Original Article

Detection and scoring of Phospholipase and Proteinase activity among the clinical isolates of *Candida*

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Abstract

Background: Extracellular enzymes phospholipase and proteinases produced by *Candida spp* perform a major part in cell membrane damage. Phospholipases play a significant role in invasion of host tissue by disrupting the epithelial cell membranes and letting the hyphal tip to enter the cytoplasm. Proteinase augments the ability of *candida* to colonise and penetrate host tissues, and also aid in evading the host's immune system. **Aim:** To determining the in *vitro* activity of phospholipase and proteinase in the candida spp isolated from the various clinical samples. **Materials and Methods:** Phospholipase production by the *Candida* was assayed using plate method .Test strains were spot inoculated (~6 mm) on the egg yolk agar and plates were incubated at 37°C for 48 hours up to 5 days. Diameters of colony and total diameters of colonies and precipitation zones together were measured. Proteinase production by *Candida* isolates was assayed using 0.2% Bovine Serum Albumin agar plates. Proteinase activity was determined by the ratio of the diameter of the clear zone to the diameter of the colony. **Results**: Out of 93 strains studied 38 (40.8%) and 21(22.6%) had PL and SAP activity respectively. A total of 46 (49.5%) had PL, SAP or both. Both PL and SAP was seen in 12 (12.9%). PL and SAP alone was seen in 26(27.9%) and 9 (9.7%) respectively. Among the 83 *C.tropicalis* isolates 32 (38.5%) and 23(27.7%) showed Phospholipase and Proteinase activity respectively. Of the 9 *candida* albicans 7(77.8%) showed phospholipase and 3 (33.3%) showed proteinase activity. Whereas *C.parapsilosis* did not have either phospholipase or proteinase activity. **Conclusion:** Increased incidence of *candidal* infections mainly in immunocompromised patients makes compulsory to know the virulence factors contributing to the pathogenicity of the diseases. Detection of phospholipase and proteinase activity among the isolates *candida* spp gives a clue in their part of relevant to infection.

Key words: *Candida albicans, Candida tropicalis*, Proteinase and Phospholipase

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INTRODUCTION

Candida infections are increasing recently due to various subsidising factors like use of higher class of antibiotics, immunosuppressive drugs, increasing incidence of immunodeficiency diseases, diabetes mellitus etc. The virulence factors of *candida* contributing for pathogenicity are adherence, dimorphism, cell surface composition, toxin and enzyme production (1). Extracellular enzymes phospholipase and proteinases produced by *candida* spp perform a major part in cell membrane damage. Phospholipases play a significant role in invasion of host tissue by disrupting the epithelial cell membranes and letting the hyphal tip to enter the cytoplasm. Proteinase augments the ability of *candida* to colonise and penetrate host tissues, and also aid in evading the host's immune system (2). The present study is aimed in determining the in *vitro* activity of phospholipase and proteinase in the *candida* spp isolated from the various clinical samples.

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MATERIALS AND METHODS

A cross sectional study was conducted between December 2010 and May 2011 after procuring approval from the institutional ethics committee in the Department of Microbiology, Sri Ramachandra Medical College and Research Institute Hospital, Porur, Chennai, a tertiary care centre with more than 1900 inpatient bed facility. A total of 93 clinically significant *candida spp* isolated from the clinical samples during the study period were included in the study. Repetitive specimen of the same patient were excluded from the study. **Table /Fig: 1** shows the breakup of clinical samples and the candida spp isolated. All the samples were subjected to KOH wet mount and Gram stain to identify budding yeast cells with or without pseudo hyphae. Samples were inoculated in Sabouraud's Dextrose Agar (SDA) and incubated at room temperature of 25^{o}C. Once yeast is isolated they were speciated by Germ tube test, colony morphology and hue formed in chromogenic Tetrazolium Reduction Medium (TRM), sugar fermentation and assimilation test.

Phospholipase production by the *Candida* was assayed using plate method (3). SDA plates supplemented with 1M NaCl, 0.0005M CaCl2 and 10% sterile egg yolk emulsion were prepared. Sterile normal saline suspensions of the test isolates were prepared to contain approximately 10^5 yeast cells (blastospores) per ml. Test strains were spot inoculated (~6 mm) on the egg yolk agar and plates were incubated at 37^{o}C for 48 hours up to 5 days. Each isolate was tested in duplicate. Diameters of colony and total diameters of colonies and precipitation zones together were measured. The Precipitation zone (Pz) value representing the ratio of the colony alone to the diameter of the colony plus precipitation zone was determined by the formula: Pz = Diameter of colony/ Diameter of colony plus precipitation zone and scored as follows:

Pz value Score:

1= Negative; 1+ = 0.9 – 1; 2+ = 0.89 – 0.80; 3+ =0.79 – 0.70; 4+ = ≤0.69

- a low Pz value indicates stronger Phospholipase activity.

