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**Original Article**

# Speciation, biofilm formation and antifungal susceptibility of *Candida* isolates

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**Abstract:** Recently, an increase in the incidence of infections caused by fungi especially non-albicans *Candida* species has been reported. Several virulence factors like biofilm formation, toxin production and presence of adhesins contribute to its pathogenesis. This study was undertaken to determine species distribution, biofilm formation and *in-vitro* antifungal susceptibility of *Candida* isolated in our tertiary care hospital. One hundred and forty-two clinical isolates obtained from various clinical specimens were subjected to KOH smear and cultured on Sabouraud's Dextrose agar medium. Conventional methods and automated identification system (Vitek 2 Compact) for yeast identification were done. Biofilm forming ability of each isolate was detected using microtitre plate method. Antifungal susceptibility against fluconazole, voriconazole, flucytosine, amphotericin B and caspofungin was tested using Vitek 2 Compact. Out of 142 *Candida* isolates, 90 (63.4%) were *C. albicans* and 52 (36.6%) were non-albicans *Candida* species. Among 52 non-albicans *Candida*, *C. parapsilosis* was found in 20 (38.5%) cases followed by *C. tropicalis* 16 (30.8%). Among all isolates, 52 (36.6%) were biofilm producers and biofilm positivity was more among non-albicans *Candida* 28 (53.8%) as compared to *C. albicans* 24 (26.7%) (p-value <0.002). The maximum positivity was observed with isolates from plastic devices (60%). The minimum inhibitory concentrations of all isolates against antifungal drugs were within susceptible range. Although *C. albicans* remains the major isolate from various clinical specimens, infections caused by non-albicans. *Candida* is on the rise and biofilm formation as a virulence factor might have a higher significance for non- albicans *Candida* species than for *C. albicans*. The changing epidemiology of *Candida* infections highlights the need for close monitoring on the distribution, biofilm production and susceptibility to optimize therapy and outcome.

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## Introduction

*Candida*, member of normal mammalian microbiota, causes a variety of superficial and deep seated mycotic infections especially in immunocompromised individuals. In the recent years, the incidence of *Candida* infections has increased dramatically because of increase in number of patients who are receiving immunosuppressive therapy, prolonged antimicrobial therapy, hyperalimentation fluids or are undergoing invasive surgical procedures and organ transplantation [1].

Although *Candida albicans* remains the most commonly identified yeast species, infections with non-albicans species are on the rise [2]. Several virulence factors like biofilm formation, toxin and enzyme production and presence of adhesions and complement receptors contribute to their pathogenicity.

Biofilms are structured microbial communities which are attached to a surface and encased in a matrix of exopolymeric material [3].

It has been implicated as a potential risk factor for some *Candida* species especially those responsible for catheter related infections [4]. Since *Candida* has been recognized as the fourth most common cause of invasive nosocomial fungal infections [5] and there is geographical variation in the distribution of its species causing various infections, the present study was undertaken to determine species distribution, biofilm formation and in-vitro antifungal susceptibility of the *Candida* isolated in a tertiary care hospital of Punjab (North India).

## Material and Methods

A total of 142 *Candida* strains isolated from clinical specimens of patients being treated in GGS Medical College & Hospital, Faridkot as a part of routine diagnostic procedures were included in the study. These patients had no history of antifungal drug exposure prior to the collection of the specimens. Out of the 142 *Candida* isolates, 66 (46.5%) were from urine, 39 (27.5%) from genital discharge, 16 (11.3%) from blood, 9 (6.3%) from sputum, 7 (4.9%) from pus and 5 (3.5%) from plastic devices (catheter tip, central line tip). All the specimens except those from blood were subjected to KOH wet mount examination and cultured on Sabouraud's Dextrose agar (SDA) medium in duplicate.

Blood was collected in biphasic brain-heart infusion agar broth medium. One of the inoculated culture medium was incubated at 37°C and another at room temperature and examined at days 1, 2, 3, 5 and 7. Yeast colonies obtained were further identified by conventional methods (germ tube test, sugar fermentation and assimilation tests) and finally by automated identification system (Vitek 2 Compact). Antifungal susceptibility against fluconazole, voriconazole, flucytosine, amphotericin B and caspofungin was also tested using Vitek 2 Compact system (bioMerieux, France).

Biofilm forming ability of each isolate was detected using microtitre plate method as described by Shin *et al*. [6]. The percentage transmittance (%T) value was measured for each isolate and subtracted from the %T value for the reagent blank to obtain a measure of the amount of light blocked while passing through the wells (%Tbloc). Biofilm production by each isolate was scored as negative (%Tbloc <5), 1+ (%Tbloc 5 to 20), 2+ (%Tbloc 20 -35), 3+ (%Tbloc 35-50) and 4+ (%Tbloc >50). *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 96142 were used as controls. The data so obtained was entered in excel worksheets and analysed using suitable statistical methods.

