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Detection of Virulence Markers Viz: Proteinase, Phospholipase, Hemolytic Activity And Biofilm Formation, From Isolates of Neonatal Systemic Candidiasis in A Tertiary Care Hospital

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Abstract :*The present study was undertaken to determine the prevalence of various Candida spp. infecting the neonates, and the virulence markers viz., proteinase, phospholipase, hemolytic activity and biofilm production expressed by the isolates. Out of the 243 blood culture samples, thirty five (35, 14.4%) showed growth of candida spp., of which thirty two (32) were Non candida albicans candida spp.(NCAC) & three (3) Candida albicans. Most common isolate among the NCAC spp. was C.krusei (19) followed by C.tropicalis (6), C.glabrata (5) & C.parapsilosis (2). Candidemia association was found to be the highest (34/35) with broad spectrum antibiotic therapy (BSA), followed by LBW (32/35), preterm (32/35) and neonates in respiratory distress (RD) (24/35). All the Candida albicans isolates expressed proteinase, phospholipase and hemolytic activity, except biofilm formation. Among the NCAC spp. hemolytic activity (27/32) was expressed maximally followed by proteinase (15/32) & biofilm formation (15/32) and lastly phospholipase (9/15) production.*
Keywords : *Biofilm, Candida, haemolysin, Neonate, Phospholipase, Proteinase*

I. Introduction

Candida species are component of normal flora of human beings. Humans are first exposed to fungus C. albicans when passing through the vaginal canal during birth. In this course the fungus colonizes the buccal cavity, and upper and lower parts of the gastrointestinal tract of the neonate, where it becomes commensal[(1)].Systemic candidiasis in neonates is increasing in frequency especially since the survival of babies with low birth weight has increased. Candida spp. are the most common fungal pathogens isolated from blood cultures of neonates[(2)]. A number of risk factors are associated with the development of neonatal systemic candidiasis such as very low birth weight, prematurity, prolonged antibiotic therapy, prolonged use of fat emulsions in total parenteral nutrition and the use of artificial ventilation. The clinical manifestations are respiratory insufficiency, apnoea, bradycardia, temperature instability, feeding intolerance and abdominal distension[(3)]. Prompt treatment with antifungals is required for these babies. Candida spp. have some virulence factors that facilitate proliferation which may result in adhesion to the epithelium and invasion of the host tissue. 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[(4)]. The secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis[(5)]. Moreover, it has been reported that biofilm formation also plays an essential role in the pathogenicity of Candida spp. In addition, it has been reported that the virulence of distinct genotypes of Candida spp. may be different. Although there are many studies about pathogenic fungi, it has been reported that studies on virulence factors are still needed.

II. Materials And Methods

Two hundred forty three (243) neonates admitted to the neonatal intensive care unit of Gauhati Medical College & Hospital, Guwahati, over a period of 1 year (July, 2014 to June, 2015) were included in the study. Detailed clinical histories were taken and risk factor assesment of very low birth weight (LBW), prematurity, prolonged broad spectrum antibiotic therapy (BSA), prolonged use of fat emulsions in total parenteral nutrition (TPN) and the use of artificial ventilation in cases with respiratory distress (RD) was done. One to 3 mL of blood was collected from the peripheral veins of the infants using standard aseptic technique and inoculated into blood culture bottles containing brain heart infusion broth. The bottles were incubated at 37˚C and were shaken periodically. On 3rd, 5th and 7th day, subcultures were done on Sabouraud dextrose agar slants with chloramphenicol. The Germtube test for rapid morphological identification of C. albicans and C. dublinensis was done. Chlamydospore and blastoconidia production was tested on cornmeal agar. The isolates from the SDA medium were then inoculated on Chrom Agar medium and incubated at 37°C in dark for 48 hours. Apart from these, various biochemical tests like the carbohydrate assimilation and fermentation tests were done for speciation.

