Neonatal sepsis due to non-*albicans Candida* species and their susceptibility to antifungal agents: first report from Bangladesh

Rafia Afreen Jalil[1*], K.M. Shahidul Islam[2], Lovely Barai[2], Shahida Akhter[3]

[1]*Department of Microbiology, Green Life Medical College, Green Road, Dhaka, Bangladesh,*
[2]*Department of Microbiology, BIRDEM General Hospital, Shahbag, Dhaka, Bangladesh,*
[3]Department *of Neonatology, BIRDEM General Hospital-2, Segunbagicha, Dhaka, Bangladesh*

Abstract

Background and objectives: Frequency of neonatal sepsis in Neonatal Intensive Care Units (NICU) has been increasing worldwide over the last decades. The emergence of non-*albicans Candida* (NAC) species and their resistance to common antifungal agents become an important preventive and therapeutic issue. The present study was undertaken to find out the role of NAC species in neonatal sepsis/candidemia in the NICUs of hospitals of Dhaka city. The susceptibility pattern of NAC species to antifungal agents was also determined.

Materials and methods: Suspected cases of neonatal sepsis admitted in NICU of four tertiary care hospitals of Dhaka city, from March to December 2018 were enrolled. In this cross sectional study, blood samples were collected from neonates with suspected sepsis for culture. Identification of *Candida* species was done by carbohydrate (CHO) assimilation tests using swab auxanographic technique, CHO impregnated yeast nitrogen base plate method (YNB), microtiter plate based miniaturized method and by HiCrome™ *Candida* Differential Media. Susceptibility of the isolated *Candida* species to antifungal agents was determined by disk diffusion (DD) and by minimum inhibitory concentration (MIC) methods. MIC was determined by broth microdilution method using RPMI 1640 and trypticase soy broth (TSB).

Results: In the present study, NAC species were isolated from 39.7% neonates. *C. tropicalis* was the predominant species (81.0%) followed by *C. parapsilosis* (12.1%), *C. auris* (5.2%) and *C. dubliniensis* (1.7%). Isolated NAC species were 98.3% sensitive to voriconazole. Sensitivity to fluconazole, ketoconazole, itraconazole, and clotrimazole was 3.5%, 15.5%, 86.2% and 56.9% respectively by DD method. All the isolates (100%) were sensitive to miconazole and nystatin. All the *C. tropicalis*, *C. auris* and *C. dubliniensis* were sensitive to amphotericin B and anidulafungin. One and four *C. parapsilosis* were found resistant to amphotericin B and anidulafungin respectively. The MIC results obtained by using RPMI 1640 and TSB as growth medium were concordant suggesting that TSB media was a good alternative to expensive RPMI 1640.

Conclusion: The advent of NAC species merits attention as they are highly resistant to most of the azoles. Therefore, speciation of *Candida* in neonatal candidemia is essential to institute appropriate antifungal therapy.

Introduction

Over the last two decades, blood stream infection (BSI) by *Candida* species has become a significant issue in neonatal intensive care units (NICUs). Candidemia is the third most common cause of late onset sepsis in neonates. It is responsible for 9-13% of BSI in neonates and is associated with high crude and attributable mortality rates [1].

*Correspondence: *Rafia Afreen Jalil, Department of Microbiology, Green Life Medical College, Green Road, Dhaka, Bangladesh. Email: rafiaafreen133@gmail.com*

Among the *Candida* species, *C. albicans* is the most commonly isolated organism. But recently non-*albicans Candida* (NAC) species have emerged as potential pathogens, particularly *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata* and *C. auris* [2-4]. Various factors such as broad spectrum antibiotics, indwelling devices, prematurity, low birth weight (LBW), total parenteral nutrition (TPN), artificial ventilation and gastrointestinal surgery contribute to the risk of fungal colonization and infection. Also, fungal colonization is associated with overcrowding in the NICU, inadequate nurse-to-patient ratio and poor hygiene practices. Approximately 10% of the newborns are colonized during the first week of life and up to 64% of them get colonized by 4 weeks stay in hospital [5-6]. *Candida* species may spread via vertical transmission from the maternal flora or by horizontal transmission from the healthcare workers (HCW) hands [7-8].

Majority of the *Candida* species become resistant to the antifungal agents, mainly to triazole compounds, by the expression of efflux pumps that minimize drug accumulation, altering the structure or concentration of antifungal target proteins and modification of membrane sterol composition [9,10]. Some NAC species are intrinsically resistant to fluconazole and newer triazoles. Therefore, speciation and antifungal susceptibility of all the yeast isolates are essential. Owing to significant regional heterogeneity, local epidemiological data is crucial in the prevention and management of invasive candidiasis.

