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Virulence Factors of Candida Species Isolated from Respiratory Tract and Urine.

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ABSTRACT

Candida species is a significant opportunistic pathogen posing a risk for the development of invasive illnesses due to its virulence properties.The purpose of the study was to evaluate the virulence factors in different *Candida* spp. isolated from respiratory specimens and urine and to compare the production of the virulence factors in each *Candida* species. This *cross*-sectional study was conducted at a tertiary care teaching hospital for a period of *2* months. A total of 80 Candida isolates included in this study were identified based on standard laboratory techniques and subjected to proteinase, phospholipase, hemolysin and biofilms production.Of these 80 isolates, 65 (81.3%) and 15 (18.7%) were from respiratory tract and urine respectively. *C. albicans* (65%%) was the predominant species followed by non-albicans candida (35%), with predominance of *C. tropicalis*(15%). In our study, prevalence of proteinase, phospholipase, hemolysin and biofilm production were 31.2%, 43.8%,76.2% and 51.3% respectively. To conclude, the present study showed *Candida albicans* predominance in different clinical samples. The phospholipase activity was more common among *C. albicans*, while the hemolysin, proteinase activity and biofilm production were more frequent among non-albicans Candida spp. The presence of various virulence factors among the non-albicans *Candida* spp. suggests that they also can be pathogenic and may emerge as important opportunistic pathogens.

Keywords: *C. albicans*, *Non albicans Candida* , Proteinase, Phospholipase

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INTRODUCTION

Candida spp. has emerged as a significant opportunistic pathogen [1].The transition of *Candida* spp. from a harmless commensal to a pathogen is mainly attributable to an extensive repertoire of virulence determinants which are selectively expressed under certain predisposing conditions [2]. The important virulence factors of the *Candida* spp. are formation of filaments, secreted aspartyl proteinases, phospholipases, hemolysins, biofilm formation [3-5]. The expression of virulence factor of Candida spp are influenced by type and site of infection and also by host immune response [6].

The phospholipases play a major role in the invasion of host tissue by disrupting the epithelial cell membranes and favouring the hyphal tip to enter the cytoplasm. Proteinase production helps *Candida* spp. to evade the host's immune system by degrading several proteins involved in host defence and thus enhances the organism's ability to colonise and penetrate host tissues [7]. The hemolysins help the *Candida* spp. destroy the heme moiety of hemoglobin and enable them to extract the elemental iron, which is of pivotal importance in their ability to establish infection in humans [8]. Formation of surface-attached microbial communities known as biofilms is a major virulence factor of *Candida* spp. as it confers the ability to adapt to a variety of habitats such as natural host surfaces or prosthetic devices [9].

The purpose of the study was to evaluate the invitro activities of proteinases, phospholipases and hemolysins and biofilm formation in different *Candida* spp. isolated from respiratory specimens and urine and to compare the production of the virulence factors in each *Candida* species

MATERIALS AND METHOD

This *cross*-sectional study was conducted at a tertiary care teaching hospital for a period for of *2* months. A total of 80 Candida isolates respiratory specimens (sputum, broncho alveolar lavage, endotracheal aspirate) and urine were included in this study. All isolates were identified based on standard laboratory techniques [10] and growth on Hicrome Candida agar (Himedia, Mumbai, India). The following methods were employed for detection of proteinase, phospholipase, hemolysin and biofilms production.

Detection of Proteinase activity

Detection of proteinase production was performed according to Aoki et al [5,6] .Each Candida isolates was inoculated into plate containing bovine serum albumin medium and incubated at 37^{o}C for 7 days, following which fixation, staining and decolourisation were done with 20% trichloracetic acid,1.25% amidoblack and 15% acetic acid respectively. Unstained area around the well denotes degradation of protein. The proteinase activity (Pz) was measured as the ratio of the diameter of the well to the diameter of the proteolytic unstained zone. Low Pz signifies a high production of the enzyme and vice versa. When Pz = 1, no proteinase activity was detected in the strain.

