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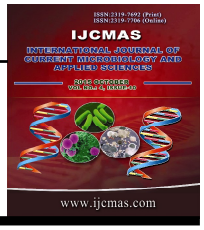


**Original Research Article**

# A Comparative Study of Virulence Factors in Clinical Isolates of *Candida* Species

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**A B S T R A C T**

Virulence factors of *Candida* species have been of great interest as targets for the development of new therapeutic interventions against candidiasis. There is an increase in incidence of infections due to non-albicans candida (NAC) species especially in hospital setups. Objective of this study was to identify clinical isolates of Candida species and evaluate hemolysin, phospholipase and proteinase activity in them. The study included 118 non-repeat clinical isolates of *Candida* species, identified by standard Microbiological techniques. Sabouraud's dextrose agar (SDA) with 3% blood, SDA with 5% eggyolk and SDA with 1% casein were used to detect and measure zone of hemolysis, precipitation and opacity respectively in them. *Candida albicans* ATCC 90028 was used as control. All the 118(100.0%) isolates were hemolysin producers, 38(32.2%) were phospholipase producers while 26(22.0%) were caseinase producers. Hemolysin (Hz) activity was more pronounced in *Candida albicans* compared to NAC, while phospholipase (Pz) and caseinase (Cz) activity was significantly more in NAC compared to *Candida albicans*. Phospholipase was more frequently associated with urine, pus and high vaginal swab isolates; while caseinase was more frequently associated with urine and pus isolates. Causative role of NAC species (66.1%) is increasing compared to *Candida albicans* (33.9%) as clinical isolates. NAC species are found to be more frequently associated with virulence factors like phospholipase and caseinase. Bringing detection of virulence factors in routine practice might improve understanding of behavior of these organisms and open doorways to better patient management and prognosis of the patients.

## Introduction

The proportion of infections due to non-albicans *Candida* (NAC) species is persistently rising and so is the need to identify clinical isolates of *Candida* up to species level (Pfaller *et al.,* 1998). Incidence of nosocomial candidemia has increased dramatically over last few decades.

Although *Candida albicans* remains the most common fungal isolate recovered, recent reports indicate a trend toward an increasing prevalence of NAC, amongst blood isolates as well (Eggimann *et al.,* 2003; McNeil *et al.,* 2001; Blumberg *et al.,* 2001; Miller *et al.,* 2001). Fungal infection

continues to increase in hospitals, as more effective and broader spectrum antibacterial agents and immunosuppressive regimens are deployed (Mark *et al.,* 2005). *Candida* species have developed putative virulence factors to assist them colonize, invade and cause pathogenesis. These virulence factors vary in relation to type, site and stage of infection (Salyers *et al.,* 1994). Virulence factors of *Candida* species have been of great interest as targets for the development of new therapeutic interventions against candidiasis (Perfect *et al.,* 1997). This study was undertaken to compare hemolysin, phospholipase and caseinase activity in the clinical isolates of *Candida* species and to detect association between virulence factors with type of infection.

## Materials and Methods

The study period was from January 2013 to June 2014. A total of 118 non repeat clinical isolates of *Candida* species from the samples received in central clinical laboratory of tertiary care hospital in Meerut were studied. The clinical samples included blood, urine, pus, high vaginal swab (HVS), vault (from hysterectomy patients), tissue and central line received from various intensive care units, wards and outpatient department.

The study has been approved by the research and ethical committee of the institute.

All the isolates of *Candida* species were identified by standard laboratory techniques (Jagdish, 2008). Hemolysin activity (Hz value) was demonstrated by zone of haemolysis around colony on Sabouraud's Dextrose agar (SDA) with 3% human blood (Manns *et al.,* 1994) (Figure 1). Phospholipase activity (Pz value) was demonstrated by zone of precipitation around colony on SDA with egg yolk (5% of mixture of egg yolk and saline in 1:1 proportion) (Price *et al.,* 1982) (Figure 2). Caseinase activity (Cz value) was demonstrated by zone of opacity around colony on SDA with 1% casein (10803, Fisher Scientific) (deDoroti *et al.,* 2002) (Figure 3). ATCC *Candida albicans* 90028 was used as control. Unless specified otherwise, all culture media, reagents, chemicals were obtained from HI MEDIA private limited, Mumbai.

**Test procedure:** A single colony was picked up from the primary isolate and subcultured on SDA with chloramphenicol and incubated for 24 hours at $37^0$ C. This growth was used to prepare a suspension of $10^8$ cells / mL in sterile saline. An aliquot of 10 µL of suspension was seeded and then streaked linearly on to above mentioned three special media. Four or less number of samples was tested on one plate. All plates were incubated for 48 hours at $37^0$ C and colony diameter and zone diameters were measured in millimeters.

**Calculations:** Enzyme activity was measured as a ratio of the colony diameter in millimeters to that of the zone. The value obtained was in fraction, which was converted into decimals. The software used for the statistical analysis was SPSS version 16.0 and Epi-info version 3.0.

