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Non-Albicans Candida Infection: an Emerging Threat

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Abstract

The nature pattern of infectious diseases has undergone profound changes in the past few decades. Fungi once considered as non pathogenic, less virulent and uncommon are now recognized as a primary cause of morbidity and mortality in immunocompromised and even severely ill immunocompetent patients. Candida spp. are amongst the most common fungal pathogens. *C.albicans* was the predominant cause of candidial infection. However, a shift towards Non-albicans Candida species has been recently observed. In the present study, we investigated the prevalence of Candida spp. in blood samples of patients and studied their virulence factors. In the present study, 2180 samples for blood culture were processed, Out of which Candida isolates were recovered in 74(3.39%) .Non albicans Candida species also demonstrated the production of virulence factors once thought to be produced exclusively by *Candida albicans*. Therefore, it can be concluded that Non-albicans Candida species also have emerged as an important cause of infections besides C.albicans.

Introduction

The incidence of fungal infections has progressively increased in the past few decades. Fungi once considered as non-pathogenic or less virulent are now recognized as one of the main causes of morbidity and mortality, worldwide [1]. Candida spp. is considered the most common fungal pathogen capable of initiating infections both in immunocompetent and immunocompromised hosts. [2] Candida spp. is responsible for various clinical manifestations ranging from muco-cutaneous overgrowth to life threatening disseminated infections like candidemia. *Candida albicans* used to be the predominant cause of Candida infections. However, a shift towards Non albicans Candida species has been recently observed.[3] Many reasons for this shift in *Candida* species have been investigated and also several risk factors have been associated with the emergence of Non-*albicans* Candida species. A widespread use of fluconazole has been associated with the development of infections due to Non albicans Candida species that are intrinsically resistant to fluconazole or have developed resistance during treatment. [4]The fluconazole exposure can be considered either on a patient level, for example, in case of long-term fluconazole prophylaxis that predisposes patients to *C. krusei* infection, or on the level of a ward or hospital, when bulk consumption can change ecology of *Candida* species. [2]Whereas the patient-level association has been confirmed in numerous studies, the hospital-level influence is more controversial and has been observed in some centers, but not in others.[5] Additionally, the relationship with a heavy consumption of an antifungal and an increase in minimum inhibitory concentration to this drug has been established in ICUs not only for azoles,

but also for echinocandin or polyenes. Risk factors reported for candidemia due to *C. parapsilosis* include the presence of in-dwelling devices and neonatal age. [6, 7]

The transition of Candida spp. from commensal to potent pathogen is facilitated by a number of virulence factors such as adherence to host tissues and medical devices, biofilm

formation, and secretion of extracellular hydrolytic enzymes [4]. Although there has been extensive research to identify these pathogenic attributes in *C. albicans*, relatively less is known about Non albicans Candida spp. [6]. The present study was conducted at Government Medical College and Hospital, Jammu (J&K), with an aim of investigating prevalence of Candidemics in clinically suspected septicemics and study their virulence factors which influence the pathogencity of Candida species.

Material & Methods

The prospective study is part of a Ph.D. research work conducted in the Department of Microbiology, Government Medical College and Hospital Jammu (J&K), India. The protocol of the study was approved by the Institutional Ethics Committee. Candida spp. isolated from blood samples between the period of January 2016 to December 2016 were included in the study. Blood samples obtained from NICU, ICU and in-door patients were collected aseptically, inoculated on Blood culture broth and incubated at 37^0C for 24 hours followed by inoculation on Blood Agar and MacConkey agar. Blood agar plates which showed no growth were further incubated for 48 hours at room temperature. . Colonies appearing pasty, opaque, slightly domed or flat, smooth and pale coloured (white or off-white) with a sweet smell reminiscent of ripe apples were suspected to be colonies of Candida. The colonies from Blood agar were examined by Gram-staining and inoculated on two tubes of Sabouraud dextrose agar slants, incubated at 25^0C and 37^0C for 24 hrs

Species Identification

For initial speciation Germ tube test was done. Isolates producing germ tubes within 2 hours of incubation were further subjected to chlamydospores formation.The germ tube negative Candida isolates were classified on the basis of colony colour on Candida CHROMagar (TM Media) theChromogenic media containing chromogenic substrates to react with enzymes secreted by target microorganisms to yield colonies of varying colours.

