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Species Distribution, Biofilm Formation and Antifungal Susceptibility of Candida Isolates in Blood Samples of NICU Patients at Tertiary Care Centre, Amritsar, India

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A B S T R A C T

To study the distribution, biofilm formation and antifungal susceptibility pattern of *Candida* species isolated from neonatal candidemia patients. *Candida* infections are a serious problem in neonatal intensive care units leads to increasing morbidity and mortality. A prospective observational study was conducted from September 2014 to June 2016. A total of 313 blood samples received in Microbiology department from NICU patients with suspected *Candida* infection were collected. Samples were processed by Gram staining, KOH mount & culture on SDA and BHI agar. Isolated yeasts were identified and speciated by germ tube test, chlamydospores formation on corn meal agar, color production on CHROM agar, sugar fermentation test and sugar assimilation test. Antifungal susceptibility testing of isolates was performed as per CLSI guidelines. Biofilm production was tested by Tube method and Tissue culture plate method. The data was statistically analysed using the statistical package for Social science (SPSS)/21.0. A total of 60 (19.17%) samples out 313 blood samples were positive for candida infections. Most common fungal isolate was *Candida tropicalis* (45%) followed by *Candida albicans* (25%), *C. parapsilosis* (11.7%), *C. glabrata* (11.7%) and *C. krusei* (6.7%.). Maximum resistance was observed to Fluconazole & Ketoconazole. Antifungal resistant was found to be more in biofilm producers. This Study highlights the emergence of *Non albicans Candida* as major isolates and increasing antifungal resistance.

Introduction

Candidiasis in neonatal intensive care units has increased steadily in incidence over the last two decades (Kossoff *et al*., 1998). The reported incidence ranges between 1.6% to 9% in very low birth weight (VLBW) and 10% to16% in extremely low birth weight (ELBW) infants with a clear association with decreasing gestational age (Johnsson *et al*., 2004). *Candida* species accounts for 9% to 13% of all hospital acquired blood stream infections (BSIs) (Beck-Sague *et al*., 1994). The incidence and associated mortality due to candidemia can be influenced by several factors including characteristic of the population at risk, standard of the health care facilities available, distribution of *Candida* species, and prevalence of antifungal resistance. Although *C. albicans*

has historically been the most frequently isolated species, infections caused by the *Non albicans Candida* have been diagnosed with increasing frequency in recent years, notably *C. tropicalis*, *C. glabrata*, *C. parapsilosis*. and *C. krusei*. There is growing evidence suggesting a role of increasing use of azole agents in this epidemiological shift. Several of these NAC species exhibit intrinsic resistance to traditional triazoles like Fluconazole (FLK) and may also demonstrate cross resistance to newer triazoles. Biofilm is an important virulence factor that can lead to antifungal resistance.

Materials and Methods

A prospective observational study was conducted from September 2014 to June 2016. A total of 313 blood samples received in Microbiology department from NICU patients with suspected *Candida* infection were collected and processed. 1-2 ml of blood sample was inoculated directly in a culture system like brain heart infusion agar and later sub cultured onto Sabourauds Dextrose Agar medium. Isolated yeasts were identified & speciated by germ tube test, chlamydospores formation on corn meal agar, color production on CHROM agar, sugar fermentation test and sugar assimilation test (Chander, 2009).

Antifungal susceptibility testing of the yeast isolates was performed by 'Disc Diffusion Method' including Amphotericin B (100 IU), Fluconazole (25µg), Nystatin (50µg), Ketoconazole (50µg), and Itraconazole (10µg) as per CLSI guidelines (Wayne, 2009).

Reference strains from quality control methods used were,

- *Candida parapsilosis* ATCC 22019
- *Candida albicans* ATCC 90028
- *Candida tropicalis* ATCC 750
- *Candida krusei* ATCC 6258

Biofilm formation ability of yeast isolates were tested by Tube Adherence Test and Tissue Culture Plate Method.

