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Original Article

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In Vitro Detection of Virulence Factors of *Candida albicans & non-albicans Candida species* Isolated from the Samples in ICU Patients

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ABSTRACT

Aim: To isolate and identify *Candida albicans and non-albicans Candida* from various clinical specimen of Intensive care Unit and to investigate some virulence factors and perform their Anti- fungal susceptibility.

Materials and Methods: Overall 32 isolates (12 *Candida albicans and 20 non- albicans Candida spp*.) were included in this study. Virulence factors were studied. In isolates, egg yolk agar was used for determining phospholipase activity, while sheep and human plasma used to determine coagulase activity, Sabouraud dextrose agar with sheep blood for hemolysin activity. Biofilm formation was detected by the tube adherence method.

Results: In both *Candida spp*., it was found that hemolytic activity were higher next to phospholipases activity. It was also found that Coagulase activity were detected in *Candida species* found to be higher with sheep plasma compared to human plasma. Biofilm production was found to be less and mostly were from blood culture isolates.

Conclusion: Knowledge of these virulence factors is an important tool to understand pathogenesis of *candidiasis* and in addition will help to explore new antifungal drug targets for improved therapeutic regimens.

Key Words: *Candida spp*., Virulence factors, *Non-albicans Candida spp.*

INTRODUCTION

Infections caused by opportunistic pathogens, such as yeasts, have become important reasons of morbidity and mortality because of alterations in the immune system and invasive hospital procedures [1]. AIDS, organ transplantation, chemotherapy, invasive procedures and radiotherapy increased the prevalence of immunocompromised individuals and also diabetes mellitus, and the over use of extended spectrum antibiotics made an increment in these infections [2,3].

Infections due to yeast like fungi have increased in intensive care units too[4]. In the last two decades, nosocomial fungal infections increased all around the world. Yeast like fungi arethe fourth most common agent in the blood stream infections. *C. albicans, C. tropicalis and C. parapsilosis* are the three most common yeast like fungi causing blood stream infection[5].

Unnecessary use of broad spectrum antibiotics, catheterization, and mucosal colonization in patients with risk factors are important predisposing factors[6]. Initiation of azole group antifungal drugs for prophylaxis and treatment led to increased isolation of resistant *non-albicans Candida* strains[7].

Candida spp. have some virulence factors that facilitate proliferation, may result in adhesion to the epithelium and invasion of the host tissue[8]. It seems that extracellular hydrolytic enzymes play an important role in candidal overgrowth[9]. The extracellular hydrolytic enzymes including secreted aspartyl proteinase and phospholipases degrade immunoglobulins and proteins of the extracellular matrix; they also inhibit the phagocytosis of polymorphonuclear neutrophils and induce inflammatory reactions[10]. Furthermore, the survivaland ability of *Candida albicans* to establish infections within humans are mainly related to its ability to procure elemental iron through hemolysin production[11].

Among *Candida spp.,* expression of virulence factors may vary depending on the infecting species, geographical origin, type of infection, the site and stage of infection, and host reaction. Knowledge of these virulence factors is an important tool to understand pathogenesis of candidiasis and in addition will help explore new antifungal drug targets for

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improved therapeutic regimens.

OBJECTIVES

With this background, we did this study to isolate and identify *Candida albicans and non- albicans Candida* from various clinical specimen of Intensive care Unit and to study the virulence factors and perform the antifungal susceptibility profile of isolated *Candida albicans and non-albicans Candida.*

MATERIAL AND METHOD

A cross-sectional study was carried out in the Department of Microbiology, MGIMS, Sevagram, during the period of April-August 2018 after approval from the Institutional Ethical Committee. Study was carried out in the Microbiology department, MGIMS, Sewagram. All the samples requested for aerobic culture and sensitivity from Intensive Care Units were processed in our laboratory and those found to be culture positive for *Candida albicans and non-albicans Candida spp.* were included in the study.

Candida albicans and non-albicans Candida spp. isolated from various clinical samples like blood, sputum, urine, wound swabs, pus, CSF, oral swabs, etc. The organisms were identified by germ tube test, morphology on cornmeal agar (HiMedia), chromagar media (HiMedia) and growthat 45°C, which were done as per standard microbiological protocols[12]. VITEK 2 YST identification card (bioMerieux, France) was used for yeast identification as per manufacturer's instructions and the antifungal susceptibility testing of *Candida spp.* was performed using VITEK

2 AST- YS08 cards. The virulence factors studied were exoenzymatic activities like phospholipase , coagulase, hemolysin production & biofilm formation.