Detection of Hemolytic activity

Hemolytic activity was determined using SDA medium supplemented with 3% glucose and 7% fresh sheep blood. Hemolysin production was evaluated after incubation in 5% CO_2 for 72 hours. A translucent halo around the inoculum site was considered as indicative of positive Hemolytic index [4, 5]. The diameters of the zones of haemolysis and the colony were measured, and this ratio was calculated as haemolytic index (HI).

Detection of Phospholipase activity

The phospholipase (Pl) was assayed according to Samaranayake et al, using a test medium prepared with Sabouraud dextrose agar (SDA) supplemented with 3% glucose, 1M NaCl, 0.005 M $CaCl_2$ and 8% sterile egg yolk emulsion[7]. Each strain was inoculated in triplicate. The Petri dishes were incubated at 37^{o}C for 4 days in a humid chamber. Pl activity produces a dense zone of precipitation around the colony. The ratio of colony alone to the diameter of colony plus precipitation zone was calculated, which gives the Phospholipase activity (Pz).Higher the Pz value means lower phospholipase activity and lower Pz value indicates higher phospolipase activity.

Detection of Biofilm formation

Biofilm formation was detected by tube adherence method proposed by Branchini *et al* [11].By this method colonies from SDA plate was taken and inoculated into the tube containing 10 ml Sabouraud's liquid medium supplemented with 8% glucose. After 24 hrs of incubation at 37°C, broth aspirated and stained with safranin. Biofilm formation was scored as negative (0+), weak positive (1+), moderate positive (2+), or strong positive (3+).

Quality control

C. albicans ATCC 10231 (which produces proteinases and phospholipases) was used as a control strain.

Statistical Analysis

Data entry and analysis was done using statistical software SPSS for Windows Version 16.0 (SPSS Inc, Chicago, IL, USA). Percentages was calculated for categorical variables. The difference in production of the virulence factors in different *Candida* species was compared using Chi-square test or Fisher's Exact test.

RESULTS

A total of 80 *Candida* spp. isolated from respiratory tract and urine were studied. Of these 80 isolates, 65 (81.3%) were obtained from respiratory tract, while 15 (18.7%) were isolated from urine. The distribution of various *Candida* species in male and female patients is shown in Table 1. The mean ± SD age of the study participants was 47.8 ± 19.7 (range, 2 to 85).

The distribution of *Candida* species isolated from respiratory tract and urine sample is shown in Table 2. *C. albicans* was the most common isolate from both respiratory tract and urine, followed by *C. tropicalis* and *C. glabrata*. Four isolates of *C. parapsilosis* was obtained from respiratory tract, while it was not isolated from the urine samples (*p* value= 1.0000, Not Significant).

The rate of biofilm production among the different *Candida* spp. is shown in Table 3. The prevalence of biofilm formation in *C. parapsilosis* was significantly higher (100%) compared to 44.2% in *C. albicans* (*p* value= 0.0478, significant).

The prevalence of proteinase production among the different *Candida* spp. is shown in Table 4. None of the *C. parapsilosis* showed proteinase activity. The prevalence of proteinase activity was only 15.4% in *C. albicans*, while it was 58-100% in other *Candida* spp. excluding *C. parapsilosis* (*p* value <0.0001, very significant).

The rate of phospholipase production among the different *Candida* spp. is shown in Table 5. The prevalence of phospholipase activity was relatively high in *C. albicans* (53.8%) and *C. krusei* (66.7%), while it was only 16-25% in *C. tropicalis, C. glabrata* and *C. parapsilosis* (*p* value= 0.0082, significant).

The rate of hemolysin production in different *Candida* spp. is shown in Table 6. The prevalence of hemolysin was only 33.3% in *C. glabrata*, while it was relatively high (50-100%) in other *Candida* spp. (*p* value= 0.0261, significant).

