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*Full Length Research Paper*

# *In vitro* evaluation of virulence factors of *Candida* species isolated from oral cavity

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**The yeast *Candida* is a normal flora of the skin and the mucous membrane and it then becomes pathogen in immunocompromised people. Various virulence factors are contributing to establishment of the infection in the host. Adherence of the pathogen to host tissues, yeast-hyphal transition and extracellular hydrolytic enzymes secretion are important virulence factors of *Candida* species. These hydrolytic enzymes play important roles in pathogenicity of *Candida* infection. The present study was conducted with an aim to determine in *vitro* phospholipase, proteinase, haemolysin, esterase activities and biofilm formation in oral *Candida* isolates. A total of 38 *Candida* species were isolated from oral cavity of patients with symptoms of oral candidiasis. The specimens were identified by standard mycological techniques up to species level and were investigated for production of hydrolytic enzymes and biofilm formation. Phospholipase activity was in 52.6% of isolates, 86.8% produced proteinase and haemolysin activity was seen in 63.1%, esterase activity was demonstrated in 50% of isolates, 78.9% of *Candida* isolates showed biofilm formation. *Candida albicans* showed more extracellular hydrolytic enzyme activity, whereas, *Candida tropicalis* showed more biofilm formation. Both the C. *albicans* and Non-*albicans Candida* (NAC) species are capable of producing extracellular hydrolytic enzymes and biofilm formation.**

**Key words:** *Candida* species, virulence factors, extracellular hydrolytic enzymes, biofilm formation.

## INTRODUCTION

The dimorphic fungus *Candida* sp. can respond rapidly to environmental changes, and this flexibility could allow this organism to take advantage of impaired immunity and facilitate establishment of disease. Although *Candida* is normal flora of skin and mucous membranes of healthy people, they cause infections that range from superficial infections to life-threatening systemic infections in immuno-compromised and immunosuppresed people. Various virulence factors are contributing to the colonization and pathogenicity of *Candida* infection, including the expression of adhesins and invasins on the cell surface, yeast-hyphal morphogenetic transformation, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes (Francois et al., 2013).

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Among the various factors, extracellular hydrolytic enzymes of which SAPs (secreted aspartyl proteinases) are considered to be one of the major virulence factors play a major role in over growth of the *Candida*, since these enzymes pave way to adhere, penetrate and for tissue invasion (Schaller et al., 2005).

Aspartyl proteinases are secreted by pathogenic species of *Candida in vivo* during infection. The enzymes are secreted *in vitro* when the organism is cultured in the presence of exogenous protein (usually bovine serum albumin) as the nitrogen source. Phospholipase enzymes, another important virulence factor, are associated with the function related to host cell damage, adherence and penetration (Kabir et al., 2012). They destroy phosphorlipids in the host cell, hence results in the damage to the cell membrane, cell lysis and facilitating tissue invasion (Bhat et al., 2011). There are four secreted phosphorlipase, A to D (PLA, PLB, PLC and PLD). Their activity is very high during tissue damage because these enzymes carry out hydrolysis of one or more ester linkages of glycerophospholipids on the host cell membrane. Furthermore, *Candida albicans* is able to acquire elemental iron from host tissues through haemolysin production, iron chelators (siderophores) and iron-transport proteins, which then is used by the fungus for metabolism, growth and establishment of infection in humans (Weinberg, 1978; Almeida et al., 2009). The ability of *C. albicans* to utilize hemoglobin as an iron source was first described by Moors et al. (1992). In humans, iron is found in some proteins, including hemoglobin (a component of erythrocytes). The first step of *C. albicans* infection *in vivo* involves binding to erythrocytes through receptors of the complement system. Next, *C. albicans* produces a hemolysis factor that induces lysis of the erythrocyte. This factor most likely corresponds to a mannoprotein bound to the cell surface of the fungus (Almeida et al., 2009; Watanabe et al., 1999). Almeida et al. (2008) observed that *C. albicans* caused greater damage to oral epithelial cells containing elevated concentrations of ferritin as compared to cells with lower iron levels.

