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Original research paper

Study of antifungal susceptibility and biofilm formation among Candida species isolated from various clinical samples

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Abstract

Objectives: To study the antifungal susceptibility and biofilm forming ability of Candida species isolated from various clinical samples.

Methods: 123 isolates of candida species from recovered from clinical specimens like vaginal discharge, blood, urine, pus, body fluids, sputum were included in the study. The identification was done by standard methods by isolating in SDA followed by Germ tube test, Chromagar candida, Corn meal agar and Vitek YST cards. Antifungal susceptibility testing was done by disc diffusion method12. Biofilm formation was detected by tissue culture plate method13. Results are summarized as frequency tables, and percentages and Statistical analysis was done by SPSS 16.0 software.

Results: In the present study, 123 Candida spp. were isolated from different clinical samples, which included C. albicans (51.2%) followed by C. tropicalis (24.3%), C. glabrata (9.75%), C. krusei (7.3%), and C. parapsilosis (7.3%). Candida albicans was the predominant isolate among all species of Candida. Out of 63 Candida albicans isolates, 24 (38%) strains were positive for biofilm production. Among the total 60 non-Candida albicans, 39 (65%) isolates were biofilm producers. Antifungal susceptibility testing of various Candida species showed that all isolates were susceptible to amphotericin. 75.55% strains were sensitive to fluconazole. 18.88% of the Candida spp. were resistant to voriconazole. Out of 63 Candida albicans isolates, 24 (38%) strains were positive for biofilm production. Among the total 60 non-Candida albicans, 39 (65%) isolates were biofilm producers.

Conclusion: Candida albicans was the most commonly isolated species identified but non Albicans Candida species as a whole were the predominant group. Candida strains isolated in our study area have not yet developed resistance to amphotericin B. The possibility of reduced susceptibility to fluconazole and voriconazole may be due to widespread and long-term use of those antifungals among the study subjects. Our study underlines the importance of performing identification of Candida species and antifungal susceptibility test to guide treatment and prevent development of antifungal drug resistance. Biofilm formation is another important virulence determinant which can lead to chronic and persistent infections. There is no statistical correlation between the biofilm formation and antifungal susceptibility ($p>0.05$).

Keywords: Candida species, antifungal susceptibility, biofilm formation

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Introduction

Candida species are yeasts that exist predominantly in a unicellular form. They are small (4-6 µm), thin-walled, ovoid cells (blastospores) that reproduce by budding. The organisms are normal commensals of humans and are commonly found on skin, throughout the entire gastrointestinal (GI) tract, in expectorated sputum, in the female genital tract, and in the urine of patients with indwelling Foley catheters [1].

Candidiasis (infection caused by yeasts of the genus Candida), can occur all over the human body ranging from cutaneous infections of the skin and nails, to systemic infections of deep organs such as infections of the gastrointestinal tract, the respiratory tract, the urinary tract and the central nervous system. Most cases of Candidiasis represent endogenous infections since these infections are caused by Candida species derived from the normal flora of the patients and its scope to produce either superficial or systemic infections depends on the host immune system and various risk factors [2]. Candidiasis also originates from exogenous sources by transmission of organisms from, for example, patient to patient and from healthcare workers to patient [2].

Candida infections were noted more during the past two decades from countries with advanced medical care. As developing countries have introduced advanced medical care, including primarily more complex surgical procedures and more comprehensive cancer treatments, there has been increasing reports of Candida infections. Candida species are ranked as the fourth leading cause of nosocomial bloodstream infection [3]. The association of the infection with several risk factors has been reported. Emerging infections have included not only bloodstream infection but also arthritis, osteomyelitis, endophthalmitis, myocarditis, pericarditis, pacemaker endocarditis, ventricular assist device infection, meningitis, peritonitis, myositis, pancreatitis and others. The rapidly growing resistance of Candida species is also an emerging problem [4].

There are more than 150 species of Candida, but only a small percentage are regarded as frequent pathogens for humans. They are C. albicans, C. guilliermondii, C. krusei, C. parapsilosis, C. tropicalis, C. pseudotropicalis, C. lusitaniae, C. dubliniensis, and C. glabrata. Candida albicans remains the major pathogen in this group. There has been a steady and significant increase in reports on the incidence and manifestations of Candida infections caused by non-albicans species [4].