Virulence Factors

Candida spp. isolated from blood were screened for the production of virulence factors which inciuded adherence to buccal epithelial

Cell (ABEC), biofilm formation, haemolytic activity, and production of extracellular hydrolytic enzymes coagulase, phospholipase, and proteinase.

1. Adherence to Buccal Epithelial Cell (ABEC)

Adherence assay was performed as described by Kimura and Pearsall with minor modifications. BECs were collected by gently rubbing the cheek mucosa. As fresh BECs were used, they were collected in the morning on the day of assay. BECs were washed thrice by phosphate buffer saline (PBS) and harvested by centrifugation. Equal volumes (1 mL) of BEC (1×10^5 cells/mL) and yeast suspension (1×10^7 cells/mL) were mixed and incubated at 37 ^{0}C for 2 hours in a shaking water bath at 40 rpm. The mixture was filtered through a 20 μm filter to remove non adherent yeast cells. The BECs on the filter were washed with 5mL of PBS and finally suspended in 5mL of PBS. A drop of this suspension was placed on glass slide. The smear was fixed by methanol, air-dried, and stained with 2% crystal violet for 1 minute. Adherence was determined microscopically by counting the mean number of yeast cells adhering to every 100 BECs.[8]

2. Biofilm Formation

Biofilm formation was determined using the tube method described by Yigit et al. with a few modifications. Colonies of *Candida* isolates to be tested for biofilm formation were inoculated in saline and incubated for 24 hours at 37 ^{0}C. 1.5mL of this saline suspension was transferred to tube containing 5mL of Sabouraud dextrose broth supplemented with glucose (final concentration, 8%).The tubes were incubated at 37 ^{0}C for 24hours. After incubation, the broth from the tube was gently aspirated using a Pasteur pipette. The tube was washed thrice with PBS (pH7.2) and stained with 1% Safranin. The stain was decanted after 15 min and the tube was rinsed with PBS to remove excess stain. Presence of visible adherent film on the wall and the bottom of the tube indicated biofilm formation by the isolate. [9]

3. Haemolytic Activity

Haemolytic activity of *Candida* spp. was screened by the method described by Luo et al. Approximately 10 μL of standard inoculums (108 *Candida* cells/ mL) was inoculated on sheep blood Sabouraud dextrose agar plate. Plates were incubated at 37^{0}C in presence of CO_2 for 48 hours. The presence of a distinct translucent halo around the inoculum site, viewed with transmitted light, indicated haemolysin production. Haemolytic activity (Hz) was determined by calculating the ratio of the diameter of the colony to that of the translucent zone of haemolysis. [10]

4. Production of Extracellular Hydrolytic Enzymes

The Candida isolates were screened for the production of enzymes like coagulase, phospholipase, and proteinase.

(i) Coagulase Production

Coagulase production was assessed by the method described by Rodrigues et al. Approximately 0.1mL of an overnight culture of Candida spp. was aseptically inoculated into a test tube containing 0.5mL of EDTA-rabbit plasma. The tube was incubated at 37 ^{0}C and observed for clot formation after 2- 4 hours. The presence of clot that could not be resuspended by gentle shaking indicated positive coagulase test. If no clot formed, the tube was re-incubated and re-examined at 24 h. [11]

(ii) Phospholipase Production

Phospholipase production was assayed according to the method of Samaranayake et al. by egg yolk agar plate method. Approximately 5 μL of standard inoculum of test strain containing 108 cells/mL was aseptically inoculated onto egg yolk agar. After inoculation, Plates were dried at room temperature and then incubated at 37 ^{0}C for 3 days. The plates were examined for the presence of a zone of precipitate around the colony (phospholipase production). Phospholipase activity (Pz) was expressed as the ratio of the colony diameter to the diameter of the colony plus the precipitation zone. A Pz value of 1 denoted no phospholipase activity, whereas Pz < 1 indicated phospholipase expression by the isolate. [12]