## Results and Discussion

A total of 118 non repeat *Candida* species were isolated from similar number of samples; which included 49 (41.5%) urine, 38 (32.2%) blood, 19 (16.1%) high vaginal swab (HVS), 7 (5.9%) pus, 3 (2.5%) vault swab and 1 (0.9%) each of tissue and central line.

The isolated *Candida* species include 40 (33.9%) *Candida albicans* and 78 (66.1%) Non- albicans *Candida* (NAC) (Table 1).
All the 118 isolates were haemolysin

producers. The mean hemolysin activity, shown by *Candida albicans* was significantly more than that shown by NAC species (p value = 0.0001)

Phospholipase producers were more amongst *Candida albicans* 24 of 40 (60.0%) than in the NAC 14 of 78 (17.9%) (p value <0.001). Phospholipase activity shown by NAC species was significantly more than that shown by *Candida albicans* (p value <0.0001). Caseinase producers were also more amongst *Candida albicans* 13 of 40 (32.5%) than amongst the NAC 13 of 78 (16.6%) (p value <0.049). Caseinase activity shown by NAC species was significantly more than that shown by *Candida albicans* (p value <0.044).

In *Candida albicans* hemolysin production was most prevalent virulence factor (100.0%) followed by phospholipase (60.0%) and then caseinase (33.3%). Among NAC species hemolysin production was (100.0%) but phospholipase producers (20.5%) and caseinase procucers (15.4%) were much less, compared to those in *Candida albicans* (Table 2).

All the isolates were haemolysin producers. Phospholipase producers were predominently from HVS 12 of 19 (63.2%), followed by pus 3 of 7 (42.9%), urine 18 of 49 (36.7%) and blood 9 of 38 (23.7%). None of vault or central line sample isolate showed phospholipase activity. Caseinase producers were predominently from pus 5 of 7 (71.4%), followed by urine14 of 49 (28.6%), blood 10 of 38 (26.3%) and HVS 3 of 19 (15.8%). One of three vault isolates exhibited caseinase activity, while central line isolate did not show any caseinase activity (Table 3).

Recent studies have documented a shift towards NAC species from *Candida albicans* (Dan, 2002). Some studies have reported increasing trend of incidences of infections caused by NACs, gradually surpassing *Candida albicans* as cause of candidemia in some regions (Pfaller, 2007). Factors like increased use of antifungal drugs and broad spectrum antibiotics, long term use of catheters and increase in the number of immunocompromised patients have contributed to the emergence of NAC species in increasing numbers (Kothavade *et al.*, 2010; Varsha *et al.*, 2013; Shivaprakasha *et al.*, 2007). NAC species cannot be overlooked as mere contaminants or non-pathogenic commensals as most of them show reduced susceptibility to commonly used antifungal drugs (Deorukhkar *et al.*, 2013).

**Table.1** Distribution of isolated *Candida* species (n=118)

| **Isolates** | | **Number** | **Percentage** |
|---|---|---|---|
| *Candida albicans* (n=40) | | 40 | 33.9 % |
| Non-albicans *Candida* (n=78) | *Candida tropicalis* | 40 | 33.9 % |
| | *Candida parapsilosis* | 22 | 18.7 % |
| | *Candida krusei* | 06 | 5.1 % |
| | *Candida glabrata* | 04 | 3.4 % |
| | *Candida kefyr* | 03 | 2.5 % |
| | *Candida guilliermondii* | 03 | 2.5 % |
| Total | | 118 | 100 % |

**Table.2** Comparison of virulence factor producers in different isolates

| Isolates | Virulence Factor | | |
|---|---|---|---|
| | **Hemolysin producer** | **Phospholipase producer** | **Caseinase producer** |
| ***Candida albicans*** **(n=40)** | 100.0 % (40/40) | 60.0 % (24/40) | 32.5 % (13/40) |
| ***Candida tropicalis*** **(n=40)** | 100.0 % (40/40) | 20.0 % (8/40) | 20.0 % (8/40) |
| ***Candida parapsilosis*** **(n=22)** | 100.0 % (22/22) | 13.6 % (3/22) | 13.6 % (3/22) |
| ***Candida krusei*** **(n=6)** | 100.0 % (6/6) | 16.6 % (1/6) | 00.0 % (0/6) |
| ***Candida glabrata*** **(n=4)** | 100.0 % (4/4) | 25.0 % (1/4) | 25.0 % (1/4) |
| ***Candida kefyr*** **(n=3)** | 100.0 % (3/3) | 00.0 % (0/3) | 00.0 % (0/3) |
| ***Candida guilliermondii*** **(n=3)** | 100.0 % (3/3) | 100.0 % (3/3) | 00.0 % (0/3) |

**Table.3** Comparison of virulence factor producers in isolates from different clinical samples

| Specimen | Virulence Factor | | |
|---|---|---|---|
| | **Haemolysin** | **Phospholipase** | **Caseinase** |
| Urine (49) | 49 | 18 | 14 |
| Blood (38) | 38 | 09 | 10 |
| HVS (19) | 19 | 12 | 03 |
| Pus (7) | 07 | 03 | 05 |
| Vault (3) | 03 | 00 | 01 |
| Central line (1) | 01 | 00 | 00 |
| Tissue (1) | 01 | 01 | 01 |