For determination of virulence factors yeast suspensions were prepared from the yeast isolates included in the study to evaluate phospholipase, proteinase, and hemolytic activity. First, a small amount of stock culture was inoculated on Sabouraud dextrose agar (SDA) (HiMedia) containing chloramphenicol by using a sterile loop and incubated at 37 °C for 24–48 h. Then the yeasts were harvested and suspended in sterile phosphate buffered solution (PBS) at turbidity equal to optical density (OD) of 0.5 McFarland. The final suspension was adjusted to contain 1×10^7 yeast cells/mL.

The Candida proteinase was detected by the modified Staib *et al*.[6] method, in which with Sabourad's dextrose agar (SDA), 2% dextrose, 0.1% KH2PO4, 0.05% MgSO4 were added and sterilised. The molten agar mixture was allowed to cool to 50ºC and then 1% Bovine seum albumin was added. The molten agar mixture was then aseptically poured in sterile petridish. Using a sterile well borer, small wells were punched out in the medium. Using a micropipette a 10 µl aliquots of the yeast suspension were innoculated into the well. The plates were then incubated at 37 ºC for 48 hours. After 48 hours, the plates were taken out and fixed with 20% Trichloroacetic acid for 20 minutes and then washed with unionized distilled water to remove the trichloroacetic acid. The plates were then stained with 1.25% Amidoblack for 10 minutes and washed with unionized distilled water. Glacial acetic acid (15%) was then poured over the plate and kept at 25ºC for 18 hours. After 18 hours, the plates were washed with unionized distilled water and observed for proteinase production. Positive proteinase production was determined by the presence of a zone of proteolysis around the wells that could not be stained with amidoblack. This indicates degradation of the protein by the proteinase enzyme. Negative Proteinase production was determined by the uniform staining of the entire plate by the Amidoblack stain. This indicates no degradation of the protein due to absence of proteinase.

The Candida phospholipase was detected by the modified method of Samaranayake *et al*.[7] according to which in 184ml of distilled water, 13g SDA, 11.7g sodium chloride, 0.111g calcium chloride were added and sterilized. To the mixture 20 ml supernatant of a centrifuged egg yolk emulsion was added. The final mixture was then poured into sterile petridish aseptically and allowed to cool. Using a sterile well borer, small wells were punched out in the medium. Using a micropipette a 10 µl aliquots of the yeast suspension were innoculated into the wells. The plates were then incubated at 37ºC for 48 hours, and then observed for phospholipase production. Positive phospholipase production was determined visually by the formation of zone of precipitation around the wells. This indicated degradation of the phospholipids by the phospholipases. Negative phospholipase production was determined visually by the absence of zone of precipitation around the wells. This indicated there was no degradation of the phospholipids due to the absence of phospholipases.

To determine hemolytic activity[8] SDA with 7% sheep blood was used. Using a sterile well borer, small wells were punched out in the medium. Using a micropipette a 10 µl aliquots of the yeast suspension were innoculated into the well. The plates were then incubated aerobically at 37 ºC for 48 hrs, and observed for Hemolysin production. Positive Hemolysin production was determined visually by the formation of a translucent zone of hemolysis around the wells. This indicated degradation of the hemoglobin by the hemolysins. Negative Hemolysin production was determined visually by the absence of translucent zone of hemolysis around the wells.

Biofilm was detected using sterile 96 well microtitre plates described by Melek at al[9]. By using a loop, a spot of each isolate was placed into tubes containing 2 mL of brain heart infusion broth (BHIB) medium with glucose (0.25%) and incubated at 37 °C for 24 hrs. After incubation all the tubes were diluted at a ratio of 1:20 by using freshly prepared BHIB. From this final dilution, 200 µL was placed into the wells of the microplate, which was then incubated at 37 °C for 24 h. After incubation, the microplates were rinsed with phosphate buffered saline (PBS) three (3) times and then inverted to blot. Now 200 µL of 1% crystal violet was added to each well, followed by incubation for 15 min. Biofilms that remain adherent to the walls and bottom of the wells will take up the stain properly. After incubation, the microplate was again rinsed with PBS three (3) times and then inverted to blot. Then 200 µL of ethanol**:** acetone mixture (80:20 w/v) was added to each well. They were read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader and the optical density (OD) was recorded for each well. Three wells were used for each strain and the arithmetical mean of three (3) readings was used in analysis. Sterile BHIB without microorganism was employed as the negative control. The cutoff value (OD_C) was determined by arithmetically averaging the OD of the wells containing sterile BHIB and

by adding +2 standard deviation. Samples with an OD higher than the cutoff value were considered positive, whereas those with lower value than cutoff were considered negative for biofilm production.

