Original Research Article

Isolation and Identification of Candida Species from Various Clinical Samples and their Biofilm Production Status

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Abstract

Candida species are the most common fungal species causing mucosal and systemic infections. Candida albicans has been the most common causative agent affecting mostly the immunocompromised. However, an increase in the prevalence of Non Albicans Candida (NAC) species is noted in the recent two decades. In this study Candida species from various clinical samples were isolated and their biofilm production status was evaluated. All the clinical samples were inoculated on to Blood agar and MacConkey agar. The suspected colonies of Candida were further confirmed by Gram stain. Germ tube test was done for the differentiation of C. albicans and C. dublinensis from the other candida species. It was further inoculated onto HiCrome Candida Differential agar for the speciation. The Biofilm production was done using the Congo-red agar. Out of a total 36 candida species that were isolated during the study, 18 (50%) were found to be Candida albicans and remaining 18 were found to be NAC (22.2% were C. tropicalis, 19.4% were C. glabrata, 8.4% were C. krusei). Among the 36 candida species, 12 (33%) were biofilm producers while the remaining 24 (64%) were non-biofilm producers. This study showed that there is an increase in the pathogenicity and prevalence of the NAC species.

Keywords
Candida species, Clinical samples, Biofilm production status

Introduction

Fungal infections are considered a serious health problem and are a major cause of morbidity and mortality worldwide. Among all the fungal pathogens, Candida species are a major cause of morbidity and mortality worldwide and thus represents a serious threat to public health (De Oliveira Santos et al., 2018). Candida is a yeast like fungus and ubiquitous human commensal (VigneshKanna et al., 2017). They are capable of initiating infections in both immunocompetent individuals and immunocompromised hosts, but the incidence of infections is more in immunocompromised individuals, hence is rightly called the ‘disease of diseased’ (Deorukhkar et al., 2014). They inhabit the gastrointestinal tract including the mouth,
oropharynx, female genital tract and skin (Sandhu et al., 2015). *Candida* species are responsible for various clinical manifestations ranging from mucocutaneous overgrowth to life threatening disseminated infections like candidemia (Deorukhkar et al., 2014). The pathogenicity of *Candida* species is attributed to certain virulence factors, such as the ability to evade host defenses, adherence, biofilm formation (on host tissue and on medical devices) and the production of tissue damaging hydrolytic enzymes such as proteases, phospholipases and hemolysin (Sardi et al., 2013).

While *Candida albicans* is still the most common causative agent of nosocomial fungal infections, some studies have reported increasing rates of species other than *C. albicans* (Aydemir et al., 2017). Shifting of trend to *Non albicans Candida* (NAC) infection with high antifungal resistance has been witnessed (Chandak et al., 2018). The *Non albicans Candida* species includes *C. tropicalis, C. glabrata, C. parapsilosis, C. krusei* and others. Several brands of chromogenic media have been developed to produce rapid yeast identification. These media contain chromogenic substrates that react with the enzymes secreted by microorganisms producing colonies showing various pigmentation. These enzymes are species specific, allowing organisms to be identified to the species level by their colour and colony characteristics (Sandhu et al., 2015).

Biofilm aids the producing organism to withstand or evade host defense mechanism and enables the organism to survive and exist as reservoir and recurrent source of infection as well as development of resistance to antimicrobial agents (Das et al., 2016). The ability of *Candida* species to form drug-resistant biofilms is an important factor in their contribution to human disease. Decision to treat such patients with antifungal drugs is complex (Naveen Saxena et al., 2014). There are various methods used to detect biofilm production like Congo red agar method, determination of dry weight, scanning electron microscope (SEM) etc. (Dhale et al., 2014).

In the recent years, the incidence of nosocomial candidiasis has increased throughout the world, starting from tertiary care centers and spreading to community hospitals (Kaur et al., 2016). The clinical manifestations of infections caused by different members of NAC species are usually indistinguishable and several NAC species are inherently resistant or acquire resistance or both, to commonly used antifungal drugs (Deorukhkar et al., 2014). This variability increases the challenge of finding an effective solution to tackle the threats of *Candida* biofilms as a unique problem (Cavalheiro et al., 2018). Hence, identification of *Candida* species and detection of biofilms becomes necessary.

Thus, this study aim to isolate and identify *Candida* species from various clinical samples and also to detect the biofilm production of isolated *Candida* species.

**Materials and Methods**

This study was conducted over a period of 2 months (May and June) in the department of Microbiology, Adichunchangiri Institute of Medical Sciences, Mandya. A total of 36 isolates were recovered from clinical samples like High vaginal swab(HVS), sputum samples, urine samples and Endotracheal tubes. Patients on any form of antifungal therapy 6 weeks prior to sample collection were excluded from the study.

All the clinical samples were inoculated onto blood agar and MacConkey agar and
incubated aerobically at 37°C for 24-48 hours. Colonies appearing pasty, opaque, slightly domed, white or off-white coloured is suspected as the colonies of *Candida* and is identified by Gram stain. The one which revealed Gram positive budding yeast cells was inoculated on SDA (Sabouraud Dextrose agar) and HiCrome *Candida* differential agar procured from Himedia Pvt. Ltd, Mumbai, India.