(iii) Proteinase Production

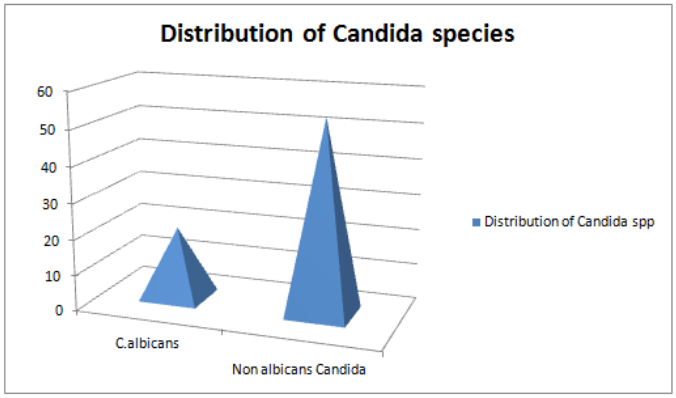
Proteinase production was performed according to the method of Aoki et al. with a few modifications using bovine serum albumin agar (BSA) plates. Approximately 10 μL of standard inoculums containing 106 cells/mL was aseptically inoculated on BSA agar plate. Inoculated plates were incubated at 37^{0}C for 7 days. Further proteinase activity was inhibited by adding 20% trichloroacetic acid and the plate was stained with 1.25% amido-black. The diameter of the colonies was measured prior to staining and the diameter of the clear zones was measured after staining.

Proteinase index (Prz) was measured in terms of the ratio of the diameter of the colony to the diameter of unstained zone. A Prz value of 1 indicated no proteinase activity; Prz < 1 denoted proteinase expression by *Candida* isolate. The lower the Prz value, the higher the activity. [13]

Result

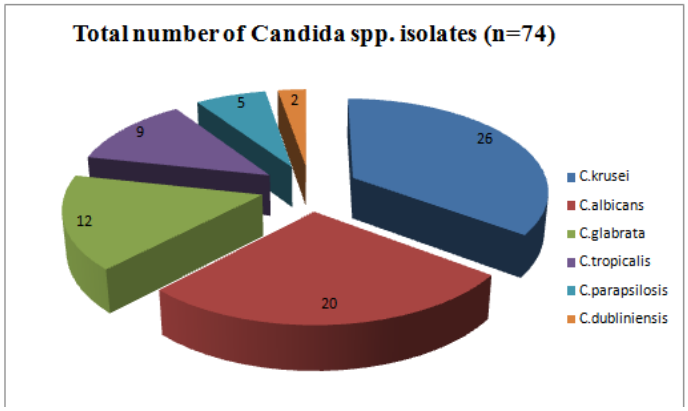
In the present study, 2180 samples for blood culture were processed, Out of which 74 (3.39%) Candida isolates were recovered. Out of 74 isolates , 16 (21.62%) Candida isolates were recovered after 48-72 hours of incubation.

Fig 1- Distribution of Candida species



Out of 74 Candida isolates, Non albicans Candida species are 54 (72.97%) whereas Candida albicans is 20 (27.0%) respectively.

Fig 2-Total number of Candida spp. isolates



Candida krusei (35.13 %) was the most common isolated species followed by *C. albicans* (27.02%), *C. glabrata* (16.21%) , *C.tropicalis* (12.16%), *C.parapsilosis* (6.75%) and C.dubliniensis (2.70%)

Table 1- Virulence factor produced by different Candida species isolated from blood samples

Organisms	No. of isolates	Adherence to Buccal epithelial cells	Biofilm Production	Haemolytic Activity	Coagulase Production	Phospholipase Activity	Proteinase Activity
C.krusei	26	22	26(100%)	25	23(100%)	19	23
C.albicans	20	18	18	16	19	20	18
C.glabrata	12	7	12	12	12	12	12
C.tropicalis	9	7	9	8	7	4	9
C.parapsilosis	5	2	4	3	3	4	3
C.dubliniensis	2	2(100%)	0	1	1	1	2(100%)

