SEEN AND NO PSEUDOHYPHAE ARE PRESENT. F. *CANDIDA KRUSEI* – FORWARD PSEUDOHYHAE WITH CROSS-MATCHSTICKS OR TREE LIKE BLASTOCONIDIA G. *CRYPTOCOCCUS NEOFORMANS*–ONLY BUDDING YEAST CELLS ARE SEEN.

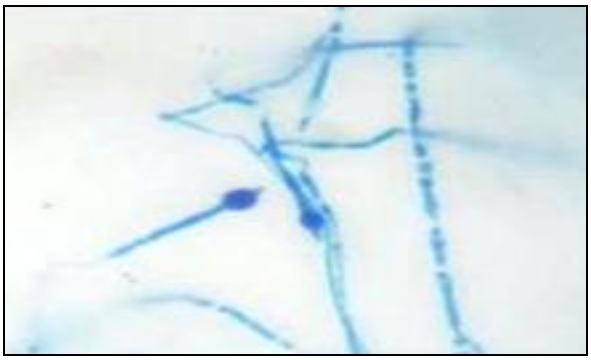
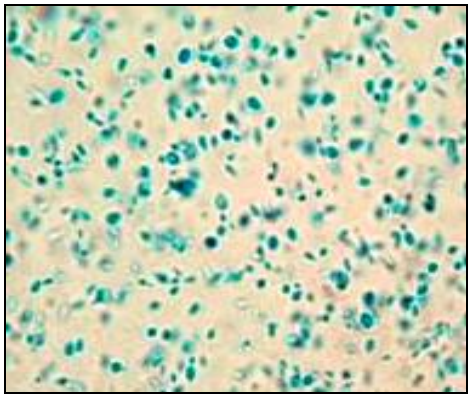
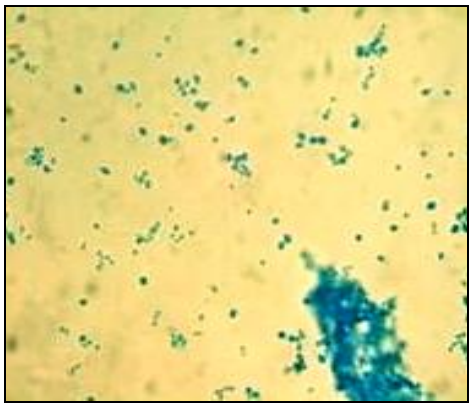


FIG. 4: GERM TUBE PRODUCED BY *CANDIDA ALBICANS*



A. *C. albicans*



B. *C. tropicalis*

FIG. 5 (A-B): MICROSCOPIC MORPHOLOGY OF DIFFERENT *CANDIDA* SPP STAINED WITH LACTOPHENOL COTTON BLUE (LCB) STAIN. SPHERICAL TO SUBSPHERICAL BUDDING YEAST LIKE CELLS OF BLASTOCONIDIA IS SEEN IN *CANDIDA ALBICANS* (A) AND *C. TROPICALIS* (B).