**Inoculation on Sabouraud Dextrose agar (SDA)**

The growth obtained on SDA culture medium was identified based on colony morphology, Gram stain and subjected to germ tube test.

**Rapid germ tube test**

This test was done to differentiate *C. albicans* and *C. dublinenses* from other *Candida* species. Small inoculums of yeast cells obtained from an isolated colony were suspended in 0.5ml of serum & was incubated at 37°C for no longer than 3 hours. A drop of this suspension was placed on a microscope slide & examined for the presence of germ tubes (Forbes *et al.*, 2007).

**Inoculation on HiCrome Candida differential agar**

Further speciation of the isolates was done by culturing it on HiCrome *Candida* differential agar. HiCrome agar was prepared as per the manufacturer’s instructions and incubated at 30°C for 24-48 hours. Species identification was done by the morphology and colour of the colony (chromogenic reaction). The isolates that remained doubtful in their appearance on HiCrome agar was considered as unidentified and excluded from the study.

The repeated isolation of *Candida* species from clinical specimen like oropharyngeal, vaginal, urinary and bronchial candidiasis was considered significant. While a single isolation was considered significant from sterile body fluids like blood, peritoneal fluid, pleural fluid and CSF.

The in-vitro screening test for biofilm production in *Candida* isolates was carried out using Congo red agar method. The Composition of the Congo red agar media is BHI (37 gm/L), glucose (80 gm/L), agar no.1 (10 gm/L) and Congo red stain (0.8 gm/L). Aqueous solution of Congo red was prepared and autoclaved separately and added to the agar after cooling it to 55°C. The identified *Candida* species were inoculated on plates of Congo red agar and incubated aerobically at 37°C for 24-48 hours. Positive biofilm production by *Candida* species was indicated by the appearance of black colonies, whereas biofilm negative *Candida* species produces white or very light pink colonies.

**Results and Discussion**

In the present study 36 *Candida* species were isolated over a period of two months (May and June 2019) from various clinical samples such as High vaginal swab (HVS), urine, sputum samples and Endotracheal tube as shown in Table 1. All the isolates were subjected to speciation and biofilm formation. HiCrome agar showed good growth of all the *Candida* isolates after 48 hours of incubation.

*Candida* species are part of normal human flora and are opportunists capable of causing a wide spectrum of infections. Colonisation of the mucocutaneous surfaces is the first step towards infection. Alteration in this balance results in growth and subsequent invasion and is supported by various risk factors leading to immunosuppression. Some of these include infections with HIV/AIDS, indiscriminate antibiotic use, use of intravenous catheters, urinary tract catheterisation, hepatic and renal
failure, prolonged hospital stay, chemotherapy, organ transplant, leukaemia, diabetes mellitus and Chronic Obstructive Pulmonary Diseases (COPD).

*Candida albicans* has been the most common species causing infection for many years but, indiscriminate use of azole group of drugs has led to increase in NAC infection. Hence, infections with NAC and overall resistance to antifungals are on the rise. This makes species identification of *Candida* very essential to prevent treatment failures (Jangla *et al.*, 2018).

Conventional *Candida* speciation methods like morphology on Corn meal agar, carbohydrate fermentation and assimilation tests are time consuming taking from 72 hours to 2 weeks and the procedures are labor intensive (Kaup *et al.*, 2016). The longer turnaround time taken by conventional methods of identification makes them less popular among the clinicians as early diagnosis is essential for initiating appropriate therapy (Shettar *et al.*, 2012). The need for rapid identification of *Candida* species and the difficulty in detecting mixed cultures of *Candida* on Sabouraud’s Dextrose Agar, has led to the development of several methods that differentiate yeast species. Newer methods which have been developed for yeast identification include CHROM agar, API systems, Vitek 2 ID system and molecular methods. Several brands of chromogenic media have been developed to produce rapid yeast identification. These media contain chromogenic substrates that react with enzymes secreted by microorganisms producing colonies with various pigmentation. These enzymes are species specific, allowing organisms to be identified to the species level by their colour and colony characteristics (Horvath *et al.*, 2003).

HiCrome *Candida* differential agar is one such medium introduced by Himedia laboratory to differentiate *Candida* species namely *C. albicans*, *C. krusei*, *C. tropicalis*, and *C. glabrata* based on colony colour. The colony color produced by *Candida albicans*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata* were typical. *Candida albicans* produces light green colonies, *Candida tropicalis* blue to metallic blue colonies, *Candida krusei* produces purple fuzzy colonies and *Candida glabrata* white to cream colored colonies (Figure 1). This medium can be recommended for identification of these species in resource limited settings as it will not require any expertise (Rachana Mehta *et al.*, 2016). Hence, the present study was undertaken to identify *Candida* isolated from various clinical specimens to the species level using HiCrome *Candida* differential agar along with their biofilm production status.