The data was statistically analysed using the statistical package for Social science (SPSS)/ 21.0 (Copyright © SPSS Inc.). Frequency of qualitative variables was calculated and correlation was tested by Chi-square test. Statistical significance was accepted at $p < 0.05$ (Park, 2011).

Results and Discussion

A total of 60 (19.17%) *Candida* isolates were obtained from 313 neonatal blood culture samples. Most common fungal isolate was *Candida tropicalis* 27/60 (45%) followed by *Candida albicans* 15/ 60 (25%), *C. parapsilosis* 7/60 (11.7%), *C. glabrata* 7/60 (11.7%) and *C. Krusei* 4/60 (6.7%).

Early neonatal period (0-6 days) 55/60 (91.67%) was most common age group followed by infantile period (2-12 month) 3/ 60 (5%) and late neonatal period (7-28) 2/60 (3.33%) found to be associated with candidemia among NICU patients.

Major risk factors determined in our study were Short for gestational age (SGA)/ Prematurity and low birth weight (≤2500 g;) 23/60 (38.33%) each followed by very low birth weight (≤1,500 g;) 10/60 (16.67%), congenital heart disease 3/60 (5%) and septicemia 1/60 (1.67%) Candidemia was found more in male babies 44/60 (73.33%) than female babies 16/60 (26.67%).

Fluconazole and Ketoconazole were main drugs found to be resistant among different *Candida* species. Resistance was more seen

among *Non albicans candida* significantly. Maximum resistance towards antifungal drug was seen in *Candida krusei* and *Candida glabrata.* Tissue culture plate method 40/60 (66.67%) was more sensitive than tube method 24/60 (40%).

A total of 40 (66%) out of 60 *Candida* isolates obtained produced biofilm. Only 26.67% (4 of 15) of *C. albicans* isolates produced biofilm, which was significantly lower than the percentage of all *Non albicans Candida* species isolates producing. slime (88.89%, 40 of 45). Strong biofilm production was seen in *C. krusei* and *C. tropicalis*. Weak biofilm production was seen in *C. albicans.* Biofilm producers were more resistant to antifungal drugs.

Candida species are frequently encountered in the normal microbiota of humans, which facilitates them to invade most implanted biomaterials and host surfaces. They are now the fourth leading cause of bloodstream infections in hospitalized patients. In our study, isolation rate of candida isolates from neonatal septicemia cases was 19.67% (60/313) which was same as several other reports showing frequency of isolation in 13.6- 19.6% cases. In the present study, *Non albicans Candida* 45/ 60 (75%) was predominantly isolated compared to *Candida albicans15/60 (*25 %). The predominance of *Non albicans Candida* species was found to be statistically significant (p <0.05). This result is collaborated well with the study by Sardana *et al*., 2012, Xess *et al*., 2007 and Baradkar *et al.,* 2008. This change in pattern has been partly attributed to increased immune suppression resulting in higher numbers of susceptible immunocompromised patients, hospitalization especially in the NICUs, placement of central venous catheters and prophylactic use of antifungal agents in critically ill patients. Among non albicans candida *C. Tropicalis* 27/60 (45%) was major isolate followed by *C. Parapsilosis* 7/60 (11.7%), *C. Glabrata* 7/60 (11.7%) and *C. Krusei* 4/60 (6.7%.). This result was found to be statistically significant (p <0.05). *C. tropicalis* was most commonly isolated in blood as the cause of candidemia in intensive care or neutropenic patients. The striking feature of the present study was the isolation of *C. parapsilosis* (11.7%) and *C. glabrata* (11.7%) as the second most common among *Non-albicans Candida* species which was in accordance with study done by Trofa *et al*., 2008. While previous study by Narain *et al*., 2003 and Narang *et al*., 1998 have documented *C. albicans* as the most common isolate from neonates. *C. parapsilosis* as an emerging fungal pathogen and a major threat for future especially among neonates in NICU's. Higher affinity of *C. parapsilosis* to adhere on foreign material and ability to form biofilms are important factors for the development of fungemia (Bonassoli *et al*., 2005).