Discussion

In recent years, Candida spp. have emerged as principal pathogens of a variety of human infections and are important causes of morbidity and mortality worldwide. [14] Advanced age, diabetes mellitus, pregnancy and use of indwelling urinary catheters were major risk factors associated with candidemia. Incidence of candidemia was high among patients admitted to the ICUs and among those who had a previous history of treatment with antibiotics. The abuse of antibiotics as a “pill for all ills,” self-medication and starting broad spectrum antibiotics as the first line treatment have led to increased selective colonization by Candida spp. As the commensal bacterial flora is suppresed[15] In the present study,out of 2180 samples for blood culture processed, 74 (3.39%) were positive for Candida spp. Out of 74 Candida isolates ,16 (21.62%) were recovered after a prolonged incubation of 48-72 .

In our study, Non albicans Candida species and *Candida albicans* were isolated at a rate of (72.97%) and (27.0%) respectively, which is similar with the findings of Sachin C. Deorukhkar *et.al*. .[16]*Candida krusei* (35.13 %) was the most common isolated species followed by *C. albicans* (27.02%), *C. glabrata* (16.21%), *C.tropicalis* (12.16%), *C.parapsilosis* (6.75%) and *C.dubliniensis* (2.70%) which is in agreement with the study conducted by Shashi .S. Sudhan *et.al*. [3]

The present study also focussed on the virulence factors which play an important role in the pathogencity of Candida spp. identifying these virulence factors in infecting pathogens and understanding their effects on the

human host are a major challenge for clinical microbiologists. Adhesion of Candida spp. to the host epithelial cells is a critical first step in the pathogenesis of infection. Binding of the Candida to host cells, host cell proteins, or microbial competitors prevents or at least reduces the extent of clearance by the host's defense mechanisms. ABEC was highest in *C. albicans* . A similar observation was reported by Mane *et al.* [16]. Biofilm formation is one of the important virulence factor of Candida spp. Candida biofilm occurs on tissues surfaces as well as the biomaterials of medical devices. As in our study Biofilm formation was mainly seen in *C.krusei* followed by *C.albicans* which is in agreement with the study conducted by Demirbilek et.al. which showed that the biofilm formation rate was higher in Non albicans Candida spp. than in higher C.albicans in their study too.[17] It was noted that the Haemolytic activity was higher in *C.krusei* (NAC) followed by *C.albicans* which is in concordance with the study conducted by K.Deepa *et. al*. [15] Enzyme coagulase binds plasma fibrinogen and activates a cascade of reactions that induce clotting of plasma .In our study, coagulase production was high in *C. albicans* as compared to NAC spp. Among NAC spp., *C. krusei* showed high coagulase expression .The result of our findings was similar to the study conducted by Sachin C. Deorukhkar *et.al* [18].Of the various hydrolytic enzymes produced by *Candida spp.*, phospholipases and proteinases are the most important. Phospholipases damage the host cell membrane and hence facilitate invasion of tissue . These enzymes hydrolyze phospholipids into fatty acids and also expose receptors on host cell membrane to facilitate adherence .In the present study, phospholipase production was high in *C. albicans* and *C.krusei*. Proteinase facilitates Candida invasion and colonization of host tissue by disruption of host membrane and by degrading important structural and immunological defense proteins. [19] In our study, although *C. albicans* demonstrated increased capability of proteinase production, significant proteinase activity was also noted in Non albicans Candida spp.

Conclusion

It is necessary to understand the pathogencity mechanism of the *Candida spp.* for the development of new antifungal strategy. Developing antifungal therapies against selective target virulence factor is very crucial now-a-days because of the multi-drug resistance developed by Candida spp. Hence our study on virulence factor of Candida spp. pave way for better understanding of the various virulence factors exhibited by Candida species.

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