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Role of Phospholipase and Proteinase as Virulence Factors of *Candida Albicans* Isolated from Clinical Samples of Inpatients in a Tertiary Care Hospital

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ABSTRACT

Multiple characteristics of Candida albicans have been proposed to be virulence factors that enable the organism to cause hematogenously disseminated infections in susceptible hosts. The phospholipases, in general, catalyse the hydrolysis of phospholipids which are the major components of cell membrane. Secreted aspartyl proteinases are capable of degrading epithelial and mucosal barrier proteins such as collagen, keratin and mucin, as well as antibodies, complement and cytokines. The objective of this study is to determine invitro phospholipase and proteinase activities as virulence factors of C. albicans isolated from various clinical samples. In the present study, the clinical samples from the respiratory tract (Bronchial wash, Tracheal Aspirates), Urine and Blood were collected and cultured. The isolates of C. albicans were identified. The phospholipase activity was carried out in egg yolk agar medium. The proteinase produced in Bovine serum albumin medium was estimated and the size of the zone of precipitation as Pz value was measured. A total of 64 C. albicans strains isolated were tested for phospholipase and proteinase activities. Fifty nine of 64 C. albicans isolates (92.2%) produced phospholipase and 50 isolates (78.1%) produced proteinase. The ATCC C. albicans 10231 strain was also positive. Analysis of the results obtained suggests that the capacity of C. albicans to produce phospholipase and proteinase may contribute to fungal virulence associated with invasive mycoses

KEY WORDS: Candida albicans, Phospholipase, Proteinase

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INTRODUCTION

Pathogenic fungi in the genus Candida cause a wide spectrum of diseases. These ranges from superficial mucocutaneous infections to invasive diseases involving internal organs as well as bloodstream infections. Technological advances in medicine have created new opportunities for Candida to gain access to the circulation and deep tissues. Candida species are emerging as an important cause of hospital-acquired (nosocomial) infections. Critically ill patients present a prime target for candidiasis as do immunosuppressed patients and those with indwelling medical devices [1]

Multiple characteristics of Candida albicans have been proposed to be virulence factors that enable the organism to cause hematogenously disseminated infections in susceptible hosts. The ability to recognize and adherence to host tissues, to respond rapidly to changes in the external environment and to secrete enzymes are all thought to be important in virulence. These putative virulence factors include adhesion, hyphal formation, phenotypic diversity and production of extracellular hydrolytic enzymes, such as Phospholipases, lipases and aspartyl proteinases (Saps) [2]

The secretion of extracellular phospholipases is considered a key attribute that aids invasion of the host mucosal epithelia. The phospholipases, in general, catalyse the hydrolysis of phospholipids which are major components of all cell membranes. Four types of phospholipases have been reported in C. abacas including phospholipase A, B, C, and D. The extracellular phospholipases of C. albicans have a significant role in the pathogenesis of infections and invasion to mucosal epithelia.[3] In addition, several studies have shown that clinical isolates of C. albicans have higher levels of extracellular phospholipase activity.[4]

Secreted aspartyl proteinases are capable of degrading epithelial and mucosal barrier proteins such as collagen, keratin and mucin, as well as antibodies, complement and cytokines. Cloning and disruption of the genes for these enzymes have shown their involvement in Candida virulence.[5, 6]

Due to the increased incidence of invasive infections by C. albicans, interest in the study of virulence factors of these species has intensified, including the production of hydrolytic enzymes, to establish strategies for the prevention and control of candidiasis and as possible targets for the development of new therapeutic interventions. Thus, the purpose of this study was to investigate the in

vitro activity of exoenzymes (Phospholipases, Acid proteases) in clinical isolates of Candida albicans, isolated from several anatomic sites.

AIMS AND OBJECTIVES

The objective of this study is to determine invitro phospholipase and protease activities as virulence factors of Candida albicans isolated from various clinical samples.

MATERIALS AND METHODS

Isolation and identification

The isolates of Candida albicans used in this study came from cultures of hospitalized patients suspected of being infected by microorganisms. Sixty four C. albicans isolates from various climical samples from hospitalized patients were studied. The collected clinical samples were streaked on Sabouraud's Dextrose Agar (SDA) and incubated at 37 ˚C for 24-48 hours. Isolates were identified and stored in the Department of Microbiology. All isolates were transferred onto fresh Sabouraud's dextrose agar plates and incubated at 37°C for 24h. Then isolates were re-identified by colony morphology, [7] germ tube formation, chlamydospore formation on Corn-meal Agar, sugar fermentation and assimilation patterns and pale pink colonies of Candida albicans on Tetrazolium Reduction Medium (TRM).