1. **Phospholipase Production:** The phospholipase activity of *Candida spp* was detected by the method of Samaranayake et al(13). Approximately 5 L of standard inoculum of test strain containing 10^8 *Candida* cells/mL was aseptically inoculated onto egg yolk agar. The plates were dried at room temperature and then incubated at 37° C for 48 h. The plates were examined for the presence of precipitation zone around the colony. The presence of precipitation zone indicated expression of phospholipase enzyme. *C. albicans* ATCC 10231 was used as positive control. The phospholipase index (Pz) is defined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone. A Pz value of 1 denoted no phospholipase activity; Pz < 1 indicated phospholipase production by the isolate. The lower the Pz value, the higher the phospholipase activity. To minimize experimental error, the assay was conducted in duplicate conditions on three separate occasions for each isolate.
2. **Coagulase Activity:** Coagulase production by *Candida spp*. was detected bythe method of Yigit et al.(14). Approximately 0.1 mL of an overnight culture of *Candida spp*. was aseptically inoculated into a tube containing 500 L of sheep plasma and human plasma separately. The tubes were incubated at 35° C and observed for clot formation after 2, 4, 6, and 24 h. The presence of a clot that could not be resuspended by gentle shaking indicated positive coagulase test. *Staphylococcus aureus* ATCC 25923 and *S. epidermidis* ATCC 14990 were used as positive and negative controls, respectively.
3. **Hemolysin Production:** Haemolytic activity of *Candida spp* was screened on sheep blood Sabouraud dextrose agar plate by the method described by Manns et al.[11]. Approximately 10 L of standard inoculum (10^8 *Candida* cells/mL) were aseptically inoculated onto the medium. The culture plates were incubated at 37° C for 48 h. *C. albicans* ATCC 90028 was used as the control strain. *Streptococcus pyogenes and Streptococcus pneumoniae* were used as positive controls for beta andalpha haemolysis, respectively. The presence of a zone of haemolysis around the colony indicated haemolysin production. Haemolytic activity (Hz) was calculated in terms of the ratio of diameter of the colony to that of the translucent zone of haemolysis (in mm)
4. Biofilm Formation: The ability of *Candida spp* isolates to form biofilms was assessed by the tube method described by Yigit et al.[15]. Colonies of *Candida spp* from Sabouraud dextrose agar was inoculated in saline and incubated overnight at 37° C. 0.5 mL of this saline suspension was added into screw capped conical polystyrene tubes containing 5 mL of Sabouraud dextrose broth supplemented with glucose (final concentration of 8%). The tubes were incubated at 35° C for 48 h without agitation. After incubation the broth from the tubes were aspirated gently using Pasteur pipette. The tubes were washed twice with distilled water and stained with 2% safranin. The stain was decanted after 10 min. The tubes were rinsed with distilled water to remove excess stain. Presence of visible adherent film on the wall and at the bottom of the tube indicated biofilm formation. Ring formation at the liquid interface was not considered as an indication of biofilm production. *Staphylococcus epidermidis* ATCC 35984 and *C. albicans* ATCC 10231 were used as positive and negative controls, respectively.

RESULTS

During the study period, a total of 32 non repeat Candida species were isolated from similar number of samples; which

included 16 (50%) blood, 7 (21.9%) urine, 5 (10.6%) sputum, 2 (6.2%) pus and 1 (3.1%) each of oral swab and CSF. All the *Candida* isolates were processed for species identification, detection of virulence factors and antifungal susceptibility testing. These *Candida* so isolated were 16 (50%) from NICU, 13 (40.6%) from MICU, 2 (6.2%) from SICU, and 1(3.1%) from PICU. (Table-1).

Out of 32 *candida* isolates, 12(37.5%) were *C. albicans*, 7(21.9%) were *C. utilis*, 4 (12.5%) *C. krusei*, 3(9.4%) *C. tropicalis,* 2(6.2%) were *C. pelliculosa* and 1(3.1%) was *C. dubliniensis*. Rest 3(9.4%) remained unidentified and grouped under *Candida species* (Table-2). In the present study, amongst 32 *Candida isolates*, phospholipase activity was seenin 16(50%) and Hemolysin activity was seen in 32(100%). (Table-3 & Figure1-4). All the 32 isolates are susceptible to caspofungin and voriconazole. 91.7% of *C. albicans* were susceptible to fluconazole,

flucytosine and amphotericine B. Furtheramong *C. utilis*, 85.7% were susceptible to flucytosine. Rest 11 isolates of *Candida species* and 2 isolates of *C. pelliculosa* were susceptible to all the antifungals tested.(Table-4)

Table 1: Distribution of Candida isolated from the ICUs, collected from different samples