Table 1.Distribution of *Candida* species in male and female patients

Candida species	Female	Male
C. albicans	32 (69.6%)	20 (58.8%)
C. tropicalis	6 (13.0%)	6 (17.6%)
C. glabrata	3 (6.5%)	3 (8.8%)
C.parapsilosis	2 (4.3%)	2 (5.9%)
C. krusei	2 (4.3%)	1 (2.9%)
C. dublinensis	1 (2.2%)	2 (5.9%)
Total	**46**	**34**

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Table 2: Distribution of Candida species isolated from respiratory tract and urine sample

Candida species	Respiratory tract(%)	Urine(%)	Total (%)
C. albicans	44 (67.7%)	8 (53.3%)	52 (65%)
C. tropicalis	9 (13.8%)	3 (20.0%)	12(15%)
C. glabrata	4 (6.2%)	2 (13.3%)	6(7.5%)
C.parapsilosis	4 (6.2%)	0	4(5%)
C. krusei	2 (3.1%)	1 (6.7%)	3(3.8%)
C.dubliensis	2 (3.1%)	1 (6.7%)	3(3.8%)
Total	**65**	**15**	**80**

Table 3: Biofilm formation of Candida species from respiratory tract and urine sample

Candida species	Biofilm producers(%)	Non Biofilm producers(%)
C. albicans (52)	23 (44.2%)	29(55.8%)
C. tropicalis (12)	7(58.3%)	5(41.7 %%)
C. glabrata (6)	4(66.7%)	2(33.3%)
C. krusei (3)	1(33.3%)	2(66.7%)
C.parapsilosis (4)	4(100%)	0
C.dubliensis (3)	2(66.7%)	1(33.3%)
Total(80)	**41(51.3%)**	**39(48.8%)**

Table 4: Prevalence of proteinase among Candida species isolates from respiratory tract and urine sample

Candida species	Proteinase	
	Positive	Negative
C. albicans (52)	8(15.4%)	44(84.6%)
C. tropicalis (12)	7(58.3%)	5(41.7%)
C. glabrata (6)	5(83.3%)	1(16.7%)
C. krusei (3)	2(66.7%)	1(33.3%)
C.parapsilosis (4)	0	4(100%)
C.dubliensis (3)	3(100%)	0
Total(80)	**25(31.2%)**	**55(68.8%)**

Table 5: Prevalence of phospholipase among Candida species isolates from respiratory tract and urine sample

Candida species	Phospholipase	
	Positive	Negative
C. albicans (52)	28(53.8%)	24(46.2%)
C. tropicalis (12)	3(25%)	9(75%)
C. glabrata (6)	1(16.7%)	5 (83.3%)
C. krusei (3)	2(66.7%)	1(33.3%)
C.parapsilosis (4)	1(25 %)	3(75%)
C. dubliensis (3)	0	3(100%)
Total(80)	**35(43.8%)**	**45(56.2%)**

Table 6: Prevalence of haemolysin among Candida species isolates from respiratory tracts and urine sample

Candida species	Hemolysin	
	Positive	Negative
C. albicans (52)	41(78.8%)	11(21.2%)
C. tropicalis (12)	11(91.7%)	1(8.3%)
C. glabrata (6)	2(33.3%)	4(66.7%)
C. krusei (3)	2(66.7%)	1(33.3%)
C.parapsilosis (4)	2(50%)	2(50%)
C. dubliensis (3)	3(100%)	0
Total(80)	**61(76.2%)**	**19(23.8%)**

DISCUSSION

Candida albicans is a yeast like fungus which is a normal commensal of oral cavity, gastrointestinal tract and genitourinary tract of humans. However in individuals with immunocompromised state, chronic infections, malignancy and on prolonged steroid, antibiotics and immunosuppressive therapy they become

opportunistic pathogen [12]. C.*albicans* commonly causes infections such as cutaneous, mucocutaneous lesions and life threatening severe systemic infections.

For a commensal to become a pathogen and to establish infections in the residing host, it has to evade the immune system, survive and divide in the host environment and spread to new tissues [13]. Production of several virulence factors like proteinases, phospholipases, hemolysins, filamentous formation, adherence to epithelial and endothelial cells contributes to the pathogenicity of *C.albicans*.

The present study showed the distribution of *Candida* species in respiratory tract and urine samples. *C. albicans* (65%) was the predominant species recovered from both respiratory tract and urine and the non-albicans candida accounting for 35%, with predominance of *C. tropicalis* (15%).