In the present study, out of the 36 *Candida* species which were isolated from the specimen, 18 (50%) were found to be *Candida albicans* and remaining 18 (50%) were identified as *Non albicans Candida*. Among the 18 (50%) NAC species, eight (22.2%) were identified as *C. tropicalis*, seven (19.4%) as *C. glabrata* and three (8.4%) as *C. krusei* (Figure 2). Similar findings were reported by Sharma *et al.*, where out of 120 *Candida* species, 58 (48.33%) were *Candida albicans* and 62 (51.66%) were identified as NAC. Das *et al.*, (2016) found similar distribution of *Candida albicans* 33 (36.7%) and NAC 57 (63.3%) from various clinical samples thus concluding that there is a notable shift in the pathogenic incidence of species from *C. albicans* to NAC species.* C. albicans* is the most frequently isolated species. Among the NAC species, *C. tropicalis* is most frequently isolated. This preponderance of *C. tropicalis* is consistent with other studies (Vignesh Kanna *et al.*, 2017; Deorukhkar *et al.*, 2014. Das *et al.*, 2016).
HiCrome agar has several advantages like rapidity, direct identification of species; thus, making it very useful in early identification and thereby early initiation of appropriate antifungal therapy (Shettar et al., 2012). A major advantage of chrome agar is the ability to detect mixed cultures of yeasts in clinical specimens (Manjunath et al., 2012). However, present study did not show any mixed isolates. In our study, Candida species were isolated from various clinical samples like HVS, urine, sputum. To rule out colonisation, a repeat sample was collected and processed to confirm the etiological significance of the isolate.

Figure 3 shows the distribution of Candida species from various clinical samples. In sputum samples, two were C. albicans and one was NAC. From HVS samples, eleven were C. albicans and five were NAC. From urine samples, five were C. albicans and eleven were NAC. One NAC was isolated from endotracheal aspiration fluid. Most of the Candida albicans species were found to be isolated from HVS samples while most of the Non albicans Candida (NAC) species were isolated from urine.

Table 1 Candida species isolated from various clinical samples

| Sample Type               | Number of samples |
|---------------------------|-------------------|
| High vaginal swabs        | 16                |
| Urine                     | 16                |
| Sputum                    | 03                |
| ET Tube aspiration        | 01                |
| **Total**                 | **36**            |

Figure 1 HiCrome agar showing colonies of various candida species producing different colors

3634
**Figure.2** Distribution of *Candida* species

![Figure 2](image2)

**Figure.3** Distribution of *Candida* species in different clinical samples

![Figure 3](image3)

**Figure.4** Biofilm producing *Candida* species

![Figure 4](image4)
Figure 5 Congo red agar showing biofilm producing *candida* species and non-biofilm producing *candida* species

All the isolated *Candida* species were studied for biofilm production on Congo red agar. Among the 36 *Candida* species, 12 (33.3%) were biofilm producers while the remaining 24 (66.7%) were non biofilm producers. The biofilm producing species includes 7 (19.4%) *C. albicans*, 3 (8.3%) *C. glabrata* and 2 (5.6%) *C. tropicalis* as depicted in Figure 4. This correlates with the study done by Saxena *et al.*, where 38.3% of the isolates were biofilm producers. However slightly higher percentage is observed in other studies done by Sharma *et al.*, (2017) and Marak *et al.*, (2018) (52.5% and 54.4% respectively). The figure 5 shows biofilm positive *candida* species with black colonies and biofilm negative candida species with pink colored colonies on Congo red agar.

Biofilms are universal, complex, interdependent communities of surface-associated microorganisms, enclosed in an exopolysaccharide matrix occurring on any surface, including medical devices. The pathogenicity of *Candida* species is associated with its ability to form Biofilm and is an essential virulence determinant during candidiasis (Mohandas *et al.*, 2011).

The formation of *Candida* biofilms carries important clinical repercussions because of their increased resistance to antifungal therapy and the ability of cells within biofilms to withstand host immune defenses. Also, biofilm formation on medical devices can negatively impact the host by causing the failure of the device and by serving as a reservoir or source for future continuing infections. The net effect is that *Candida* biofilms adversely impact the health of these patients with increasing frequency and severity and with soaring economic sequel. Hence detection of biofilms becomes necessary (Bansal *et al.*, 2016).

In conclusion, the present study shows the predominance of NAC species over *C. albicans*. This helps us understand that the pathogenicity and susceptibility to NAC has increased over the years. Hence, this knowledge is very important in the early initiation of anti-fungal therapy especially in patients suffering from systemic candidiasis such as blood stream infections which have a high mortality. The present study also highlights the fact that HiCrome agar can be cost-effective alternative to the other conventional methods which are time
consuming. However, the major drawback of this medium is the subjective nature of the identification and inability to differentiate few species. With further modifications in the agar, the use of HiCrome agar may obviate the need for conventional identification methods in routine laboratories.

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