III. Results And Observations

The prevalence of Candidiasis in the blood culture of 243 neonates was found to be 14.4% (n=35). TABLE:1 reveals the profile of different Candida spp. isolated from the 35 culture positive cases. It is evident that NCAC species (n=32, 91.4%) outnumbered the C.albicans (n=3, 8.5%). Among the NCAC species,

Candida krusei was the most common isolate (n=19, 54.2%), followed by C.tropicalis (n=6, 17.1%), C.glabrata (n=5, 14.2%) and C.parapsilosis (n=2, 5.7%). TABLE:2 enumerates the predisposing factors that possibly led to neonatal systemic candidiasis based on an analysis of clinical histories. Of the 35 culture positive infants 34 received systemic broad spectrum antibiotic therapy, 32/35 were low birth weight, 32/35 were preterm and 24/35 suffered from respiratory distress syndrome. In TABLE:3 the virulence factor profiles of the 35 Candida spp. isolated from the neonates were evaluated. It was observed that the Candida spp. expressed the Hemolytic activity maximally (30, 85.7%), followed by proteinase (18, 51.4%), biofilm formation (15, 42.8%) and phospholipase (12, 34.2%). The TABLE 4 reveals the relationship of the virulence factors, viz: proteinase, phospholipase, hemolysin production and biofilm formation among the various Candida spp. isolated. Among the three (3) Candida albicans isolates, all expressed proteinase, phospholipase and hemolytic activity, except biofilm formation. Among the C.krusei isolates (n=19), 16 (84.2%) were positive for hemolytic activity, 10(52.6%) positive biofilm formation, 7(36.8%) positive phospholipase and 10 (52.6%) positive proteinase. Among the C.tropicalis isolates (n=6), 5(83.3%) showed hemolytic activity, 4(66.6%) positive for proteinase, 3(50%) positive biofilm formation and 1(16.6%) positive phospholipase. Among the C.glabrata isolates (n=5), none (0) were positive for proteinase, phospholipase and biofilm formation, whereas 4 (80%) showed hemolytic activity. Among the C.parapsilosis isolates (n=2), 100% were positive for hemolytic activity, 100% positive biofilm formation, 100% positive proteinase, and 1(50%) positive for phospholipase.

Figures and Tables

Table 1 Profile of Candida species in study population

SPECIES	TOTAL(n)	%
Candida albicans	3	8.5
C.tropicalis	6	17.1
C.krusei	19	54.2
C.parapsilosis	2	5.7
C.glabrata	5	14.2

Table 2 Risk factor association of culture positive cases

Risk Factor	Total(N=35)
Broad Spectrum Antibiotic Therapy	34
Low Birth Weight	32
Preterm	32
Respiratory Distress Syndrome	24

Table 3 Profile of Virulence factors detected among the Candida species

Virulence Factor	Total Positive	%
Proteinase	18	51.4
Phospholipase	12	34.2
Hemolytic Activity	30	85.7
Biofilm Formation	15	42.8

table 4 profile of virulence factors detected among the various candida species

species	virulence factors							
	PROTEINASE		PHOSPHOLIPASE		HEMOLYSIS		BIOFILM	
	+(%)	-	+(%)	-	+(%)	-	+(%)	-
C.albicans n=3	3(100)	0	3(100)	0	3(100)	0	0(0)	3
C.glabrata n=5	0(0)	5	0(0)	5	4(80)	1	0(0)	5
C.krusei n=19	10(52.6)	9	7(36.8)	12	16(84.2)	3	10(52.6)	9
C.parapsilosis n=2	2(100)	0	1(50)	1	2(100)	0	2(100)	0
C.tropicalis n=6	4(66.6)	2	1(16.6)	3	5(83.3)	1	3(50)	3