In recent years has been the evolving studies on the role of biofilm as a pathogenesis factor for Candida. Biofilms are bacterial populations that are enclosed in a matrix of extracellular polymeric substances. Biofilm formation can occur on mucosal surfaces and plastic surfaces of indwelling devices [5]. Biofilms are genetically resistant to antifungal agents including amphotericin B (AMB) and fluconazole (FLU). Biofilm formation varies depending on the Candida species [5, 6]. This study is undertaken to isolate Candida species from various clinical specimens, detect biofilm formation and to study their antifungal susceptibility pattern.

Materials and Methods

This study was undertaken over a period of one year from December 2020 to November 2021 at Dr. B.R. Ambedkar Medical College and hospital, Bangalore. A total of 123 isolates of Candida recovered from clinical specimens like vaginal discharge, blood, urine, pus, body fluids, sputum, etc. were included in the study. Both outpatient and inpatient samples were included in the study. The genus Candida was identified by colony morphology, Gram-staining, and other standard biochemical reactions. Candida species were identified to their species level by using Chromagar Candida, Cornmeal agar and VITEK 2 YST ID cards as follows-

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Procedure

1. Using phenotypic methods.

a) Gram stain [7]

After gram staining, we looked for gram positive budding yeast cells with hypae and/or pseudohypae. This is to distinguish a candida infection from a bacterial infection.

b) Isolation on Sabouraud dextrose agar (SDA) [7]

Samples were cultured on 2 tubes of SDA, one of which contained cycloheximide, at a temperature of 28'C and 45'C (being able to grow at both high and low temperatures will distinguish C. albicans from other species) in an aerobic environment for 48 hours. If there is no growth after 48 hours, they were further inoculated for a week. The different species were identified crudely using the following colony characteristics.

C. albicans	Smooth, creamy, pasty, glistening
C. glabarata	Cream coloured, soft, glossy, smooth colony
C. tropicalis	White to cream coloured colonies with peripheral fringe
C. parapsilosis	Soft, smooth, white sometimes lacy
C. krusei	Colonies are flat, dry becoming dull, smooth or wrinkled
C. kefyr	Smooth, creamy appearance

c) Germ tube test [8]

Using a sterile loop, a colony of yeasts was transferred to a test tube containing 3 drops of human serum and emulsified. After incubating for 3 hours, a drop of solution was placed on a clean dry glass slide and covered with a cover slip. It was observed under 10 and 40x of microscope to see the presence or absence of germ tube. This a quick method to differentiate C. albicans from other species.

d) Corn meal agar [9]

Subcultures were made by furrowing the Corn meal agar plates with coverslips were applied on the streak line and incubated at 28:C. After 2-5 days the plates were examined directly under a microscope to look for the following morphological characteristics of different candida species:-

C. albicans	Terminal and intercalary chlamydospores
C. glabarata	No pseudohyphae, only blastoconidia
C. tropicalis	Branching pseudohyphae and blastoconidia
C. parapsilosis	Curved pseudohyphae and blastconidia
C. krusei	Pseudohyphae and blastoconidia resembles crossed match stick
C. dublinensis	Terminal and intercalary chlamydospores

e) CHRO Magar [10]

A single yeast colony was streaked on to the plate after which it was inoculated for 48-72 hours. After this time, the species were identified due to their characteristic colours:-

C. albicans	Light green
C. glabarata	Pink to purple
C. tropicalis	Blue with pink halo

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C. parapsilosis	Cream to pale pink
C. krusei	Pink
C. dublinensis	Dark green

f) Using automated methods for Identification [11]

VITEK YST (Biomerieux)-ID cards were used. Cards were held at 35.5 °C for 18 h inside the Vitek 2 instrument which will take optical density readings automatically at every 15 min. Based on these readings, the species was identified according to a specific algorithm.

f) Antifungal susceptibility test using disk diffusion [12]

It was carried out by disk diffusion method on using Mueller-hinton agar with 2% glucose and 0.5 µg/ml Methylene blue for Fluconazole (25µg), Itraconazole (10µg), Amphotericin B (100 U) and Nystatin (10µg).Each inoculum was standardized to 0.5 McFarland units and the zone break points were interpreted as per the following table.
Phenotypic Determination of Biofilm Formation-

Tissue culture plate method [13]

This quantitative test described by Christensen *et al* is considered the gold-standard method for biofilm detection. Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose. Broths were incubated at 37 ^{o}C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37 ^{o}C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done as follows-
OD ≤ ODc-Strain not producing biofilm.
ODc< OD ≤ 2x ODc-Strain weakly producing biofilm.
2x ODc < OD ≤ 4x ODc-Strain moderately producing biofilm.
4x ODc< OD-Strain strongly producing biofilm.