Samples	NICU	MICU	SICU	PICU	Total
Blood	16	0	0	0	16
Sputum	0	5	0	0	5
Urine	0	6	0	1	7
Pus & Wound swab	0	0	2	0	2
Others(CSF, Oralswab)	0	2	0	0	2
Total	16	13	2	1	32

Table 2: Distribution of isolated Candida species (n=32)

Isolates		Number	%
Candida albicans		12	37.5%
Non-albicans Candida species	*Candida utilis*	7	21.9%
	Candida krusei	4	12.5%
	Candida tropicalis	3	9.4%
	Candida dubliniensis	1	3.1%
	Candida pelliculosa	2	6.2%
	Not identified	3	9.4%

Table 3: Comparison of virulence factor producers in isolates from different samples

Samples	Phospholipase activity	Coagulase test	Hemolysin activity	Biofilm formation
Blood (n=16)	8	4	16	6
Sputum(n=5)	2	0	5	0
Urine(n=7)	6	3	7	2
Pus & Wound swab (n=2)	0	0	2	0
Others(CSF,Oral swab) (n=2)	0	0	2	0
Total	16	7	32	8

Table 4: Antifungal sensitivity on Candida isolates

Species	Fluconazole	Voriconazole	Caspofungin	Amphotericin B	Flucytosin
Candida albicans	91.7%	100%	100%	91.7%	91.7%
Non- albicans Candida	100%	100%	100%	100%	85.7%

DISCUSSION

Candida is an asexual, dimorphic fungi present as normal flora in humans. A small number of *candida species* are pathogenic for humans. Its infections may be primary or secondary. These organisms are capable of causing superficial and deep seated infections such as cutaneous, mucocutaneous, subcutaneous and systemic candidiasis. They are commensals and act as pathogens when host defenses are interrupted. Risk factors include prolonged stay in ICU's, diabetes mel-

litus, prolonged usage of antibiotics, immunosuppression, defects in CMI etc. In the present study, the phospholipase, hemolysin, Coagulase and Biofilm production of *Candida species* in the Intensive Care Units of a tertiary care hospital were studied. In our study, a total of 32 *Candida* were isolated out of which 12(37.5%) were *C.albicans* and 20(62.5%) were non- *albicans Candida.* These results are in agreement with those of Ruchika et al whose study also showed the same results.[16]. In the recent studies, observation of a shift towards *non-albicans Candida species* from *Candida albicans* is seen[17]. Some studies have reported increasing trend ofincidences of infections caused by *non-albicans Candida,* thus gradually changing the cause of candidemia to be *non-albicans Candida* and not *Candida albicans* in some regions[18]. Contribution of factors like increased use of antifungal drugs and broad spectrum antibiotics, long term use of catheters and increase in the number of immune-compromised patients has led to the emergence of *non-albicans Candida species* in increasing numbers[19,20,21]. *Non-albicans Candida species* cannot be overlooked as mere contaminants or non-pathogenic commensals as mostof them show reduced susceptibility to commonly used antifungal drugs (22). In *Candida*, the transition from commensalism to pathogenicity, is attributed to the selective expression of different virulence factors that act synergistically under favourable conditions. The type, stage and infection site in addition to the immune response, determine which virulence factors are expressed. Among these virulence factors, phospholytic, coagulase, hemolytic activity and biofilmformation seems to play a major role in the pathogenicity of these microorganisms. Not only the absence or presence of a virulence factor but when present the amount of its activity is also an important factor contributing to pathogenicity. Research on prevalent *Candida species* along with their virulence factors in a givenset up would be an important tool to prove the relation between the infective species of *Candida* and infection.

Phospholipase activity in our study was demonstrated in 16 out of 32(50%) *Candida* isolates.These results correlate closely with those of Faris et al.[23] who reported phospholipase activity in 44% of *Candida* isolates. Our study also agrees with Deepthi et al.[24] who reported 53.4% of *Candida* isolates showing phospholipase activity. *Candida albicans* is a stronger hemolysin producer compared to *non albicans Candida species*. In our study 32 out of 32 isolates were observed to be hemolysinproducers, i.e. 100% of the *Candida* isolates were positive for hemolysin production. 100% hemolysin producers have also been reported in other studies[25].

Coagulase tests are routinely used to detect the presence of *S. aureus*. The coagulasetube test is the most frequently used method for *Staphylococci* because of greater accuracy and its ability todetect both bound and free coagulase. In the present study, sheep and human plasma showed different sensitivities when used to test the coagulase activities of *Candida* isolates. Sheep plasma showed more sensitivity with 21.87 % of all *Candida* isolates being positive for Coagulase activity whereas human plasma expressed no activity for any *Candida spp.* tested. This study agrees well with Yigit et al.[14] which showed no sensitivity to human plasma and in sheep plasma 20.8% *Candida* showed Coagulase activity. Biofilm formation was carried out by tube adherence.