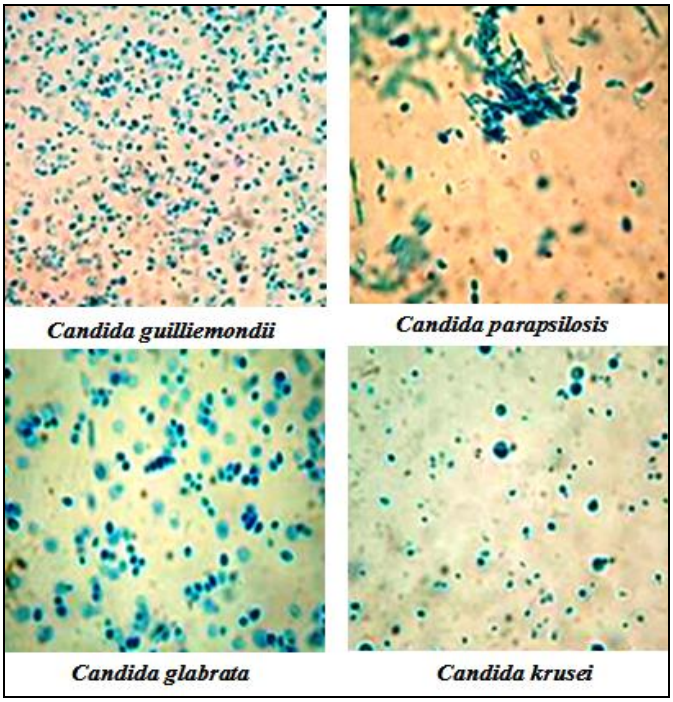


FIG. 5 (C-F) MICROSCOPIC MORPHOLOGY OF DIFFERENT *CANDIDA* SPP. STAINED WITH LACTOPHENOL COTTON BLUE (LCB) STAIN. SPHERICAL TO SUBSPHERICAL BUDDING YEAST LIKE CELLS OF BLASTOCONIDIA ARE SEEN IN *CANDIDA GUILLERMONDII* (C), PREDOMINANTLY SMALL, GLOBOSE TO OVOID BUDDING YEAST CELLS ARE SEEN IN *C.PARAPSILOSIS* (D) NUMEROUS OVOID, BLASTOCONIDIA ARE SEEN IN (E) *C. GLABRATA*, SMALL, ELONGATED TO OVOID BLASTOCONIDIA ARE SEEN IN *C .KRUSEI* (F).

FIG. 6: MICROSCOPIC EXAMINATION OF *CRYTOCOCCUS NEOFORMANS* STAINED WITH INDIA INK. DISTINCT, WIDE GELATINOUS CAPSULES ARE VISIBLE IN THE PREPARATION

Biochemical tests:

1. **Sugar Fermentation:** For Sugar fermentation, production of gas in the tubes was taken as fermentation positive while acid production alone was indicative of carbohydrate assimilation.

   All the *Candida albicans* and other *Candida* spp. fermented glucose whereas maltose was fermented by *C. albicans, C. tropicalis*. Sucrose was fermented by *C. tropicalis* and *C. guillermondii* only but lactose was not fermented by any Candida strains. None of the sugars were fermented by Cryptococcus neoformans st.

2. **Sugar Assimilation:** Results of assimilation of different sugars by various strains are presented in **Table 1**. The presence of growth around the disc was considered as positive for sugar assimilation (**Fig-7 a-b**).

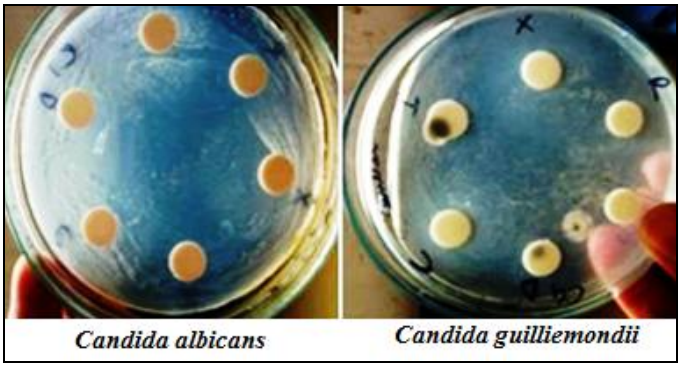


FIG. 7(A-B): ASSIMILATION OF SUGARS BY A. *CANDIDA ALBICANS* AND B. *C. GUILLERMONDII* STRAINS. THE PRESENCE OF GROWTH AROUND THE DISCS CONTAINING DIFFERENT SUGARS IS SUGGESTIVE OF SUGAR ASSIMILATION

Virulence Factors:

1. **Growth on blood agar:** All *Candida spp.* and *Cryptococccus neoformans* isolates showed growth. Among the *Candida* isolates, *C. parapsilosis* alone showed β-hemolysis and *Cryptococcus neoformans* showed α-hemolysis after an incubation of 72h at 37˚C.

