

***In vitro* evaluation of proteinase, phospholipase and haemolysin activities of *Candida* species isolated from clinical specimens**

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ABSTRACT

Background: Adherence to host tissues, morphological changes and secretion of extracellular hydrolytic enzymes are important virulence attributes of *Candida* species. These enzymes play pivotal roles in pathogenicity of candida infection. **Aim:** The present study aimed to determine phospholipase, proteinase and haemolysin activities in *Candida* species isolated from various clinical samples. **Material and Method:** A total of 110 *Candida* species isolated from various clinical specimens were identified up to species level by standard mycological techniques and were tested for extracellular hydrolytic enzyme activity. **Results:** Phospholipase activity was in 60.9% of isolates, 59.1% produced proteinase and haemolysin activity was demonstrated seen in 51.8% of *Candida* isolates. Maximum strains of *Candida albicans* produced extracellular hydrolytic enzymes. Among Non-*albicans* *Candida* (NAC) species, phospholipase and proteinase activity was higher in *C. tropicalis* whereas, haemolysin production was more in *C. dubliniensis*. **Conclusion:** From the present study it can be concluded, that both *C. albicans* and NAC species produce of extracellular hydrolytic enzymes. Since these enzymes are important to understand the co-relation between the species and infection their detection is extremely important.

Key words: Extracellular hydrolytic enzymes, phospholipase, proteinase, haemolysin, non-*albicans* *Candida* species

INTRODUCTION

The incidence of invasive fungal infection has increased steadily over the past two decades.^[1] With recent advances in medical and surgical intervention and increasing population of immunocompromised patients, the diversity and list of fungal pathogens continue to grow.^[2] The yeasts of the genus *Candida* are opportunistically invasive in individuals whose defense mechanisms are impaired and they cause diseases ranging from superficial mycoses to disseminated and often fatal infections.^[3,4] The individuals at risk include intensive care and

postsurgical patients; human immunodeficiency virus (HIV) infected hosts, patients with hematological malignancies, elderly patients and premature infants.^[3,4]

Extracellular hydrolytic enzymes seem to play an important role in candidal overgrowth, as these enzymes facilitate adherence and tissue penetration and hence invasion of the host.^[5] Among the most important hydrolytic enzymes produced by *Candida* are phospholipases and secreted aspartyl proteinases (Saps).^[6] Haemolysin is another putative virulence factor thought to contribute to candidal pathogenesis. In

particular, the secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis.^[6]

Most of the studies on extracellular hydrolytic enzymes of *Candida* are focused on *Candida albicans*.^[7,8] Information on the extracellular enzymes of other medically important *Candida* species is limited.^[9,10] Although *C. albicans* is the most frequently isolated yeast associated with human infection, changing patterns of the *Candida* species detected among clinical isolates in the last decade are evident.^[9,11] Reports from the studies have postulated the significant contribution of the widespread use of antifungal drugs to an increased incidence of Non albicans *Candida* (NAC) species.^[10,11] The present study was conducted with an aim to determine *in vitro* phospholipase, proteinase and haemolysin activities in *Candida* species isolated from various clinical samples.

MATERIALS AND METHODS

A total of 110 *Candida* isolates obtained from various clinical specimens like blood, vaginal swab, oral swabs and urine were screened for extracellular enzymes production. Speciation of *Candida* isolates were done by evaluating the germ tube formation, assimilation, and sugar fermentation.^[12] Culture on cornmeal agar was for demonstration of chlamydospores while culture on candid chromagar was for the identification of the species.^[12]

Determination of phospholipase activity

The *Candida* phospholipase activity was detected by measuring the size of precipitation zone after the growth on egg yolk agar.^[13] The egg yolk medium consisted of 13.0 g Sabouraud's dextrose agar (SDA), 11.7 g NaCl, 0.11 g CaCl₂ and 10% sterile egg yolk (all in 184 ml distilled water).^[6,12] First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500 g for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium.^[6] Standard inocula of the test and control *Candida* isolates [5 µl, with 10⁸ yeast cells (ml saline)⁻¹] were deposited onto the egg yolk agar medium and left to dry at room temperature.^[6] Each culture was then incubated at 37°C for 48 h. The assay was conducted in duplicate on three separate occasions for each *Candida* isolate tested.