Traditionally, antifungal drugs were developed either to inhibit or to kill the pathogenic organism. Because of the development of anti-fungal resistance to various anti-fungal drugs by the pathogen, there is a need to develop new antifungal strategy which specifically targets the virulence factors. The study of virulence factors provides a way to specifically target virulence of *Candida* sp. Therefore, the present study was conducted with an aim to determine *in vitro* phospholipase, proteinase, haemolysin, esterase activities and biofilm formation in oral *Candida* isolates.

## MATERIALS AND METHODS

### Collection of samples

A total of 38 clinical samples were obtained from patients attending Tertiary Care Hospitals, Coimbatore, TamilNadu, India, with symptoms of oral candidiasis. Oral swabs were collected with all aseptic precautions using sterile swabs from tongue and buccal mucosa by gently rubbing over the lesional tissue. The swabs were then dispensed in a test tube containing sterile SDA broth. Then, they were identified by Gram staining, lactophenol cotton blue test, germ tube test, carbohydrate fermentation test, urease test, morphology on HiCHROM agar and corn-meal agar with Tween-80. Culture on *Candida* HiCHROM agar was for the species identification whereas corn-meal agar was for demonstration of chlamydospores.

### Preparation of the yeast suspension

Yeast suspension was prepared from the isolates. A small amount of stock culture was inoculated on Sabouraud dextrose agar (SDA) containing chloramphenicol by using a sterile loop and incubated at 37°C for 24-48 h. Then, the yeasts were harvested and suspended in sterile phosphate buffered solution (PBS) at turbidity equal to optical density (OD) of 0.5 McFarland. The final suspension was adjusted to contain $1 \times 10^7$ yeast cells/ml.

### Determination of phospholipase activity

The extracellular phospholipase activity of *Candida* sp. was determined by growing them on egg yolk agar and measuring the size of zone of precipitation by the method prescribed by Samaranayake et al. (1984). The egg yolk medium was prepared according to Tsang et al. (2007) and Mohandas (2011). A 10 ml suspension of yeast cells per ml saline was placed on the egg yolk medium and left to dry at room temperature. The culture was then incubated at 37°C for 48 h, after which the diameter of the precipitation zone around the colony was determined. Phospholipase activity was measured by dividing colony diameter by the diameter of the precipitation zone (pz) around the colony formed on the plate. The pz was scored as follows: pz = 1, negative phospholipase activity; pz = 0.64-0.99, positive phospholipase activity; and pz ≤ 0.63, very strong phospholipase activity (Price et al., 1982). The lower the pz value, the higher the enzymatic activity.

### Determination of proteinase activity

Extracellular proteinase activity of *Candida* sp. was analyzed in terms of bovine serum albumin (BSA) degradation by the technique described by Staib et al. (1965). To determine proteinase activity, bovine-serum albumin agar (0.1% $KH_2PO_4$, 0.05% $MgSO_4$, 4% agar and 1% bovine serum albumin) was employed (Tsang et al., 2007). The final pH was adjusted to 4.5. Ten microliters of previously prepared yeast suspension was inoculated into the wells punched onto the surface of the medium onto the plates; these were then incubated at 37°C for 10 days in both aerobic and anaerobic conditions. After incubation, the plates were fixed with 20% trichloracetic acid and stained with 1.25% amidoblack. Decolourization was performed with 15% acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the wells that could not be stained with amidoblack, indicated degradation of the protein. The presence of proteinase activity was determined by the formation of a transparent halo around the yeast colonies. The diameter of unstained zones around the well was considered as a measure of proteinase production. The proteinase activity (Prz) was determined in terms of the ratio of the diameter of the well to the diameter of the proteolytic unstained zone. Proteinase activity (Prz) was determined by the method described by Price et al. (1982). Prz was scored as follows: Prz = 1, negative proteinase activity, Prz =