2. **Biofilm formation:** Biofilm production was determined by microtiter plate method. All the randomly selected strains 6 out of 10 strains of *Candida* and 7 out of 8 strains of *Cryptococcus neoformans* produced biofilms in microtiter plates (**Table 2**).

3. ***In-vitro* antifungal activity of amphotericin B deoxycholate (AMB-d) against *Candida spp.*:** Antifungal activity of AMB-d was determined by the micro-broth dilution method as per CLSI M27-A2 protocol. The MIC values of Amphotericin B deoxycholate against *Candida spp*. as determined by this method are presented in **Table 3**. The MIC Amphotericin B deoxycholate against *Candida* spp. are presented in Table 3.The MIC values were recorded as 0.5mg/l for eight strains, 2mg/l for eleven strains and 8mg/l for five strains.

4. ***In-vitro* antifungal activity of amphotericin B deoxycholate (AMB-d) against *Cryptococcus neoformans*:** Antifungal activity of AMB-d was determined by the micro-broth dilution method as per CLSI M27-A2 protocol. The MIC values of Amphotericin B deoxycholate against *Cryptococcus neoformans* are presented in **Table 4**. It is evident from this table that all the isolates were susceptible to AMB-d with MIC values of 0.125mg/l, 0.25mg/l and 0.5mg/l except one strain which showed the MIC value of 1mg/l.

5. ***In-vitro* antifungal activity of Fungisome™ against *Candida* species:** Antifungal activity of Fungisome™ was determined by the micro-broth dilution method as per CLSI M27-A2 protocol. The MIC values of Fungisome™ against *Candida* spp. presented in **Table 5**. The drug showed efficient antifungal activity against all *Candida spp.* with MIC values of 0.125mg/l, 0.25mg/l and 0.5mg/l.

6. ***In-vitro* antifungal activity of Fungisome™ against *Cryptococcusneoformans*:** Antifungal

activity of Fungisome™ was determined by the micro-broth dilution method as per CLSI M27-A2 protocol. The MIC values of Fungisome™ against *Cryptococcus neoformans* presented in **Table 6**. This antifungal drug showed efficient activity against all the *Cryptococcus neoformans* with MIC values of 0.125mg/l and 0.25mg/l.

7. **Comparative analysis of MIC ranges of Conventional Amphotericin B (AMB-d) vis a vis Liposomal Amphotericin B (Fungisome™) against *Candida spp.* and *Cryptococcus neoformans*:** The MIC ranges of AMB-d and Fungisome™ against different strains of *Candida* spp. and *Cryptococcus neoformans* were compared. The MIC ranges of Fungisome™ ranged from 0.25-0.125 mg/l as against 0.5-8.0 mg/l in case of AMB-d for *Candida* spp. and the MIC ranges of Fungisome™ ranged from 0.25-0.125 mg/l as against 0.125-1.0 mg/l in case of AMB-d for *Cryptococcus neoformans*. It is thus clear that Fungisome™ was more efficacious against both the fungal spp.

DISCUSSION: The most common organisms implicated in human fungal infections are: *Candida* spp. and *Cryptococcus neoformans*. In our country, *Candida albicans* is the most predominant fungus involved in human infections [6]. This organism is the leading agent associated with blood stream infections [23]. Reports of increasing incidence of infection with *Candida non albicans* species like *C. glabrata, C. parapsilosis, C. guillermondii* and *C. tropicalis* are pouring in. In this group, the prevalence of *C. tropicalis* and *C. glabrata* is more as compared to other species.