Phospholipase activity of the isolate was considered positive when a precipitation zone was visible around the colony on the plate.^[14] The value of phospholipase activity (Pz) was measured by the ratio of the diameter of the

colony to the total diameter of the colony plus the precipitation zone.^[14]

A Pz value of 1 denotes no activity, and less than one (Pz < 1) indicated the phospholipase activity. The lower the Pz value, the higher the enzymatic activity.^[14] Reference strains of *C. albicans* (ATCC 10231 and ATCC 24433) served as positive controls.^[6]

Determination of proteinase activity

Extracellular proteinase activity of *Candida* isolates was analyzed in terms of bovine serum albumin (BSA) degradation according to the technique described by Staib *et al.*^[15] In short, an 18 h yeast suspension of 1X10⁶ cells ml⁻¹ was prepared, and 10 µl suspension was inoculated onto a 1% BSA medium plate (dextrose 2%, KH₂PO₄ 0.1%, MgSO₄ 0.05%, agar 2% mixed after cooling to 50°C with 1% BSA solution).^[6] The plate was incubated at 37 °C for 5 days. The plates were then fixed with 20% trichloroacetic acid and stained with 1.25% amidoblack. Acetic acid was used for decolourisation. Degradation of the protein was seen as opaqueness of the agar, corresponding to a zone of proteolysis around the colony which could not be stained with amidoblack. The assay was done in duplicate on three different occasions for each *Candida* isolate tested.

Proteinase activity (Pr_z) was determined as the ratio of the colony to the diameter of the proteolytic unstained zone. A Pr_z value of 1 signifies no activity, and less than one (Pr_z<1) means proteinase activity. The lower the Pr_z value, the higher the enzymatic activity. Reference strains of *C. albicans* (ATCC 10231 and ATCC 10261) served as positive controls.

Determination of haemolysin activity

Haemolysin activity was evaluated with a blood plate assay as described by Manns *et al.*^[16] Media were prepared by adding 7 ml fresh sheep blood to 100 ml SDA supplemented with glucose at a final concentration of 3% (w/v).^[16] A standard inoculum of both the test and the control *Candida* isolates [10 µl, with 10⁸ yeast cells (ml saline)⁻¹] was deposited onto the medium.^[6] The plate was then incubated at 37°C in 5% CO₂ for 48 h.

The ratio of the diameter of the colony to that of the translucent zone of haemolysis (in mm) was used as the haemolytic index (H_z value) to represent the extent of haemolysin activity by different *Candida* isolates.^[6] The assay was conducted in duplicate on three separate occasions for each yeast isolate tested.^[6] A reference strain of *C. albicans* (ATCC 90028) served as a positive control.^[6] In addition, one

strain each of *Streptococcus pyogenes* (Lancefield group A) and *Streptococcus sanguis*, which induce beta and alpha haemolysis, were used as positive controls.^[6]

RESULTS

The present study aimed at determining *in vitro* phospholipase, proteinase and haemolysin activities in 110 *Candida* isolates. The distributions of the isolates are shown in table 1. Positivity of phospholipase activity was found in 67 (60.9%) and positivity for proteinase activity in

65 (59.1%) *Candida* isolates. Haemolysin activity was seen 57 (51.8%) isolates. Maximum phospholipase (92.3%), proteinase (82.1%) and haemolysin (94.8%) production was seen in *C.albicans*. Among non-albicans *Candida* (NAC) species, maximum activity of phospholipase was seen in *C.tropicalis* (76%) followed by *C.dubliniensis* (40%). Proteinase activity among NAC species was more in *C.tropicalis* (80%) followed by *C.glabrata* (42.8%). Haemolysin production was more in *C.dubliniensis* (60%). Haemolysin production was not noted in *C.kefyr* isolates (Table 1).