**Table 1.** Various virulence factors exhibited by *Candida* sp.

| Organism | No. of isolates | Phospholipase ($P_z$) activity No. (%) | Proteinase ($Pr_z$) activity No. (%) | Haemolytic ($H_z$) activity No. (%) | Esterase activity No. (%) | Biofilm formation No. (%) |
|---|---|---|---|---|---|---|
| *C. albicans* | 22 | 22 (100) | 20 (90.9) | 14 (63.6) | 13 (59) | 19 (86.3) |
| *C. tropicalis* | 9 | 4 (44.4) | 9 (100) | 7 (77.7) | 3 (33.3) | 8 (88.8) |
| *C. krusei* | 6 | 2 (33.3) | 3 (50) | 2 (33.3) | 2 (33.3) | 2 (33.3) |
| *C. glabrata* | 1 | 1 (100) | 1 (100) | 1 (100) | 1 (100) | 1 (100) |
| Total | 38 | 20 (52.6) | 33 (86.8) | 24 (63.1) | 19 (50) | 30 (78.9) |

0.64-0.99, positive proteinase activity; and Prz ≤ 0.63, very strong proteinase activity. Thus, a low Prz indicated high production of the enzyme.

#### Determination of haemolysin activity

To determine hemolytic activity, SDA (Oxoid) containing 7% sheep blood and 3% glucose with a final pH adjusted to 5.6 ± 0.2 was employed. Ten microliters of yeast suspension was inoculated onto plates; these were then incubated at 37°C for 48 h in aerobic condition. After incubation, a transparent/semitransparent zone around the inoculation site was considered as positive hemolytic activity (Manns et al., 1994). The ratio of the diameter of the colony to that of the translucent zone of haemolysis (mm) was used as the haemolytic index (Hz value).

#### Determination of esterase activity

To determine esterase activity, Tween-80 opacity test medium was used. The test medium with a pH adjusted to 6.8 consisted of 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$ and 1.5% agar. After cooling the medium (50°C), 0.5% of Tween-80 was added. Ten microliters of previously prepared suspension was carefully deposited on the Tween-80 opacity test medium. This was then incubated at 30°C for 10 days in aerobic conditions. Esterase activity was considered as positive in the presence of a halo pervious to light around the inoculation site (Slifkin, 2000).

#### Determination of biofilm formation

*Candida* sp. was evaluated for biofilm formation using the method described by Melek et al. (2012). Sterile 96-well microplates were used to evaluate biofilm formation. Yeast culture was inoculated using a loop into a tube containing 2 ml of brain heart infusion broth (BHIB) medium with glucose (0.25%) and incubated at 37°C for 24 h. Then, all tubes were diluted at a ratio of 1:20 by using freshly prepared BHIB. From this final solution, 200 µL was placed into the microplate, which was then incubated at 37°C for 24 h. After incubation, the microplate was rinsed with PBS 3 times and then inverted to blot. Then 200 µL of 1% crystal violet was added to each well, followed by incubation for 15 min. After incubation, the microplate was again rinsed with PBS 3 times. Then 200 µL of ethanol : acetone mixture (80:20 w/v) was added to each well. They were read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader and the OD was recorded for each well. Three wells were used for biofilm formation and the arithmetical mean of 3 readings was used in analysis. *Enterococcus faecalis* ATCC 29212 was employed as the control strain. Sterile BHIB without microorganism was employed as the negative control. Samples with an OD higher than the cutoff value were considered positive, whereas those with lower value than cutoff were considered negative.

## RESULTS AND DISCUSSION

Of the 38 isolates, 22 isolates (58%) were identified as *C. albicans*, while 9 (24%) were identified as *Candida tropicalis*, 6 (16%) as *Candida krusei*, 1 (2%) as *Candida glabrata* (Table 1). These isolates were studied for the production of hydrolytic enzymes such as phospholipase, proteinase, esterase, haemolytic activity and for the biofilm formation (Figure 1).