Suspension preparation

Each isolate was inoculated onto test tubes contained 10ml SDA and incubated at 37°C for 18h shaking. Each tube centrifuged for30min and the sediment was washed by PBS for 30min. Supernatant was removed and sediment was re-suspended in sterile distilled water. A suspension with turbidity according to the McFarland standard #2 of yeast cells was prepared in distilled water[8]

Estimation of Phospholipase Activity

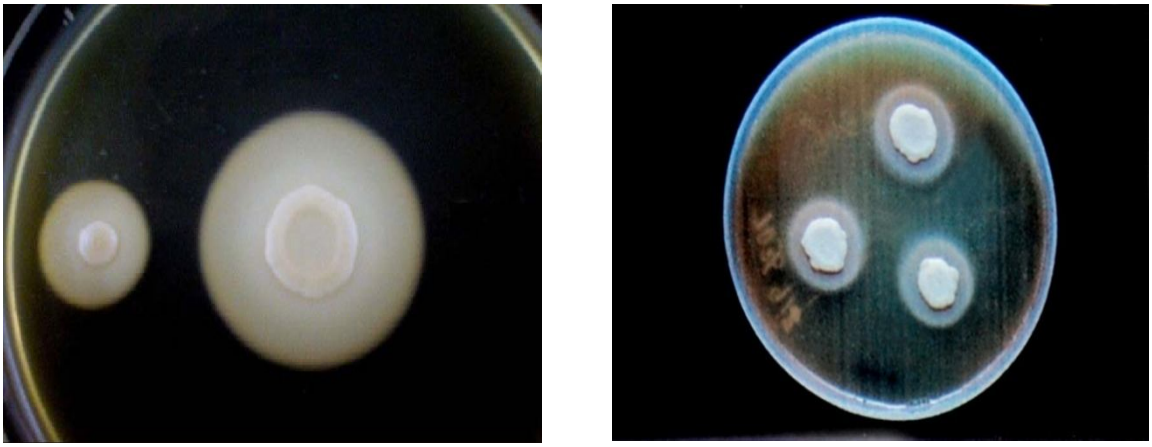
The isolates were screened for their extra cellular phospholipase activity by growing them on egg-yolk agar and measuring the size of the zone of precipitation by the slightly modified method of Samaranayake et al.The egg-yolk medium consisted of 13.0g Sabouraud's dextrose agar (SDA), 11.7g sodium chloride, 0.11g Calcium chloride and 10% sterile egg yolk (all in 184ml distilled water). First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500g for 10 minute at room temperature and 20ml of the supernatant was added to the sterilized

medium. Extracellular phospholipase activity was detected by inoculating 10 µl of yeast suspension onto the surface of the egg-yolk medium and left to dry at room temperature.[9] Control Candida albicans and each test strain were inoculated in Triplicate. Each culture was incubated at 37˚C for 5-6 days.

Calculation of the zone of phospholipase activity was performed according to Price et al.[8] method. Phospholipase activity was measured by dividing colony diameter by the diameter of precipitation zone (Pz) around the colony formed on the plate.

$$\frac{\text{Colony Diameter}}{\text{Colony Diameter + Zone of precipitation}} = Pz$$

Five classes were described for phospholipase activity including; Pz value = 1 means that the test strain is negative for phospholipase (-), while a value of Pz <0.90- 0.99 = weak phospholipase activity (+), 0.80-0.89 = mild phospholipase activity (++), 0.70-0.79= moderate phospholipase activity (+++) and <0.69 = strong phospholipase activity (++++).The ATCC Candida albicans 10231 strain was used as control. Thus, again, a low Pz indicated high production of the enzyme.