TABLE 1: SUGAR ASSIMILATION BY YEASTS SPECIES OF ISOLATED FROM CLINICAL SPECIMENS

Name of the Test	Sugars Used for Test	Species of the Fungi						
		C. albicans	*C. tropicalis*	*C. parapsilosis*	*C. guillermondii*	*C. glabrata*	*C. krusei* ATCC 6258	*Cryptococcus neoformans*
ASSIMILATION	Glucose	+	+	+	+	+	+	+
	Maltose	+	+	+	+	+	_	+
	Sucrose	+	+	+	+	+	_	+
	Lactose	_	+	_	_	_	_	_
	Galactose	+	+	+	+	_	_	+
	Mellibiose	+	_	_	+	_	_	_
	Cellobiose	+	+	_	+	+	_	+
	Inositol	_	_	_	_	_	_	+
	Xylose	+	+	+	+	_	+	+
	Raffinose	+	_	_	+	_	_	+
	Trehalose	+	+	+	+	_	_	+
	Dulcitol	_	_	_	_	_	_	+

Note: (-) = Negative, (+) = Positive; *Grading according to size of zone of sugar assimilation.

TABLE 2: BIOFILM FORMATION BY *CANDIDA* SPECIES AND *CRYPTOCOCCUS NEOFORMANS* STRAINS

S. No.	Strains	Biofilm formation
1	CA/B-1622109	_
2	*C. albicans* – 23	_
3	*C. albicans* – 30	_
4	*C. albicans* – 26	_
5	*C. parapsilosis* (B-1597109)	++
6	*C. glabrata* (B-1366109)	+++
7	*C.glabrata*– 15	+++
8	*C. parapsilosis*– 9	++
9	*C. glabrata*– 17	++
10	*C. albicans* ATCC 90028	++
11	*Cryptococcus neoformans* (25:110)	++

12	*Cryptococcus neoformans* (25:52)	_
13	*Cryptococcus neoformans* (25:104)	+++
14	*Cryptococcus neoformans* (25:84)	+++
15	*Cryptococcus neoformans* (25:909)	+
16	*Cryptococcus neoformans* (25:391)	++
17	*Cryptococcus neoformans* (25:61)	+++
18	*Cryptococcus neoformans* ATCC 34664	++

Note: (-) = Negative (% $Transmittance_{block}$<5), (+) = Weak (% $Transmittance_{block}$5-20), (++) = Moderate (% $Transmittance_{block}$ 20-50), (+++) = Strong (% $Transmittance_{block}$ ≥50)

TABLE 3: *IN-VITRO* ANTIFUNGAL ACTIVITY OF AMPHOTERICIN B DEOXYCHOLATE (AMB-D) AGAINST STRAINS OF *CANDIDA SPP*.

Organisms		**N= 16**	**(AMB-d) amphotericin B deoxycholate**
S. No.	***Candida albicans* strains**		**Minimum Inhibitory Concentration (MIC) mg/l**
1	CA/MCG/10B		0.5
2	CA/2/040/20		0.5
3	CA/30/028/14		0.5
4	CA/B-1622109		0.5
5	CA/B-1599019		0.5
6	CA/3HD/29		8.0
7	CA/AGK3		2.0
8	CA/GMK3		8.0
9	CA/21042/21/A		2.0
10	CA/03/074/37		2.0
11	CA/GMC/6		2.0
12	CA/03/028/4		8.0
13	*C. albicans* – 23		2.0
14	*C. albicans* – 30		0.5
15	*C. albicans* – 24		0.5
16	*C. albicans*– 26		8.0
	***Candida* non *albicans* strains**		
17	*C. tropicalis* (B-1410109)		2.0
18	*C. tropicalis* (B-1384109)		2.0
19	*C. parapsilosis*(B1597109)		2.0
20	*C. glabrata* (B-1366109)		2.0
21	*C. guillermondii* (B-1343109)		2.0
22	*C. glabrata*– 15		0.5
23	*C.parapsilosis*– 9		2.0
24	*C. glabrata*–17		8.0

N= No. of strains tested

TABLE 4: *IN-VITRO* ANTIFUNGAL ACTIVITY OF AMPHOTERICIN B DEOXYCHOLATE (AMB-D) AGAINST *CRYPTOCOCCUS NEOFORMANS* STRAINS

Organisms		**N=7**	**Amphotericin B deoxycholate (AMB-d)**
S. No.	***Cryptococcus neoformans* strains**		**Minimum Inhibitory Concentration (MIC) mg/l**
1	*C. neoformans* (25:110)		0.125
2	*C. neoforman s* (25:52)		0.5
3	*C. neoformans* (25:104)		0.125
4	*C. neoformans* (25:84)		0.125
5	*C. neoformans* (25:909)		0.25
6	*C. neoformans* (25:391)		1.0
7	*C. neoformans* (25:61)		0.125