Phospholipase activity was found in 20 (52.6%) isolates and positivity for proteinase activity was found in 33 (86.8%) *Candida* isolates. Hemolysin activity was seen in 24 (63.1%) isolates and esterase activity was found in 19 (50%). About 30 (78.9%) isolates gave positive result for biofilm formation. Maximum phospholipase (100%) activity and esterase activity (59%) was seen in *C. albicans* whereas maximum proteinase (100%) activity, haemolysin (77.7%) production and biofilm formation (88.8%) was seen in *C. tropicalis*. *C. krusei* and *C. glabrata* also showed positive results for all the activities (Table 1).

*C. albicans* is an opportunistic pathogenic micro-organism that has developed several virulence factors facilitating the invasion of host tissues (Schaller et al., 2005). The ability of *Candida* species to persist on mucosal surfaces of healthy individuals is an important factor contributing to its virulence. This is particularly important in the oral cavity, where the organism has to resist the mechanical washing action of a relatively constant flow of saliva toward the esophagus (Sitheeque and Samaranayake, 2003). Various virulence factors contribute to the colonization and pathogenicity of *C. albicans* infection, including the expression of adhesins and invasins on the cell surface (Cannon and Chaffin, 1999), yeast-hyphal morphogenetic transformation, phenotypic switching (Francois et al., 2013), the secretion of hydrolytic enzymes (Schaller et al., 2005), iron acquisition from the environment (Manns et al., 1994), the ability to form biofilm on various surfaces (Williams and Lewis, 2011).

Many different hydrolytic enzymes are identified in

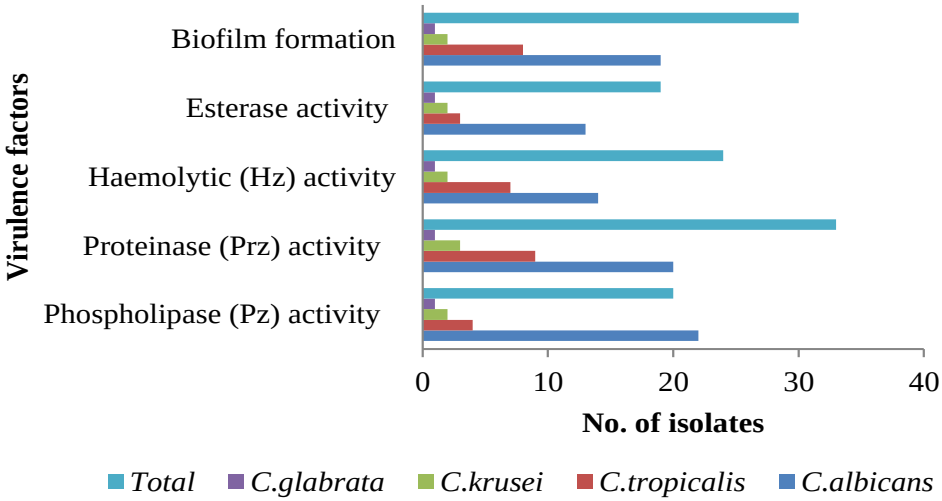


**Figure 1.** Virulence factors produced by different *Candida* sp. isolated from oral cavity.

*Candida* sp. including secreted aspartyl proteinase, phospholipase, lipase and esterase. The production of hydrolytic enzymes helps in colonization of host surfaces, increase adhesion by degrading host surface molecules, allow penetration into host tissues by digesting host cell membranes or evasion of host defense mechanism by digesting cells and molecules of the host immune system hence modulate host immune responses (Calderone et al., 2002).