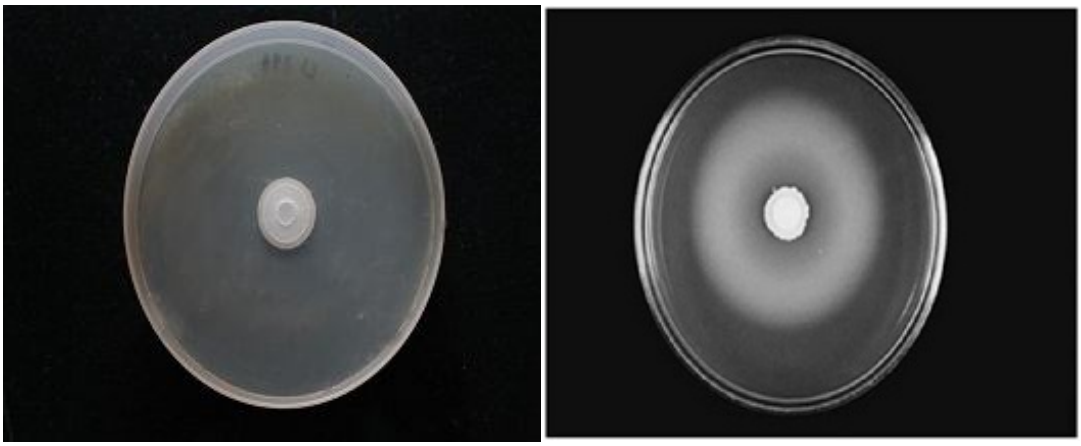


Low Pz group (Pz = 0.625) **High Pz group (Pz = 0.842)**

Fig 1: Phospholipase activity of C. albicans showing low and high Pz value

Estimation of Proteinase Activity

Candida proteinase was detected by the slightly modified staib method(1965)[10] using Bovine Serum Albumin medium (Dextrose 2%,Dihydrogen potassium phosphate(KH2Po4) 0.1%, Magnesium sulphate(MgSo4) 0.05%, Agar 2% mixed after cooling to 50˚c with 1% bovine serum albumin solution). Proteinase activity was detected by inoculating 10µl of yeast suspension onto the surface of the sterile paper disk placed on the surface of bovine serum albumin agar medium (pH 5.0). The plates were incubated at 37˚C for 6days. The plates were observed each day for an increasing opacity around the disks caused by growing Candida. Subsequently, clearing of the opacity by hydrolysis of precipitated albumin was affected by acid protease of the Candida; this was recorded. The millimetric zone measurement were evaluated as negative (-) for no clearance, 1+ for mild activity (a lysis zone 1-2mm around the zone), and 2+ for strong activity (a lysis zone of 3 – 5mm around the disk), 3+ for very strong activity (a lysis zone of <6mm around the disk). The ATCC Candida albicans 10231 strain was used as control.



Opacity around the disc **Clearing by Proteinase**

Fig 2: Proteinase activity of C. albicans shown by clearing of the opacity around the disc

RESULTS

A total of 64 Candida albicans isolated from different anatomical sites of hospitalized patients were tested for phospholipase and proteinase activities in this study. The distribution of Candida albicans isolates according to their sources is shown on Table 1.

TABLE 1: Distribution of isolated C. albicans strains according to the clinical sample

Clinical sample	Candida albicans	
	Number	**%**
Urine	42	65.6
Tracheal Aspirate	12	18.8
BAL	5	7.8
Blood	5	7.8
Total	64	100.0

BAL – Bronchoalveolar lavage

Fifty nine of 64 C. albicans isolates (92.2%) produced phospholipase and 50 isolates (78.1%) produced protease as shown on Table 2. The ATCC Candida albicans 10231 strain was also positive.

TABLE 2: Phospholipase and acid Proteinase activity of isolated C. albicans according to the site of origin.

Species	Origin site	Phospholipase					Proteinase				
		Positive	**%**	**Negative**	**%**	**Total**	**Positive**	**%**	**Negative**	**%**	**Total**
Candida albicans	Urine	40	95.2	2	4.8	42	34	81	8	19	42
	Tracheal Aspirate	9	75	3	25	12	9	75	3	25	12
	BAL	5	100.0	0	0.0	5	4	80	1	20	5
	Blood	5	100.0	0	0.0	5	3	60	2	40	5
	Total	59	92.2	5	7.8	64	50	78.1	14	21.9	64

The isolates tested demonstrated varying degrees of phospholipase activity (Pz value: 0.4-0.8) and protease activity (Pz value: 0.62-0.86) with most significant enzymatic activity. The distribution of the Pz values (mm) among the 64 isolates of Candida albicans analyzed is presented in Table 3.