N= No. of strains tested

TABLE 5: *IN-VITRO* ANTIFUNGAL ACTIVITY OF FUNGISOME™ AGAINST STRAINS OF *CANDIDA* SPECIES

Organisms		N*	Fungisome™
S. No.	*Candida albicans* with strain number	16	Minimum Inhibitory Concentration (MIC) mg/l
1	CA/MCG/10B		0.125
2	CA/2/040/20		0.125
3	CA/30/028/14		0.125
4	CA/B-1622109		0.125
5	CA/B-1599019		0.125
6	CA/3HD/29		0.125
7	CA/AGK3		0.125
8	CA/GMK3		0.125
9	CA/21042/21/A		0.125
10	CA/03/074/37		0.125
11	CA/GMC/6		0.125
12	CA/03/028/4		0.125
13	*C. albicans*– 23		0.125
14	*C. albicans*– 30		0.125
15	*C. albicans*– 24		0.125
16	*C. albicans*– 26		0.25
	***Candida* non *albicans* strains**		
17	*C. tropicalis* (B-1410109)		0.125
18	*C. tropicalis* (B-1384109)		0.125
19	*C. parapsilosis*(B-1597109)		0.125
20	*C. glabrata*(B-1366109)		0.5
21	*C. guillermondii* (B-1343109)		0.125
22	*C. glabrata* – 15		0.125
23	*C.parapsilosis*– 9		0.5
24	*C. glabrata*– 17		0.5

N= No. of strains tested

TABLE 6: *IN-VITRO* ANTIFUNGAL ACTIVITY OF FUNGISOME™AGAINST STRAINS OF *CRYPTOCOCCUS NEOFORMANS*

Organisms		N=7	Fungisome™
S. No.	*Cryptococcus neoformans* strains		Minimum Inhibitory Concentration (MIC) mg/l
1	*C. neoformans* (25:110)		0.125
2	*C. neoformans* (25:52)		0.125
3	*C. neoformans* (25:104)		0.25
4	*C. neoformans* (25:84)		0.125
5	*C. neoformans* (25:909)		0.25
6	*C. neoformans* (25:391)		0.125
7	*C. neoformans* (25:61)		0.25

N= No. of strains tested

The incidence of invasive mycosis associated with *Candida* species has increased rapidly in immuno-compromised patients [24]. The tropical climate of the Indian sub-continent also offers a suitable environment for *Cryptococcus neoformans*, and the onslaught of the AIDS pandemic since the early 1990s has led to a sharp increase in the number of reported cases of cryptococcosis during past decade [25]. Being, the most densely populated country in the world, India is facing a significant increase in the incidence of candidaemia, cryptococcosis and AIDS cases which warrant for a detailed investigation of the fungal strains in the country [26]. There is an urgent need to develop fast and cost effective methods for identification of the wide spectrum of *Candida* species and *Cryptococcus* species of clinical significance.

In light of the above stated facts, we planned to characterize the *Candida* species and *Cryptococcus neoformans* strains obtained from clinical cases using various phenotypic methods and selective virulence factors. Two strains of each *Candida* species i.e. *Candida albicans*, *C. tropicalis*, *C. guillermondii*, *C. parapsilosis*, *C. glabrata*, and two strains of *Cryptococcus neoformans* were selected randomly for this purpose. The samples

originated from tissue biopsies, blood, pus, sputum, bronchoalveolar lavage, corneal scrapings, etc. These strains recovered by NCCPF (PGIMER), Chandigarh, India were studied for their growth characteristics on different media such as Sabouraud's dextrose agar (SDA), Hicrome *Candida* differential agar, corn meal agar, blood agar, etc. All the *Candida* spp. and *Cryptococcus neoformans* isolates produced growth on SDA.