Proteinase production by *Candida* isolates was assayed using 0.2% Bovine Serum Albumin (BSA) agar plates containing 1.17% yeast carbon base, and 0.01% yeast extract. Sterile normal saline suspensions of the test isolates were prepared containing approximately 10^5 yeast cells (blastospores) per ml. Test strains were spot inoculated (~6 mm) on the BSA agar plates and incubated at 37^{o}C for 5 days. Each isolate was tested in duplicate. Post incubation, plates were stained with 0.5% amido black and the zone of clearance around the colony was recorded. Scoring was carried out by determination of the Proteinase zone (Prz) value. Proteinase activity was determined by the ratio of the diameter of the clear zone to the diameter of the colony.

Scoring was done as follows-

Prz value Score:

1: Negative; 0.9 – 1=1+; 0.89 – 0.80=2+; 0.79 – 0.70=3+; ≤0.69= 4+ accordingly, a low Prz value indicates stronger enzyme activity.

RESULTS

Table 1: Break up of clinical isolates

Specimen	*Candida tropicalis*	*Candida albicans*	*Candida parapsilosis*	Total
Urine	62	8	1	71
Exudates	14	-	-	14
Blood	4	1	-	5
Respiratory	3	-	-	-
Total	**83**	**9**	**1**	**93**

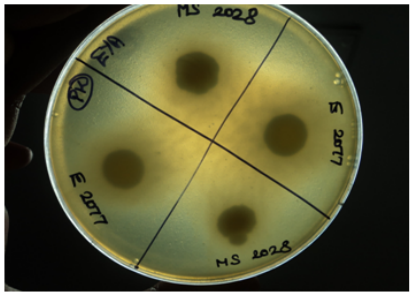

Figure 1

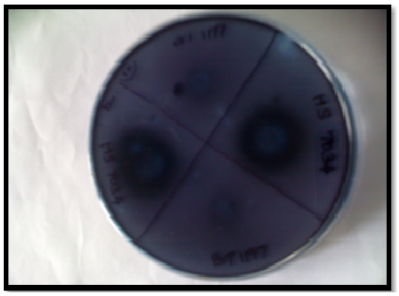

Figure 2

Legend

Figure 1: Phospholipase production in Egg Yolk Agar

Figure 2: Protienase production in Bovine Serum Agar

Table 2: Age wise distribution of the clinical samples (n=93)

Age in years	Number of patients	Percentage
0-10	4	4.3%
11-20	1	1.1%
21-30	11	11.8%
31-40	5	5.4%
41-50	12	12.9%
51-60	27	29%
>60	33	35.5%

Table 3: Phospholipase activity among the *Candida spp*

Candida spp	Scoring of Phospholipase activity(Pz value) No of isolates(%)				
	4+	3+	2+	1+	Negative
C.tropicalis (n=83)	2(2.4)	6(7.2)	8(9.6%)	16(19.3)	51(61.5%)
C.albicans(n=9)	0	1(11%)	2(22.4%)	4(44.4%)	2(22.2%)
C.parapsilosis(n=1)	0	0	0	0	1(100%)
Total	**2**	**7**	**10**	**20**	**54**

Table 4: Proteinase activity among the *Candida spp*

Candida spp	Scoring of Proteinase activity(Prz value) No of isolates(%)				
	4+	3+	2+	1+	Negative
***C.tropicalis* (n=83)**	o	1(1.2%)	3(3.6%)	19(22.9%)	60(72.3%)
C.albicans(n=9)	0	0	0	3(33.3%)	6(66.7%%)
C.parapsilosis(n=1)	0	0	0	0	1(100%)
Total	**0**	**1**	**3**	**22**	**67**

Table 5: Proteinase activity among the *Candida spp*

Candida spp	Phospholipase activity scoring: No of isolates (%)					Proteinase activity scoring : No of isolates(%)				
	4+	3+	2+	1+	Negative	4+	3+	2+	1+	Negative
Urine Isolates										
C.tropicalis(n=62)	2(3.2%)	6(9.7%)	5(8.1%)	9(14.5%)	40(64.5%)	0	0	2(3.2%)	11(17.8%)	49(79%)
C.albicans(n=8)	0	1(12.5%)	2(25%)	3(37.5%)	2(25%)	0	0	0	2(25%)	6(75%)
C.parapsilopsis(n=1)	0	0	0	1(100%)	0	0	0	0	0	1(100%)
Blood										
C.tropicalis(n=4)	0	0	1(25%)	3(75%)	0	0	0	0	2(50%)	2(50%)
C.albicans(n=1)	0	0	0	1(100%)	0	0	0	0	0	1(100%)
Exudate										
C.tropicalis(n=14)	0	0	2(14.3%)	3(21.4%)	9(64.3%)	0	0	1(7.1%)	5(35.8%)	8(57.1%)
Respiratory										
C.tropicalis(n=3)	0	0	0	1(33.3%)	2(66.7%)	0	1(33.3%)	0	0	2(66.7%)