Table 1: Phospholipase, proteinase and haemolytic activity of *Candida* isolates

Species	No. of isolates	Phospholipase activity No. (%)	Proteinase activity No. (%)	Haemolytic activity No.(%)
<i>C.albicans</i>	39	36 (92.3)	32 (82.1)	37 (94.8)
<i>C.tropicalis</i>	25	19 (76)	20 (80)	12 (48)
<i>C.glabrata</i>	14	04 (28.5)	06 (42.8)	03 (21.4)
<i>C.krusei</i>	13	02 (15.3)	02 (15.3)	01 (7.6)
<i>C.guilliermondii</i>	08	03 (37.5)	02 (25)	01 (12.5)
<i>C.kefyr</i>	06	01 (16.6)	01 (16.6)	00
<i>C.dubliniensis</i>	05	02 (40)	02 (40)	03 (60)
Total	110	67 (60.9)	65 (59.1)	57 (51.8)

DISCUSSION

Fungi, once considered microbiological curiosities without pathogenic potential, have emerged as important opportunistic human pathogens.^[15] Opportunistic fungal pathogens are becoming increasingly important cause of community acquired and nosocomial infections. The yeasts of the genus *Candida* are the most important opportunistic fungal pathogens associated with the rising incidence in immunocompromised individuals.^[16]

The pathogenicity of *Candida* depends on several putative virulence factors, including germination, adherence to host cells, phenotypic switching and production of extracellular hydrolytic enzymes.^[17] Among these virulence attributes of *Candida* species, the present study targeted extracellular phospholipase, proteinase and haemolysin activities in *Candida* species isolated from various clinical samples.

Out of 110 *Candida* species studied phospholipase activity was seen in 67 (60.9%) isolates. Kantarcioglu and Yucel^[18] also reported the same positivity rate of phospholipase activity, in samples from patients with invasive *Candida*

infection. Previous studies have reported phospholipase activity in 30 to 100% of *Candida* isolates.^[6,19,20] As shown in table 1, 92.3% of *C.albicans* produced phospholipase, among the NAC species *C.tropicalis* followed by *C.dubliniensis* showed maximum phospholipase production. Thangam *et al.*^[20] also reported high phospholipase activity in *C.tropicalis*. 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.

In the present study, the positivity for proteinase production was found in 65(59.1%) of *Candida* isolates. Maximum proteinase production was seen in *C.albicans* (82.1%), followed by *C.tropicalis* (80%), which is similar to the observation of Ruchel *et al.*^[21] High rates of proteinase production in *C.albicans* have been reported by other workers.^[6,7,22] Complement C₃ and some specific endogenous proteinase inhibitors involved in regulating inflammation can be also by *C.albicans* proteinase.^[17]

In our study, 42.8% *C.glabrata* isolates produced proteinase, although little is known of proteinase production by *C.glabrata*, a single study has shown that isolates of *C.glabrata* are at least capable of proteinase production.^[23] However, the high mortality and rapidity of the spread of the disease would argue the contrary; only few studies have been conducted on virulence of *C.glabrata*.^[9]

Studies on the activity of haemolysin in *Candida* are limited. We noted that haemolysin activity was higher in *C.albicans* (94.8%) and *C.dubliniensis* (60%). Manns et al^[14] defined the condition under which *C.albicans* can display haemolytic activity, but found that haemolysis is non-existent when no glucose is available in the culture medium. On the other hand, Luo et al^[24] have tested 80 *Candida* isolates from clinical sources in different geographical locales and detected only alpha, and no beta haemolysis in experiments with glucose-free sheep blood agar.

In conclusion, the keen interest in *Candida* species is a reflection on the incidence of *Candida* infections. Species of *Candida* other than *C.albicans* which were previously considered as less or non virulent are now implicated in human disease. In our study extracellular hydrolytic enzyme activities was shown by *C.albicans* and NAC species including *C.glabrata*. Detection of virulence factors like phospholipase, proteinase and haemolysin will help to provide sufficient data in the establishment of a relationship between the infection and *Candida* species isolated.

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