The average OD values are calculated (from the inoculated triplets). The cut-off value (ODc) was established; the ODc is defined as three standard deviations (SD) above the mean OD of the negative control. The OD value of the tested strain is expressed as average OD value of the strain reduced by ODc value. ODc value should be calculated for each microtiter plate separately.
ODc= Ø OD negative control+ 3 x SD of negative control.
OD = Ø OD tested strain-ODc Ø=Average, SD=Standard deviation.
ATCC Staphylococcus epidermidis-35984 was used as positive control and ATCC Staphylococcus epidermidis-12228 was used as negative control.

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Results

In the present study, 122 Candida spp. were isolated from different clinical samples, which included C. albicans (51.2%) followed by C. tropicalis (24.3%), C. glabrata (9.75%), C. krusei (7.3%), and C. parapsilosis (7.3%). All the isolates were tested for antifungal susceptibility. Biofilm detection was done by tissue culture plate method. Results are summarized as frequency tables, and percentages were worked out. SPSSV.16.0 was used for Statistical analysis. Proportions and association were calculated by the descriptive study test and unpaired Student's t-test. Table 1 shows various Candida species isolated from clinical specimens. Clinical history was obtained from the medical records. Table 1 shows distribution of Candida species among various clinical samples. Candida albicans was the predominant isolate among all species of Candida. Out of 63 Candida albicans isolates, 24 (38%) strains were positive for biofilm production. Among the total 60 non-Candida albicans, 39 (65%) isolates were biofilm producers (Table 2). Antifungal susceptibility testing of various Candida species showed that all isolates were susceptible to amphotericin B similar to findings of Munmun B *et al*. The result of antifungal susceptibility of different Candida spp. is shown in Table 3.

Table 1: Sample wise distribution of Candida albicans and non Albicans Candida species

S. No	Clinical specimens	Candida albicans	Non albicans Candida species	Total
1.	BAL/Sputum	39	18	57
2.	Urine	18	36	54
3.	Blood	0	3	3
4.	High Vaginal swab/Vaginal discharge	6	0	6
5.	Wound swab	0	3	3
6.	Total	63	60	123

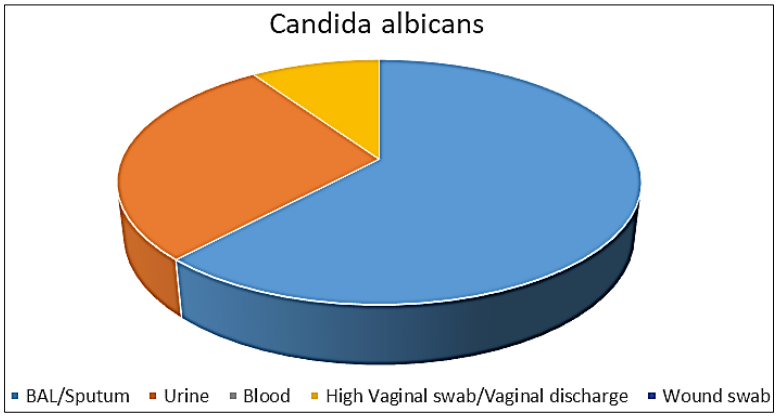

Chart 1

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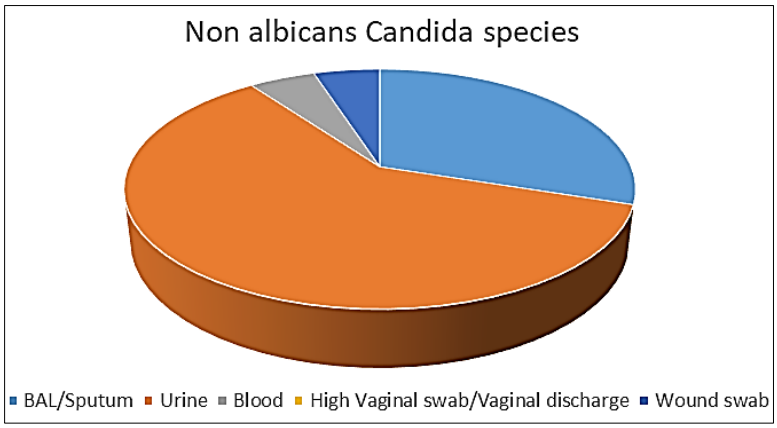