The most common age group to be infected with candidemia was early neonatal period (0-6 days) (91.67%) which was in accordance to the study done by Juval *et al*., 2013. In our study this correlation was found to be statistically significant (p <0.05). Candidemia was found more in male babies (73.33%) than female babies (26.67%).

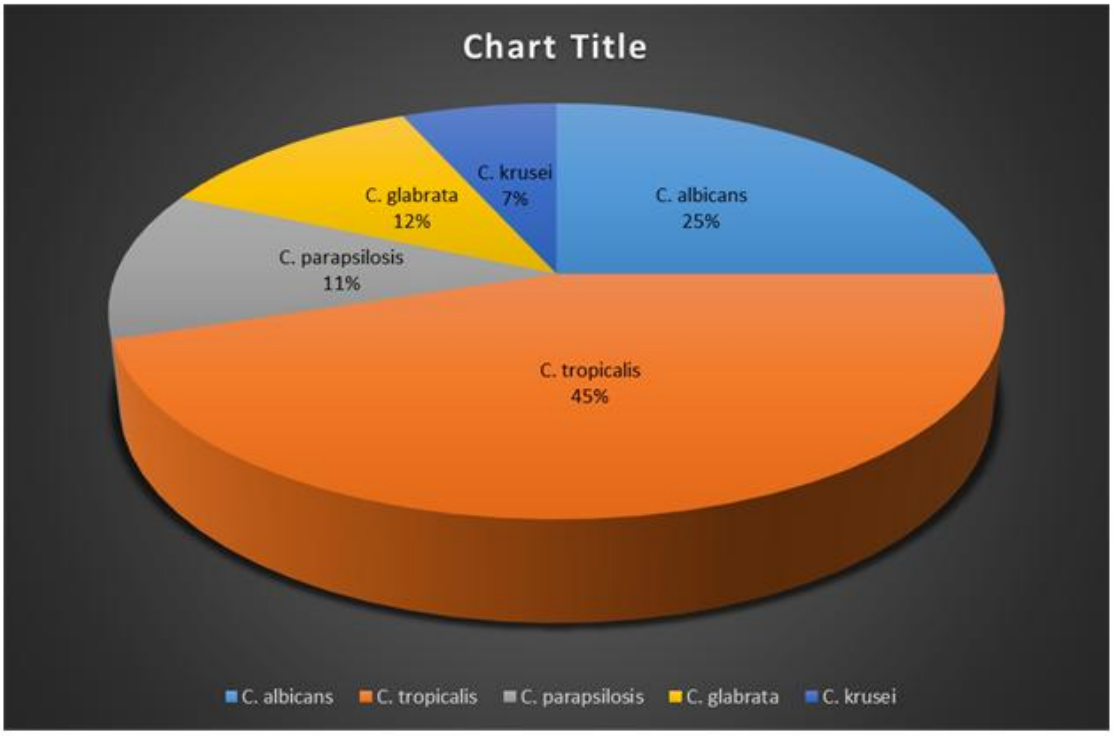
Short for gestational age (SGA)/prematurity and low birth weight were most common risk factors associated with neonatal candidemia followed by followed by very low birth weight (≤1,500 g;) (16.67%), congenital heart disease (5%) and septicemia (1.67%). In our study this correlation was found to be statistically significant (p <0.05). This was in accordance to studies by Juval *et al*., 2013 and Narain *et al*., 2003. Preterm, low birth

weight babies are more vulnerable to acute fungal sepsis, primarily because of an immature immune system, invasive interventions and prolonged use of antimicrobials that serve as risk factors for fungal colonization. Preceding colonization is an important risk factor for subsequent dissemination and invasive disease.