It has been reported that the enzymatic activity of *Candida* sp. may vary depending on the species and source of isolates (Mohandas and Ballal, 2002). In this study, out of 38 isolates, phospholipase activity was detected in 100% of the *C. albicans*. Tsang et al. (2007) also reported the same positivity rate of phospholipase activity, in samples from patients with oral *Candida* infection. Previous studies have reported phospholipase activity in 30 to 100% of Candidal isolates from various groups of patients and from various sites (Price et al., 1982; Wu et al., 1996). As shown in Table 1, 100% of *C. albicans* produced phospholipase, among the NAC species, *C. tropicalis* followed by *C. krusei* showed maximum phospholipase production. Phospholipase enzyme digests the host cell membrane phospholipid causing cell lysis and changes in the surface features that enhance adherence and consequent infection and hence phospholipase production may be used as one of the parameters to distinguish virulent invasive strains from non-invasive colonisers. About 90.9% of *C. albicans* showed proteolytic activity in the present study. *C. tropicalis* showed 100% proteolytic activity followed by *C. krusei.* This observation was similar to the reports given by previous workers (Marcos-arias et al., 2011).

It was noted that haemolysin activity was higher in *C. tropicalis* (77.7%) followed by *C. albicans* (63.63%). Manns et al. (1994) defined the condition under which *C. albicans* can display haemolytic activity and found that haemolysis is non-existent when no glucose is available in the culture medium. Rossoni et al. (2013) found that non-*Candida* species also produced same haemolytic activity as *C. albicans. C. albicans* secretes a haemolytic factor that causes the release of haemoglobin, which is then used as an iron source by the organisms. Watanabe et al. (1999) demonstrated that mannoprotein released from *C. albicans* bound to the band 3 protein on RBCs, thereby promoting their disruption. They detected the haemolytic activity in the culture supernatant of C. *albicans in vitro*. In the oral cavity, extracellular iron is bound mainly to lactoferrin, a protein present in saliva, while intracellular iron is stored as ferritin. Although, this element is bound to proteins and/or is present in the cytoplasm of cells, oral infections with *C. albicans* are frequent, suggesting that this yeast is able to take up different forms of iron from the oral cavity (Almeida et al., 2008).

About 59% of *C. albicans* expressed esterase activity (Pakshir et al., 2013; Aktas et al., 2002). It has been reported that both *C. albicans* and non-*albicans Candida* sp. express esterase activity. Rudek et al. (1978) demonstrated that esterase activity would appear to be a common feature of *Candida* species that are frequently isolated from clinical specimens. Kumar et al. (2006) reported that Tween 80 opacity test cannot be used as the sole phenotypic trait in the differentiation of *C. albicans* and *C. dubiliancis* though it appears to be simple, economical and easy method to perform for use in small clinical laboratories. Melak et al. (2012) detected that C. *albicans* showed esterase activity in aerobic conditions but not in anaerobic conditions.

Biofilm formation is one of the most important virulence factors of *Candida* sp. (Figure 1). *Candida* biofilms occur on tissue surfaces as well as the biomaterials of medical devices. As reported by Gultekin et al. (2011), no biofilm formation was detected in any *C. albicans* strains by

microplate method, while it was found in 50% of non-*albicans Candida* sp. Demirbilek et al. (2007) also detected that the biofilm formation rate was higher in non-*albicans Candida* sp. than in *C. albicans* strains by the microplate method. In our study, the biofilm formation rates were found to be higher in *C. tropicalis* (88.8%) than *C. albicans* (86.3%). According to the above mentioned studies, the biofilm formation rate was higher in non-*albicans Candida* species as compared to *C. albicans* isolates.

## Conclusion

It is necessary to understand the pathogenicity mechanisms of the *Candida* sp. for the development of new antifungal strategy. Developing anti-fungal therapies against selective target virulence factor is very crucial nowadays because of the multi-drug resistance developed by *Candida* sp. Hence, our study on virulence factors of *Candida* sp. pave way for the better understanding of the various virulence factors exhibited by *Candida* sp.

## Conflict of interests

The authors did not declare any conflict of interest.

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