Chart 2

Table 2: Biofilm formation by various Candida species

Candida species	Biofilm positive	Biofilm negative	Total
Candida albicans	34(53.9%)	29(46.1%)	63
Candida parapsilosis	6(67%)	3(33%)	9
Candida tropicalis	24(80%)	6 (20%)	30
Candida krusei	3(33%)	6 (67%)	9
Candida glabrata	6(50%)	6(50%)	12
Non albicans Candida species Total	39(65%)	21(35%)	60

Table 3: Antifungal susceptibility pattern of isolated Candida species

Antifungal agents	Sensitivity	Candida albicans	Candida parapsilosis	Candia tropicalis	Candida krusei	Candida glabrata	Total
Nystatin	Sensitive	45(71.4%)	6(66.7%)	24(80%)	3(33.3%)	9(75%)	87(70.7%)
	Resistant	18(28.5%)	3(33.3%)	6(20%)	6(66.7%)	3(25%)	36(29.2%)
Amphotericin B	Sensitive	63(100%)	9(100%)	30(100%)	9(100%)	12(100%)	123(100%)
	Resistant	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Ketoconazole	Sensitive	0(0%)	6(66.7%)	12(40%)	0(0%)	9(75%)	27(21.9%)
	Resistant	63(100%)	3(33.3%)	21(60%)	9(100%)	3(25%)	96(78%)
Itraconazole	Sensitive	0(0%)	6(66.7%)	9(20%)	0(0%)	12(100%)	27(21.9%)
	Resistant	63(100%)	3(33.3%)	24(80%)	9(100%)	0(0%)	99(80.4%)
Clotrimazole	Sensitive	3(4.76%)	6(66.7%)	9(20%)	0(0%)	12(100%)	30(24.3%)
	Resistant	60(95.2%)	3(33.3%)	24(80%)	9(100%)	0(0%)	96(78.%)
Miconazole	Sensitive	9(14.2%)	6(66.7%)	9(20%)	0(0%)	12(100%)	36(29.2%)
	Resistant	54(85.7%)	3(33.3%)	24(80%)	9(100%)	0(0%)	90(73.1%)

Table 4: Sample wise distribution of biofilm production-TCP method

S. No.	Sample	Biofilm positive	Biofilm negative	Total
1.	BAL/Sputum	27(47.3%)	30(52.7%)	57
2.	Urine	30 (55.5%)	24(44.5%)	54
3.	Blood	0	3(100%)	3
4.	High Vaginal swab/Vaginal discharge	5 (83.3%)	1(16.7%)	6
5.	Wound swab	1(33.3%)	2(66.7%)	3
6.	Total	63	60	123

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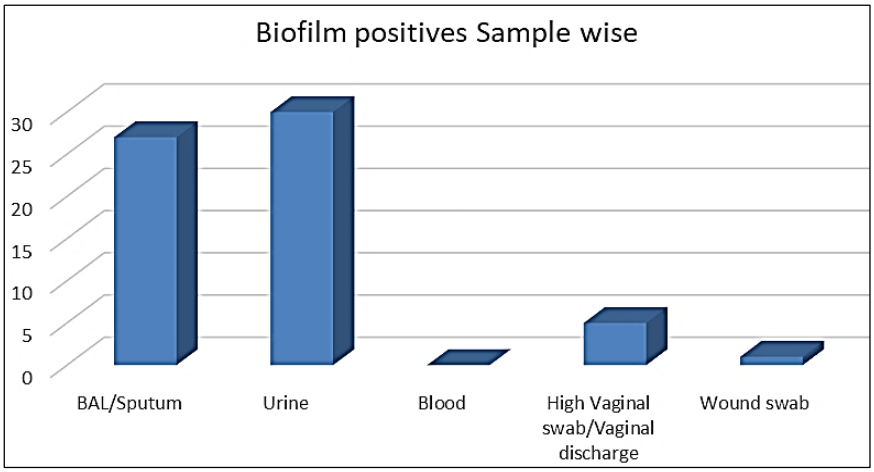
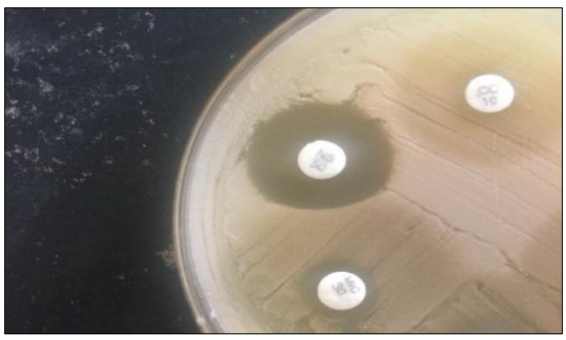
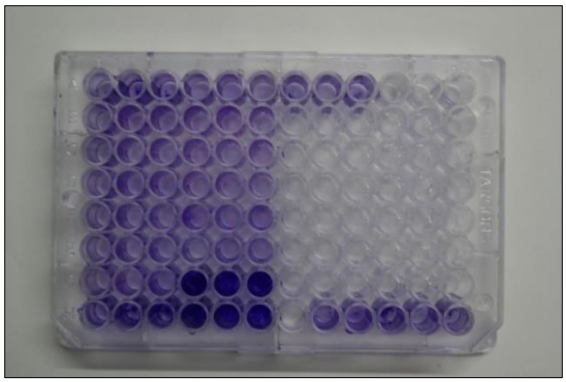