The colonies could be distinguished by their color, texture and other morphological features (Fig 1 a-e). Hicrome *Candida* differential agar has been useful in discriminating *Candida* species. *Candida albicans* strains produced green color on this medium after 48hrs of incubation at 35^{o}C while all other species of *Candida* produced different colors. Similar observations have been made by others [19, 20]. These workers used this medium for examination of mixed infestations of *Candida*. This method is thus, a reliable and sensitive method for presumptive identification of most *Candida* species. The *Candida albicans* and non albicans could be differentiated on corn meal agar by pseudohyphae formation, production of different types of spores and mycelium formation. Similar features for differentiation have been reported by others [27, 19, 28] . These workers isolated different *Candida* species from bloodstream or other body fluids. On blood agar, all *Candida* spp. and *Cryptococccus neoformans* isolates showed growth. Among the *Candida* isolates, *C. parapsilosis* alone showed β-hemolysis and *Cryptococcus neoformans* showed α-hemolysis after an incubation of 72h at 37^{o}C.

On microscopic examination, Lactophenol Blue stained preparations of all the *Candida* spp. exhibited typical features as given in Fig. 5 a-e. i.e. formation of different types of blastoconidia by different *Candida* species. Distinct, wide gelatinous capsules characteristic of *Cryptococcus neoformans* were observed by India ink method (Fig-6). The phenotypic traits such as colony characteristics on different growth media and the microscopic features, fermentation of sugars by *Candida* spp. and sugar assimilation pattern of both these organisms confirmed their identity (Table 1). In addition, *Candida aibicans* produced germ tubes (Fig. 4).

In the present study, the development of biofilms by different fungal strains was determined by microtiter plate methods; 6 out of 10 strains of *Candida* and 7 out of 8 strains of *Cryptococcus neoformans* produced biofilms. The biofilm production serves as virulence factor of the fungus and also contributes to resistance to antimicrobial agents. It is interesting to note that in our study none of the *Candida non albicans* were biofilm negative in microtiter method. This points out to the fact that the *Candida non albicans* species are becoming more virulent, that certainly speaks of the increasing clinical significance of this group of *Candida species*. This finding is in agreement with that reported by others [21]. However, those workers additionally studied protinease and phospholipase activity as additional virulence factor for *Candida albicans* from bloodstream. However, we did not perform such tests.

Amphotericin B binds to the fungal membrane sterol (ergosterol) resulting in the formation of aqueous pores through which essential cytoplasmic materials leak out. In a similar manner, due to the interaction of this drug with mammalian membrane, toxicity can be produced especially in the kidneys. Despite being one of the most potent antifungal agents, the clinical use of amphotericin B is impeded owing to its poor aqueous solubility and toxicity [13]. Mutations in the ERG genes have been reported to be responsible for drug resistance in *Candida* spp,: a non-sense mutation in the ERG6 gene have been implicated in reduced susceptibility of clinical isolates of *Candida glabrata*. Other genes such as ERG3 and ERG11 have also been reported to be associated with the resistance to polyene drugs [29].

Recently, newer antifungal agents with broader antifungal activity and having fewer harmful effects and minimal resistance have become available. Fungisome^{TM}, which is an L-AMB formulation indigenously developed in India [15, 17] is commercially available and has been reported to be a safe, effective drug for the treatment of systemic fungal infections [30, 31]. In clinical trials, Fungisome^{TM} exhibited more than 90 % efficacy, and a negligible toxicity [16]. Both the drugs were evaluated in the present study for their efficacy by determining their MIC values of these drugs.

The MIC values of Fungisome™ were recorded as 0.125, 0.25 and 0.5 mg/l against all the *Candida* strains studied. MIC value of 0.125 mg/l was recorded against fifteen out of sixteen strains of *Candida albicans*, except one strain i.e. *C. albican*-26 for which a higher concentration of the drug was required (0.25 mg/l). Similarly MIC value of 0.125 mg/l was recorded in respect of five out of eight *Candida non albicans* strains. The MIC value of 0.5 mg/l. with respect to other three strains i.e. *C. glabrata* (B-1366109), *C. glabrata*-17, and *C. parapsilosis* was recorded.