TABLE 3: Phospholipase and Proteinase production by candida albicans

S. No	Candida species	Number of Isolates	PZ values for Phospholipase production					PZ values for Proteinase production				
			4+	3+	2+	1+	0	4+	3+	2+	1+	0
1	Candida albicans	64	45	8	5	1	5	33	9	6	2	14

The C. albicans isolates that showed enzymatic activity were considered to have very strong activity (++++), both regarding phospholipase activity (70.3%) and protease activity (51.6%) as shown on Table: 4

TABLE 4: Distribution of the Pz* value among the isolates of Candida albicans.

Pz Value	Result	Candida albicans	
		Phospholipase	Proteinase
< 0.69	++++	45 (70.3%)	33 (51.5%)
0.7 – 0.79	+++	8 (12.5%)	9 (14.1%)
0.8 – 0.89	++	5 (7.8%)	6 (9.4%)
0.9 – 0.99	+	1 (1.6%)	2 (3.1%)
1.0	Negative	5 (7.8%)	14 (21.9%)
* 0 – Negative,1+ Weak Positive, 2+ Moderate Positive, 3+ Strong Positive, 4+ Very Strong positive			

DISCUSSION

Candida is an asexual, diploid, dimorphic fungus that is present on humans and in their environment. These organisms are capable of causing a variety of superficial and deep-seated mycoses such as cutaneous, mucocutaneous, subcutaneous, or systemic candidiasis. Candida species are pervasive pathogens capable of causing both local and systemic, acute and chronic infections in hospitalized patients. Candidiasis has emerged as an alarming opportunistic disease as there is an increase in number of patients who are immunocompromised, aged, receiving prolonged antibacterial and aggressive cancer chemotherapy or undergoing invasive surgical procedures and organ transplantation.

Phospholipase and proteinase activities are considered to play important roles in the pathogenesis of opportunistic fungi. The roles of these two hydrolytic enzymes in C. albicans and other yeast species seem to be related to species virulence.[10] The present study aimed to determine in vitro phospholipase and proteinase activities in 64 Candida isolates, which were collected from several anatomically distinct sites of hospitalized patients.

There are a number of publications available examining phospholipase and protease production in C. albicans isolates from various sources [4, 11, 12] and on their role in the pathogenesis of invasive candidiasis. Pinto et al.[13] reported 99.4% of isolates of C.albicans with phospholipase activity. Samaranayake et al.[14] found that 73% of the isolates they studied proved to be phospholipase positive however; Price et al.[15] observed a lower degree of positivity among their isolates. They observed 55% of blood isolates, 50% of wound isolates, and 30% of urine isolates to be phospholipase positive. In the present study, 100% of blood and BAL isolates, 95.2% of urine isolates and 75% of tracheal aspirate isolates of C. albicans had detectable phospholipase activity (Table 2). These discrepancies in percentages of positivity may be considered as being relevant to the amounts of the different species tested together. Borst and Fluit[16] also found difference phospholipase activity between samples originated from urine, blood and wound. He believed that virulence factors could be associated with geographical region and infection type.

Among the virulence factors of C. albicans, the production of acid proteinases has been extensively studied in view of its important role in the pathogenesis of candidiasis. The role of secreted aspartic proteases of C. albicans in experimental and clinical candidiasis has been demonstrated. The enzymatic production of C. albicans and other species isolated from different clinical conditions and

anatomical sites was studied and variations of 62.5-100% for proteinase activity were determined. [14,17,18,19] In the present study, 81% of urine isolates, 80% of BAL isolates, 75% of tracheal aspirate isolates and 60% of blood isolates of C. albicans had detectable proteinase activity (Table 2).

CONCLUSION

In the present study, 92.2% and 78.1% of clinical isolates of C. albicans from various samples demonstrated phospholipase and proteinase activities which may contribute to fungal virulence associated with invasive mycoses. Analysis of the results obtained indicates differences in phospholipase and acid proteinase production by C. albicans isolates from different sources. This study suggests that the pathogenicity of Candida might be related to the site of infection. Early detection of virulence factors by Candida is useful in clinical decision making.

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