**Figure.1** Hemolysin activity on Sabouraud's Dextrose Agar with 3% blood

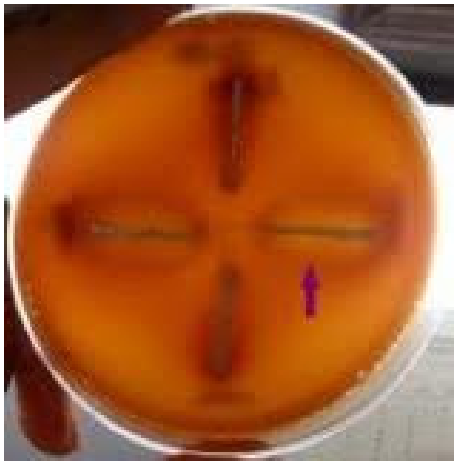

**Figure.2** Phospholipase activity on Sabouraud's Dextrose Agar with egg yolk

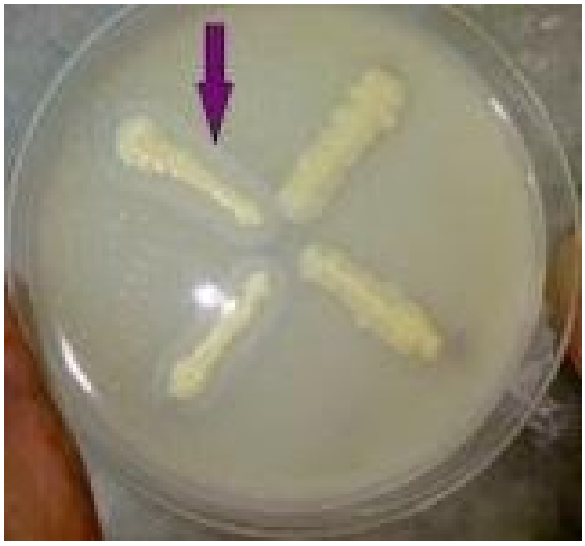

**Figure.3** Caseinase activity on Sabouraud's Dextrose Agar with casein

In the present study, the clinical isolates of *Candida* species (118) consisted of NAC (78) which by far exceeded the *Candida albicans* (40), in concordence with various other studies (Pfaller *et al.,* 1998; Jennifer *et al.,* 2008).

In candida the transition from commensalism to pathogenicity, is attributed to the selective expression of different virulence factors that act synergistically under favourable conditions. The type, stage and infection site in addition to the immune response, determine which virulence factors are expressed. Among these virulence factors haemolytic, lipolytic or proteolytic activity seems to play a major role in the pathogenicity of these microorganisms. Not only the absence or presence of a virulence factor but when present the amount of its activity is also an important factor contributing to pathogenicity. In the present study all the isolates of candida were haemolysin producers, although in variable amounts.

*Candida albicans* is a stronger haemolysin producer (mean Hz value 0.274) compared to NAC species (mean Hz value 0.338). Hundred percent haemolysin producers have

also been reported in other studies (Nachimuthu *et al.,* 2011). The percentage of phospholipase and caseinase producers was significantly higher in *Candida albicans* compared to percentage of NAC isolates. Other studies have also similarly reported higher number of producers of these two enzymes in *Candida albicans* compared to producers in NAC species (Nachimuthu *et al.,* 2011; Ozkan *et al.,* 2005). Although the phospholipase and caseinase producing NAC isolates were less in number; the amount of activity observed in NAC was found to be significantly stronger (mean Pz value 0.067 and Cz value 0.051) compared to *Candida albicans* (mean Pz value 0.223 and mean Cz value 0.089).

Research on prevalent *Candida* species along with their virulence factors in a given set up would be an important tool to prove the relation between the infective species of *Candida* and infection.

This changing trend of causative role of *Candida* in different studies from different parts of the world and from India and the emergence of NAC species and their association with virulence factors cannot be overlooked (Jennifer *et al.,* 2008). Hemolysin appears to be associated with pathogenicity in all clinical conditions caused by candida species. Phospholipase was associated with significantly more number of isolates from HVS isolates, compared to caseinase. Both phospholipase and caseinase were found to be more associated with isolates from pus and urine, compared to less number of blood isolates and were absent in central line isolate. Thus their action of degrading cell membrane appears to be important in case of tissues or epithelial cells but not so on endothelial lining.

In conclusion, reports of rising NAC emergence may be the result of increased isolation and complete identification of *Candida* species in more microbiology laboratories. More multi-locational studies on larger sample size will definitely go a long way in revealing epidemiology, emergence and spread of NAC.

Presence or absence of the virulence factors and when present the amount produced, decides the degree of pathogenicity of *Candida* species. Bringing detection of virulence factor in routine practice might help in better understanding of behaviour of these organisms in a given set up and also open doorways to better management and prognosis of the patients. The changing patterns of *Candida* isolated from various clinical samples has made, identification of *Candida* species producing virulence factors an important procedure for any diagnostic microbiology service.

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