Antifungal susceptibility testing in our study revealed that Fluconazole (80%) and Ketoconazole (70%) were most common antifungal drug found to be resistant which was in accordance to study done by Shin *et al*., 2002. Non albicans candida species were more resistant to antifungal drugs. The possibility of increase in the percentage of the resistance to antifungal agents among *Non-albicans Species* is due to widespread use of antifungal drugs, long-term use of suppressive azoles and the use of short courses of antifungal drugs (Ng *et al*., 2001). Maximum resistance towards various antifungal drugs was found in *C. krusei* and *C. glabrata*. Similar results were obtained in study done by Sobel and Babin *et al*., 2013. In *C. krusei*, there is intrinsically resistance to azoles.

Table.1 Correlation of antifungal resistance with different *Candida* species

Candida species	Ketoconazole		Fluconazole		Itraconazole		Amphotericin B		Nystatin	
	No.	%	No.	%	No.	%	No.	%	No.	%
C. albicans	8	53.33	10	66.67	1	6.67	0	0.00	1	6.67
C. tropicalis	21	77.78	22	81.48	3	12.00	3	11.11	4	14.81
C. parapsilosis	4	57.14	4	57.14	0	0.00	1	14.29	1	14.29
C. glabrata	6	85.71	6	85.71	1	14.29	1	14.29	2	28.57
C. krusei	4	100.00	4	100.00	1	25.00	1	25.00	2	50.00



S. no.	Patient Name	Age	Sex	ward	sample type	Risk factor
263	kaka of harjinder	1	M	NICU	blood culture	SGA/Prematurity
12	Kaka s/o kashmir	1	M	NICU	blood culture	very low birth wt.
27	kaka s/o sikandar	1	M	NICU	blood culture	low birth wt.
90	jashan	1	M	NICU	blood culture	prematurity
199	gudiya of sita	1	F	NICU	blood culture	low birth wt.
230	kaka of ranjit	1	M	NICU	blood culture	SGA/Prematurity
239	kaka of dilbagh singh	1	M	NICU	blood culture	SGA/Prematurity
240	kaka of vijay	1	M	NICU	blood culture	SGA/Prematurity
242	gudiya of vikas	1	F	NICU	blood culture	SGA/Prematurity
243	kaka of bunty	1	M	NICU	blood culture	SGA/Prematurity
253	gudiya of ranjit	1	F	NICU	blood culture	SGA/Prematurity
257	kaka of gurprit	1	M	NICU	blood culture	very low birth wt.
258	shubham	1	M	NICU	blood culture	very low birth wt.
259	kaka of jewan	1	M	NICU	blood culture	very low birth wt.
264	kaka of vijay	1	M	NICU	blood culture	SGA/Prematurity
266	gudiya of kashmir	1	F	NICU	blood culture	low birth wt.
272	gudiya of sujita	1	M	NICU	blood culture	low birth wt.
282	kaka of akash	1	M	NICU	blood culture	SGA/Prematurity

A total of 40 (66%) out of 60 *Candida* species isolates obtained from the clinical isolates produced biofilm. Tissue culture plate method (66.67%) was found to be more sensitive than Tube method (40%) ($p < 0.05$). Only 26.67% (4 of 15) of *C. albicans* isolates produced biofilm, which was significantly ($p < 0.05$) lower than the percentage of all non albicans *Candida* species isolates producing slime (88.89%, 40 of 45) which was according to the findings obtained in studies done by Shin *et al*., 2002 and Gokce *et al*., 2007. Strong biofilm production was seen in *C. krusei* and *C. tropicalis*. Weak biofilm production was seen in *C. albicans.* Biofilm producers were more resistant to antifungal drugs.

In conclusion, there is significant shift from *C. albicans* to *Non albicans Candida* species. *Non albicans Candida* species are more commonly associated with antifungal resistance and biofilm production. So, it has become imperative for us to take timely steps for early speciation and antifungal susceptibility pattern of the clinically significant *Candida* isolates prevalent in our society. Early empirical antifungal therapies can also reduce the ICUs/NICUs associated mortality.

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