## Results

Out of 142 *Candida* isolates, 90 (63.4%) were found to be *C. albicans* and 52 (36.6%) non-albicans *Candida* and the difference between the two was statistically significant (p value <0.001). **Table 1** shows the distribution of various *Candida* species. Urine was the most common specimen for the isolation of both *C. albicans* and non albicans *Candida* species which was followed by vaginal discharge and blood. Among the 52 non-albicans *Candida*, *C. parapsilosis* was the most common species 20 (38.5%) followed by *C. tropicalis* 16 (30.8%)

**Table 1: *Candida* species isolated from different clinical specimens (n=142)**

| ***Candida* species** | **Clinical specimens** | | | | | | **Total (%age)** |
|---|---|---|---|---|---|---|---|
| | Urine (n=66) | Vaginal discharge (n=39) | Blood (n=16) | Sputum (n=9) | Pus (n=7) | Plastic devices (n=5) | |
| *C. albicans* | 38 | 31 | 7 | 7 | 5 | 2 | 90(63.4%) |
| *C. parapsilosis* | 14 | 3 | 0 | 1 | 2 | 0 | 20(14.1%) |
| *C. tropicalis* | 10 | 3 | 1 | 0 | 0 | 2 | 16(11.3%) |
| *C. krusei* | 0 | 1 | 4 | 0 | 0 | 0 | 5(3.5%) |
| *C. dubliensis* | 2 | 1 | 0 | 1 | 0 | 0 | 4(2.8%) |
| *C. famata* | 0 | 0 | 2 | 0 | 0 | 1 | 3(2.1%) |
| *C. pelliculosa* | 0 | 0 | 2 | 0 | 0 | 0 | 2(1.4%) |
| *C. lusitaniae* | 1 | 0 | 0 | 0 | 0 | 0 | 1(0.7%) |
| *C. utilis* | 1 | 0 | 0 | 0 | 0 | 0 | 1(0.7%) |

**Table 2** analyzes the results of biofilm production with respect to various *Candida* species. Of the 142 *Candida* isolates, 52 (36.6%) were biofilm producers and among these 52, biofilm positivity was significantly more (p-value <0.002) among non-albicans *Candida* species 28 (53.8%) as compared to *C. albicans* 24 (26.7%). Biofilm production was also studied with respect to clinical specimens.

The maximum positivity was observed with isolates from plastic devices 3/5 (60%) followed by those from urine 31/66 (46.9%), pus 1/7 (14.3%) and sputum 1/9 (11.1%) (**Table 3**). *In vitro* antifungal susceptibility testing by Vitek 2 showed that the Minimum Inhibitory Concentrations of all the isolates against fluconazole, voriconazole, flucytosine, amphotericin B and caspofungin was within the susceptible range.

**Table 2: Biofilm production by various *Candida* species**

| *Candida* spp. | Biofilm production | | | | |
|---|---|---|---|---|---|
| | Positive | | | | Negative |
| | 3+ | 2+ | 1+ | Total | |
| *C. albicans* (n=90) | 0 | 7 | 17 | 24(26.7%) | 66(73.3%) |
| *C. parapsilosis* (n=20) | 0 | 5 | 7 | 12(60.0%) | 8(40.0%) |
| *C. tropicalis* (n=16) | 1 | 6 | 3 | 10(62.5%) | 6(37.5%) |
| *C. krusei* (n=5) | 0 | 0 | 0 | 0 | 5(100.0%) |
| *C. dubliensis* (n=4) | 0 | 2 | 2 | 4(100.0%) | 0 |
| *C. famata* (n=3) | 0 | 1 | 0 | 1(33.3%) | 2(66.7%) |
| *C. pelliculosa* (n=2) | 0 | 1 | 0 | 1(50.0%) | 1(50.0%) |
| *C. lusitaniae* (n=1) | 0 | 0 | 0 | 0 | 1(100.0%) |
| *C. utilis* (n=1) | 0 | 0 | 0 | 0 | 1(100.0%) |
| **Total** (n=142) | 1 | 21 | 30 | 52(36.6%) | 90(63.4%) |

**Table 3: Biofilm production in various clinical samples**

| Specimen | Biofilm production | | | | |
|---|---|---|---|---|---|
| | Positive | | | | Negative |
| | 3+ | 2+ | 1+ | Total | |
| Urine (n=66) | 1 | 11 | 19 | 31(47.0%) | 35 (53.0%) |
| Blood (n=16) | 0 | 3 | 2 | 5(31.2%) | 11 (68.8%) |
| Sputum (n=9) | 0 | 1 | 0 | 1(11.1%) | 8 (88.9%) |
| Pus (n=7) | 0 | 0 | 1 | 1(14.3%) | 6 (85.7%) |
| Plastic Devices (n=5) | 0 | 1 | 2 | 3(60.0%) | 2 (40.0%) |
| Vaginal Discharge (n=39) | 0 | 5 | 6 | 11(28.2%) | 28 (71.8%) |
| **Total** (n=142) | 1 | 21 | 30 | 52(36.6%) | 90 (63.4%) |