IV. Discussion

The current study reveals the profile of different Candida spp. isolated from the 35 culture positive neonates where the prevalence was found to be 14.4%. Study by Sankarankutty Jaya and Vipparti Harita[(10)] in 2013, found 16% prevalence from blood. It is evident that in the present study NCAC species (n=32, 91.4%) outnumbered the C.albicans (n=3, 8.5%). These statistics shows close resonance with studies conducted by Mohandas V, Ballal M[(11)] in 2011 and Sachin C. Deorukhkar, Santosh Saini, and Stephen Mathew[(12)] in 2014.

Among the NCAC species, Candida krusei was the most common isolate, followed by, C.tropicalis, C.glabrata and C.parapsilosis. The study conducted by Mohandas V, Ballal M[(11)] was in close conjunction. It was observed in the present study that the Candida spp. expressed the Hemolytic activity maximally (85.7%), followed by proteinase (51.4%), biofilm formation (42.8%) and phospholipase (34.2%). In a similar study by Melek et al(2012)[(13)] very close resemblance was seen. In the present study, the factors that possibly led to candidaemia were broad-spectrum antibiotic therapy, low birth weight, prematurity and ventilator therapy. Similar finding were reported in a case control study.

In the present study all Candida albicans isolates expressed proteinase, phospholipase and hemolytic activity, except biofilm formation. Among the C.krusei isolates (84.2%) were positive for hemolytic activity, (52.6%) positive biofilm formation, (36.8%) positive phospholipase and (52.6%) positive proteinase. Among the C.tropicalis isolates (83.3%) showed hemolytic activity, (66.6%) positive for proteinase, (50%) positive biofilm formation and (16.6%) positive phospholipase. Among the C.glabrata isolates, none (0) were positive for proteinase, phospholipase and biofilm formation, whereas (80%) showed hemolytic activity. Among the C.parapsilosis isolates all were positive for hemolytic activity, biofilm formation, proteinase, and (50%) positive for phospholipase. Similar findings were found in the studies conducted by Deepa K et al(2015)[(14)], Sachin et al(2012)[(15)] and A.A.Shehabi et al.(2004)[(16)].

V. Conclusion

From the present study findings, it is clear that the frequency of candidal infection rate is increasing. The keen interest in Candida species is a reflection on the incidence of Candida infections. Species of Candida other then Candida albicans which were previously considered as less or non virulent are now implicated in human disease. The study indicates higher incidence of Candida krusei then other NCAC isolates, from the neonatal intensive care unit (NICU). It has its implications, as it is known that C.krusei is intrinsically resistant to fluconazole and it has been associated with the use of fluconazole during hospitalization. And C.krusei fungemia is associated with higher mortality. The results of the present study indicate that the production of the virulence factors varies widely between the Candida spp.and within the same species. The production of hemolysin as a putative virulence factor was expressed maximally and almost equally by all the Candida isolates. Candida albicans was the forerunner in regards to production of proteinase and phospholipase then the NCAC species. Of the NCAC species C.glabrata were negative for proteinase, phospholipase and biofilm formation. Phospholipase production may be used as one of the parameters to distinguish virulent invasive strains from non invasive colonizers. An important finding was the increased production of biofilm by the NCAC species as compared to Candida albicans.

Candidal pathogenesis is a multifactorial phenomenon; therefore the nature of the Candida spp., their virulence factors, and there interaction with the host defence mechanisms need to be explored for the development of more effective antifungal therapy. Further studies on a larger cohort prospectively would be of value in confirming the findings of this study. Although phenomenal progress has been made in this field; this issue needs further investigation in order to know the exact contribution of each virulence factor, under different disease conditions.

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