Biofilms are the structured microbial communities that are encased in a matrix of extracellular polymers and are important for the development of clinical infection. Most often fungi form biofilm on surface of device used in clinical practices such as intravascular catheters, dentures, heart valves, implanted devices and contact lenses .They are indeed difficult to eradicate by host immune mechanism and the associated organisms are highly refractile to antimicrobials [14].

In our study, biofilm formation rate of *Candida* species was 51.3%. Several studies on biofilm formation of *Candida* species in different clinical samples showed a similar percentage of 44.7%, 64.9% and 73% comparable to our study [3, 15, 16]. The prevalence of biofilm formation in *C. parapsilosis* was significantly higher (100%) compared to 44.2% in *C. albicans* (*p* value 0.0478). Our study showed that the biofilm formation rate of *C.albicans* isolates was significantly lower than non-albicans *Candida* spp .Similar study by Mohandas et al reported that biofilm positivity occurred most frequently in isolates of *C. krusei* followed by *C. tropicalis*, *C. kefyr*, *C. guilliermondii*, C. *parapsilosis*, *C. glabrata*, and *C. albicans*[3].

Aspartyl proteinases are secreted by the majority of strains of *C.albicans, C. tropicalis* and *C. parapsilosis*, reflecting the sequence of virulence of *Candida* spp. for man. Secreted aspartyl proteinases which promote in adhesion, tissue damage and invasion of host immune mechanism are by degradation of proteins such as immunoglobulins, complements and cytokines at the site of infections [17].

In our study, proteinase activity was detected in 25(31.2%) isolates. Study by D'EçaJúnior A et al on proteinases of clinical isolates of *Candida* showed similar percentage of 44.4%[18]. Several other studies showed proteinase production greater than 75% (74.5%, 90.5% & 100%) [6,16,19]. The prevalence of proteinase activity was only 15% in *C. albicans*, while it was 58-100% in other *Candida* spp. excluding *C. parapsilosis* (*p* value <0.0001). The proteinase-producing capacity of *C. albicans* 8(15.4% %) was less than that of non-albicans Candida (58-100%) excluding *C. parapsilosis* in this study (*p* value <0.0001). Another similar study by Mohandas et al showed highest proteinase producers were *C. kefyr ,C. guilliermondii* , followed by *C. albicans* whereas the least producer in the group was *C. glabrata*[3].

Phospholipases are group of heterogeneous enzymes which hydrolyse the phospholipid of epithelial cell membranes and allowing the hyphal tip to enter the cytoplasm, leading to cell lysis; direct host cell damage and lysis has been proposed as a major mechanism contributingto microbial virulence [20].

Prevalence of phospholipase activity was noted in 35(43.8%) isolate in our study similar to other studies by Mohandas et al and Basu et al with 44.14% and 48.7% respectively[3,21]. Phospholipase activity of 80 to 100% have been reported from other similar studies done from various sites and in different groups of patients [22,23,24]. The prevalence of phospholipase activity was relatively high in *C. albicans* (53.8%) and *C. krusei* (66.7%), while it was only 16-25% in *C. tropicalis, C. glabrata* and *C. parapsilosis* (*p* value 0.0082). The result of this study is in agreement with the reports of Mohandas et al [3] which showed 46.93% positivity among *C. albicans* and 42% positivity of non-albicans Candida isolates.

Candida possesses another putative virulence factor, hemolysin. Hemolysin contributes to its pathogenesis, by lysis of erythrocytes and iron acquisition which facilitates hyphal invasion in disseminated Candidiasis [25].

In our study hemolysin production was noted in 61(76.2%) of candida isolates. The prevalence of hemolysin was only 33.3% in *C. glabrata*, while it was relatively high (50-100%) in other *Candida* spp. (p value 0.0261).Luo G et al, in their study noted 91.2% of hemolysin activity, where by majority of the isolates showed 100% hemolysin activity [8].

To conclude, the present study showed predominance of Candida albicans in different clinical samples. The virulence factors such as biofilm formation and hemolysin production were frequently observed among both C. albicans and non-albicans Candida isolates. However, the phospholipase activity was more common among C. albicans, while the proteinase activity was more frequent among non-albicans Candida spp. The presence of various virulence factors among the non-albicans Candida spp. suggests that they also can be pathogenic and may emerge as important opportunistic pathogens.

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