In our study, 8 out of 32(25%) *Candida* isolates showed biofilm formation in which 75% were from blood samples. In the study by Tortorano et al.[26] showed 39% of *Candida* isolates showing biofilm formation. Also Faris et al.[23] reported 40% of *Candida* isolates positive for biofilm production. In this study we also conducted antifungal susceptibility tests. The study showed that all *Candida* isolates are susceptible to Voriconazole and Caspofungin. Susceptibility of *C. albicans* to Amphotericin B was 91.7% and to Fluconazole was 91.7%. Shivanand(27), Araj[28] got similar results for voriconazole but differed in the results where they got 100%susceptibilty to AMP-B. Also, Amina[29] got a susceptibility of 78.3% only for AMP-B and against Voriconazole too Amina found only 30.4% susceptibility, alongwith only 30.4% susceptibility to fluconazole whichis very less than other studies. However, our study agrees with the study by Deepthi et al.[24] which showed 100% susceptibility to voriconazole and 92.3% to fluconazole.

CONCLUSION

Infections due to yeast like fungi have increased in Intensive Care Units. In this study, most of the *Candida* were isolated from neonatal intensive care units(50%) and from blood samples(50%). This may be due to invasive procedures, prolonged hospitalization, chronic antibiotic uses and low birth weight. It is necessary to understand the pathogenic mechanism of *Candida spp.* for implementation of new antifungal therapy. Hence our study on virulence factors of *Candida spp.* highlighted the way for better patient management and prognosis of patients.

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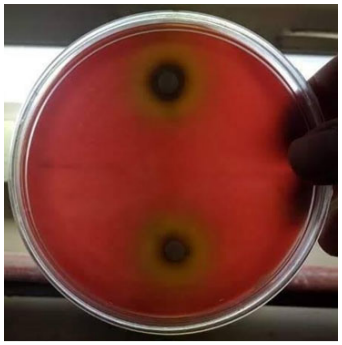
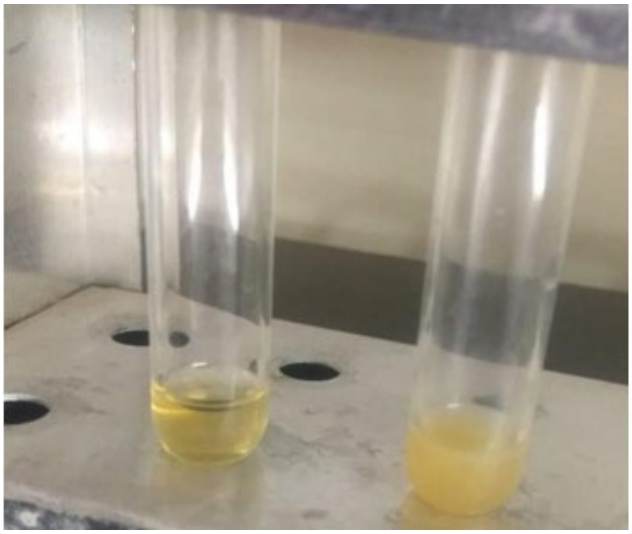


Figure 1: Hemolysin activity on Sabouraud s Dextrose Agar with 3% blood.

Figure 2: Phospholipase activity on Sabouraud's Dextrose Agar with egg yolk.



Fiure 3: Coagulase activity by Tube Method.

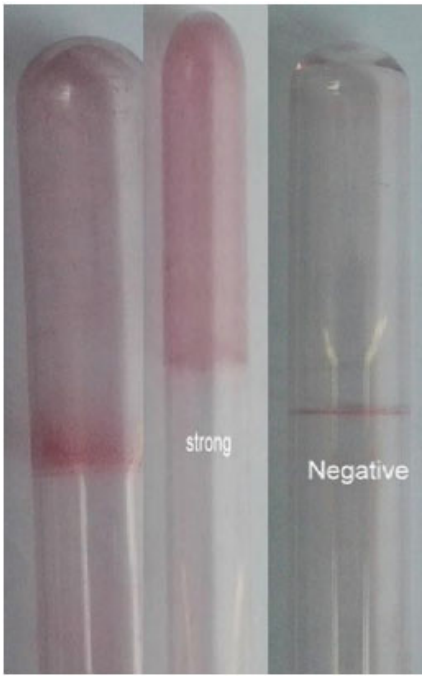


Figure 4: Biofilm production by Tube method.