Out of 93 patients 54 were males and 39 were females. Among them 7 were outpatients and the remaining 86 were inpatients. Age wise distribution of clinical sample is shown in Table/Fig: 4. Among the inpatients, majority of the patients with *candidal* infections were from the Medicine ward 36 (41.9%), followed by Surgery ward 30 (34.9%) and Intensive care unit 20 (23.2%). The underlying risk factors for *candidal* infections were studied and a total of 60 (64.5%) patients had indwelling urinary catheters. This was followed by other risk factors such as antibiotic usage in 34 (36.6%), diabetes 33 (35.5%), post-surgery 30(32.3%), mechanical ventilation 20 (21.5%), renal disease 19 (20.4%), steroids and neutropenia 8 (8.6%) each, malignancy (6.5%), and pregnancy (2.2%). A sum of 51 (54.7%) patients had more than two risk factors. Out of 93 strains studied 38 (40.8%) and 21(22.6%) had PL and SAP activity respectively. A total of 46 (49.5%) had PL, SAP or both. Both PL and SAP was seen in 12 (12.9%). PL and SAP alone was seen in 26(27.9%) and 9 (9.7%) respectively. The PL and SAP activity among the *candida* spp is illustrated in the Table/Fig: 5 and Table/Fig: 6.

Relationship of enzyme activity with respect to the clinical specimen is shown in the Table/Fig: 7. Among the 83 *C.tropicalis* isolates 32 (38.5%) and 23(27.7%) showed Phospholipase and Proteinase activity respectively. Of the 9 *candida* albicans 7(77.8%) showed phospholipase and 3 (33.3%) showed proteinase activity. Whereas *C.parapsilosis* did not have either phospholipase or proteinase activity.

DISCUSSION

Recent advances in medical care have made patients more vulnerable to fungal infections. These patients include those on immunosuppressive therapy, prolonged antibiotic therapy, indwelling catheters and patients on assisted ventilation. In the present study, we observed that nosocomial infections due to Candida spp were more common above 50 years of age (64.5%) and also mostly seen in male than females. This could be due to lowered host defences at old age and this finding is supported by the study conducted by Jain M(4)and his colleague's in 2011, where nosocomial candiduria was more in extremes of age i.e., below 14yrs and above 50 years (80%). In our study23.2% of Candida isolated were from patients admitted to various ICUs. This could probably be explained by the fact that patients in ICU are on more than one antibiotic and also have underlying metabolic, respiratory or cardiac disorders which enable various species of Candida to cause secondary or opportunistic infection. Jain M (4) study showed antibiotic usage as a major risk factor contributing to 75.7% cases, followed by post-surgery (52.9%) and diabetes mellitus (38.6%). Virulence attributes of Candida species include adherence to host tissues, morphological changes, and secretion of hydrolases, e.g., phospholipases and proteinases. Secreted aspartyl proteinases (SAPs) of pathogenic Candida spp. have been studied extensively (3, 5 & 6). C. albicans, C. tropicalis, C. dubliniensis, C. guilliermondii, and C. parapsilosis are known to possess SAP gene families. In the present study 22.6% of Candida isolates were proteinase producers. This finding is lower than the 78.9% reported by Kantaricoglu S A (2) and also the 42.2% reported by Shehabi A A(7) in 2004. High proteolytic activity among C.albicans strains have been previously reported (8&9). In a study by Dostal J(10), 100% C. albicans, showed SAP activity. Kumar G C P(11) in 2006 studied proteinase activity in HIV and cancer patients and reported 94.1% of C.albicans and 70.3% of non C.albicans showing proteinase activity. Similarly OksuzS(12) and his colleagues reported proteinase activity in 56.7% of C.albicans. However, in the present study proteinase activity was observed among 27.7% of C.tropicalis and 33.3% of C. albicans but not with C.parapsilosis isolated from urine. Phospholipase activity observed by Kumar G C P(11) was 100% for C.albicans and 29.6% for non-C.albicans. Whereas Oksuz S (12)and his colleagues reported 53.8% phospholipase activity in C.albicans. Whereas in the present study 77.8% of C.albicans and 38.5% of C.tropicalis showed phospholipase activity. Price M F (3) in his study had 55% of blood isolates positive for phospholipase. Borst A and Fluit C A (13) reported 71% of blood isolates to be phospholipase producers. However Kumar G C P (11) had only 45.83% of blood isolates as phospholipase producers. In the present study 100% of blood isolates were phospholipase producers. This was higher than those reported in previous studies.

CONCLUSION

The production of phospholipase as virulence factor is verified by the relatively high percentage of phospholipase producers among the blood isolates in this study. The fact that many of the *Candida* species isolates were negative for either of the enzymes especially proteinase, underscores the fact that various other mechanisms are also in operation in pathogenesis. With growing incidence and reported shift towards infections by Non albicans species, it becomes imperative for conducting further studies probing virulence mechanisms in a larger scale and improvising the techniques and systems for the detection of virulence factors.

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