Chart 3

Chromagar Candida



Antifungal susceptibility test



Biofilm production by tissue culture plate method

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Discussion

Candida species are usually considered to be normal commensals among most samples and lowering of standard host defenses is required for them to act as pathogens. As already stated, with increase in advanced medical care, the population of patients with immunocompromised states, receiving antibacterial and aggressive cancer chemotherapy, or undergoing invasive surgical procedures and organ transplantation has increased. With this trend, Candidiasis has emerged as an alarming opportunistic disease.

Also another trend is the emergence of more non albicans Candida species as pathogens. In our study also we can find almost half of the species (48.7%) were non Albicans Candida species. Candida tropicalis (50%) was the most common among non albicans Candida species.

Biofilms are communities of cells developed on living tissues or on abiotic surfaces. Biofilm forming cell can also be attached to each other and are enclosed in a self-produced extracellular polymeric substance. This survival mode of growth allows cells to grow under hostile environmental conditions, for example, treatment with antibiotics and exposure to the immune response. Consequently, Candida biofilms have a significant impact on treatment in medical settings. In the present study, 65% were of non Albicans Candida species which is similar to findings S. Golia *et al*. [14] and R K Mukhia *et al*. [15] Among Candida albicans, 53.9% showed biofilm formation which is similar to findings of Melek Inci *et al*., [16] RK Mukhia *et al*. [15].

Among the non-Candida albicans spp. studied for biofilm production in the present study, 80% of C. tropicalis showed biofilm formation, followed by C. parapsilosis (66.7%) and C. glabrata (50%).

In our study, biofilm production was most frequently seen in vaginal swabs (83.3%) followed by urine (55.5%), sputum (47.3%), and pus (33.3%) which correlated well to findings of R K Mukhia *et al*. [15].

It is crucial to monitor antifungal resistance among new resistant strains that help in empirical treatment. Among the 123 Candida isolates tested, all were susceptible to amphotericin B in our study which is similar to the results reported by Arora *et al*. [15] and Munmun *et al*. Therefore, Candida strains isolated in our study area have not yet developed resistance to amphotericin B. 75.55% strains were sensitive tofluconazole, and 24.44% were resistant. Among the nonsusceptible Candida spp. 40.90% were C. albicans, 36.36% were C. tropicalis, and 22.72% were C. krusei. 18.88% of the Candida spp. were resistant to voriconazole, among which 17.64% were C. krusei and 41.17% were C. albicans and C. tropicalis. However, Yenisehirli *et al*. reported 34%and 14% resistance rates of fluconazole and voriconazole among C. albicans, respectively [17]. Jayalaksmi *et al*. published a resistance rate of 34.3% to fluconazole among 105 Candida isolates recovered from different clinical specimens [18]. A study conducted by Pelletier *et al*. revealed that 42 out of 295 Candida isolates were showing reduced susceptibility to fluconazole [19]. Our resistance rates of fluconazole and voriconazole are in line with those of earlier studies. The possibility of reduced susceptibility to fluconazole and voriconazole may be due to widespread and long-term use of those antifungals among the study subjects. There is no statistical correlation between the biofilm formation and antifungal susceptibility ($p>0.05$).

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