In the present study, the MIC values of Amphotericin B against *Candida* spp. were recorded as 0.5 mg/l for eight strains, 2 mg/l for eleven strains and 8 mg/l for five strains, only eight strains were susceptible to Amphotericin B with MIC values of 0.5 mg/l i.e. CA/MCG/10B, CA/2/040/20, CA/30/028/14, CA/B-1622109, CA/B-1599019, *C. albicans*- 30, *C. albicans*- 24, *C. glabrata*- 15. It is interesting to note that in our study; most of the *Candida* isolates (16nos.) were resistant to Amphotericin B out of total (24nos.), in which nine *Candida albicans* and seven non-*Candida albicans* were found resistant with MIC value ≥ 2 mg/l. In previous studies, which have been done by other workers [18, 32, 33, 34].

These workers have done the antifungal susceptibility testing with same protocol as used in present study, and the MIC value of Amphotericin B against *Candida* spp. were recorded as i.e. ≥ or ≤ 1 mg/l. Repeated exposure to Amphotericin B against *Candida* spp. and the MIC range were recorded as 0.5-2 mg/l [35].

MIC values of 0.25 and 0.5 mg/l of Fungisome™ were recorded against all *Cryptococcus neoformans* strains, of these two strains (25:104 and 25:61) showed MIC values of 0.25mg/l while rest of the strains showed MIC values of 0.125 mg/l. Fungisome™ thus, showed considerable inhibitory activity against all Candida spp. and *Cryptococcus neoformans* strains. Similar observations have been made by other workers[34] who observed the MIC value of Fungisome™ against yeast spp. as 0.125, 0.25 and 0.5mg/l respectively. Also, in the present study, the MIC values of Amphotericin B against *C. neoformans* were recorded as 0.125. 0.25 and 0.5 mg/l except one strain which showed the MIC value of 1 mg/l i.e. *C. neoformans* (25:391). The susceptibility of C. neoformans strains were reported susceptible to Amphotericin B by other workers [36]. While MIC values of ≤4mg/l of Amphotericin B has been correlated with resistance to Amphotericin B recorded by other group [37].

The comparative analysis of Fungisome™ and Amphotericin B in the present study, reveals that Fungisome™ has better activity against different isolates of *Candida* spp. and *Cryptococcus neoformans*. The studies can be further extended to evaluate the recently developed Lipid–AMB formulations such as PAMBO (Amphotericin-B loaded Polymersomes). This formulation should be evaluated *in vitro* before the clinical trials are conducted to control a wide range of emerging and re-emerging fungal infections. Furthermore, the MIC of Fungisome™ was nearly 2 to 3 dilutions lower than AMB-d.

The remarkable difference in the efficacy of Fungisome™ may be attributed to the size of the liposomal carrier, structure and lipid composition of this formulation and consequently a variation in the rate of AMB release from the liposome. Only 20 % of AMB is released after incubation of an aqueous solution containing 0.05 mg L-AMB/L for one hour [38]. In contrast, the average median particle diameter of the Ambisome (Gilead) is 77.8 nm, as determined by dynamic light scattering [39].

This smaller size of the liposomal carrier molecule in Fungisome™, achieved by sonicating the drug formulation prior to use, may enable a convenient transfer of AMB to the hydro-lipophylic fungal cell membrane. Furthermore, the number of liposomes/mg of drug is presumably higher in Fungisome™ in comparison to other liposomal preparations. L-AMB may therefore, be more efficient in targeting a fungal cell in greater numbers, and also in targeting a greater number. The clinical efficacy of liposomal amphotericin B was effective as empirical therapy for confirmed invasive fungal infections as revealed by several randomized, double-blind trials in adult and pediatric patients [40].

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