## Discussion

The frequency of invasive opportunistic mycosis has increased significantly over the past two decades [7]. We studied 142 *Candida* strains isolated from various clinical specimens, which showed significant predominance (p value <0.001) of *C. albicans* over non-albicans *Candida* species, although 36.6% strains were those of non-albicans *Candida* species. Similar to our findings, studies from other regions of India have also reported *C. albicans* as the most common isolated species with the trends towards increasing prevalence of infections caused by non-albicans *Candida* species [4, 8, 9]. However, Golia *et al.* from Bangalore observed higher rate of isolation of non-albicans *Candida* species as compared to *C. albicans* [10].

In the present study, *C. albicans* was the predominant isolate from vaginal discharge (79.5%), respiratory (77.8%) and urine specimens (57.6%) which is similar to the findings of Emam *et al*. [11] and Nayman *et al*. [12]. However, Jain *et al*. showed predominance of non albicans *Candida* species in urine specimens [13]. We observed higher rate of isolation of non albicans *Candida* species from blood specimens which is well correlated with the studies from different parts of India. Amongst the non albicans *Candida* species, *C. tropicalis* has been reported as the predominant species in most of the Indian studies [14, 15, 16]. However, the present study showed the predominance of *C. krusei* in these specimens. This could be because of an outbreak of *C. krusei* septicaemia in neonates during the period of the study.

Biofilm production may help the *Candida* species in establishing the infection by evading host immune mechanisms, resisting antifungal treatment, and withstanding the competitive pressure from other organisms. The reported biofilm forming capability of *Candida* is variable in different studies. We observed that of the 142 *Candida* isolates, 52 (36.6%) were biofilm producers and this is in concordance to the results obtained by Shin *et al* [6] and Dag *et al*. [9]. However, some authors have reported higher percentage (64% to 73%) of biofilm producing *Candida* isolates [2, 4, 10].

In the present study, it was found to be more among non-albicans *Candida* isolates (28/52=53.8%) than *C. albicans* (24/90=26.7%) and the difference was statistically significant (p-value <0.002). This corroborates with the findings of many other authors [4, 17] and suggests that *C. albicans* may be possessing mechanisms other than biofilm production to establish infection. However, a study done by Alka *et al*. showed that more of *C. albicans* isolates were biofilm producers than non-albicans species of *Candida* [8]. Among the non-albicans *Candida* species, 62.5% of *C. tropicalis* and 60% of *C. parapsilosis* were found to be biofilm producers. Only four isolates of *C. dubliensis* were obtained and all were biofilm producers. One strain of *C. tropicalis* showed very high biofilm intensity (3+) which was not observed in any other *Candida* isolate. In contrast to other studies which reported *C. krusei* to be strong biofilm producer [10, 18], *C. krusei* isolates didnot show biofilm production in the present study. Further analysis showed that isolates from plastic devices had higher positivity (60%) for biofilm formation which is similar to the study of Sahar *et al*. [1].

This could be because the *Candida* which are the normal flora of human's colonies the various devices such as stents, shunts, prostheses, implants, endotracheal tubes, pacemakers, and indwelling catheters and form biofilm.

Isolates from plastic devices were followed by those from urine (46.9%), blood (31.2%), vaginal discharge (28.2%), pus (14.3%) and sputum (11.1%). The isolates from urine were from catheterized patients and 46.9% of them were found to be biofilm producers. Percentage of bloodstream isolates forming biofilm in our study was less than those from other studies [1, 10]. The least biofilm producers were the isolates from pus and respiratory tract and this is similar to the study done by Golia *et al*. [10]. The limitation of the present study was that we could not study virulence factors other than biofilm formation in the isolates of respiratory tract. Data from western countries show that the most of the *Candida* species had remained reliably susceptible to the polyenes, flucytosine, azoles and echinocandins. In the present study, we also observed that all the isolates (*C. albicans* as well as non-albicans *Candida* species except *C. krusei*) were susceptible to fluconazole, voriconazole, flucytosine, amphotericin B and caspofungin.

However, some Indian studies have reported resistant *Candida* strains especially among non-albicans *Candida* species suggesting that azoles could no longer be used for treatment of candidiasis [1]. The 100% susceptibility of our strains might be because of the judicious use of antifungal drugs in our set up. However, we should be vigilant and monitor resistance trends regularly as resistance to antifungal agents especially azoles are being reported.

To conclude, although *C. albicans* remains the major isolate from various clinical specimens, infections caused by non-albicans *Candida* is on the rise and biofilm formation as a virulence factor might have a higher significance for non-albicans *Candida* species than for *C. albicans*. Therefore, the changing epidemiology of *Candida* infections highlights the need for close monitoring on the distribution, biofilm production and susceptibility to optimize therapy and outcome.

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