No study has yet been carried out in Bangladesh on the frequency and the types of NAC species responsible for sepsis in neonates admitted at the NICUs of different hospitals. The present study was undertaken to determine the NAC species and their antifungal susceptibility pattern causing neonatal sepsis in the NICUs of four tertiary care hospitals of Dhaka city.

Materials and methods

This cross sectional hospital based study was carried out in the Department of Microbiology, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) in collaboration with Department of Neonatology of four tertiary care hospitals of Dhaka city. The study period was from March, 2018 to December, 2018. The study was approved by the Institutional Ethical Committee of each institution and written informed consents from patients' guardian were obtained prior to collection of samples.

Study population and collection of blood samples: Neonates admitted in respective NICUs with suspected septicemia were included in the study. About 1-2 ml of peripheral venous blood samples were collected aseptically from enrolled neonates. Immediately, 0.5-1 ml of blood was inoculated in BacT/Alert PF plus bottle and remaining 0.5-1 ml blood inoculated in the lytic blood culture tube. The specimens were transported immediately to microbiology laboratory of BIRDEM. Since *Candida* could be part of skin flora of neonates admitted in hospital, its isolation from blood culture might reflect contamination from skin flora. To rule out this contamination, a second blood sample was collected from the culture positive cases.

Candidemia was diagnosed by isolation of *Candida* species from at least two consecutive blood samples with clinical features of septicemia.

Isolation and identification of NAC species: Culture was performed using standard microbiological techniques [11,12]. *Candida* was identified by colony morphology, wet film and Gram stain. Species identification was done by germ tube test, carbohydrate (CHO) assimilation tests using swab auxanographic technique, carbohydrate impregnated yeast nitrogen base plate method and microtitre plate based miniaturized method, modified enrichment broth growth assay and HiCrome™ *Candida* Differential Media [13-16]. Yeast nitrogen base, bromocresol purple and eleven types of carbohydrates were used in all the three methods of CHO assimilation tests. In swab auxanographic method, carbohydrate was incorporated in individual discs. In the mictotitre plate and CHO impregnated YNB plate methods, the carbohydrates were incorporated in the media. Growth in the media and turning the bromocresol purple to yellow indicated utilization of particular

carbohydrate. *C. auris* was further confirmed by modified enrichment broth growth assay with salt yeast nitrogen base broth. Growth at 42^0C and development of a yellow colour in the medium indicated C*. auris*. The *Candida* isolates were inoculated on HiCrome^TM *Candida* Differential Media and incubated at 37^0C for 24 hours and the species were identified by colour of the colonies as per manufacturer's instructions.

Antifungal susceptibility tests: Antifungal susceptibility test was performed by disk diffusion method using Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue dye. Inhibition zones for fluconazole and voriconazole were interpreted according to validated CLSI (M44-A) [17], while for other drugs the inhibition zones were adopted from published studies [18-20]. Broth microdilution was done to determine minimum inhibitory concentration (MIC) of fluconazole, amphotericin B and anidulafungin as per NCCLs M27-A2 and EUCAST v 7.3.1 [21,22] using both RPMI 1640 and trypticase soy broth as growth medium. For determination of minimal fungicidal concentration (MFC), 2 µl of broth was withdrawn from the optically clear MIC well of respective antifungal agent (concentrations above the MIC) and plated on Sabouraud dextrose agar plate and incubated at 35^0C for 72 hrs. MFC was defined as the lowest drug concentration that yielded less than three colonies, a killing activity of ~ 99% [23].

Results

A total of 146 neonates with suspected sepsis were enrolled in the study. Out of 146 suspected cases of neonatal sepsis, 91 (62.3%) yielded positive blood culture. NAC species was isolated from 58 (39.7%) cases and remaining 33 (22.6%) yielded growth of bacteria (Table-1). Detail rate of isolation of NAC species from different categories of study population is shown in Table-1. Rate of isolation of NAC species from term and preterm babies were 19.1% and 43.2% respectively. Fungal culture positivity among normal birth weight (NBW), low birth weight (LBW) and very low birth weight (VLBW) babies were 26.3%, 46.8% and 33.3% respectively. Of the 58 NAC species isolated, 3 were from neonates with early-onset sepsis and the rest 55 were from cases with late-onset sepsis. The ratio of isolation of NAC species in neonates of diabetic and non diabetic mother was 2:1. Table-2 shows that *C. tropicalis* was the predominant species (81.0%) followed by *C. parapsilosis* (12.1%), *C. auris* (5.2%) and *C. dubliniensis* (1.7%).

Susceptibility of NAC species to fluconazole, ketoconazole, itraconazole, clotrimazole and nystatin by disc diffusion method is shown in Table-3. Out of 58 NAC isolates, 96.6%, 84.5%, 13.8% and 43.1% were resistant to fluconazole, ketoconazole, itraconazole and clotrimazole respectively. Except one *C. parapsilosis* isolate, none was resistant to voriconazole. None of the isolated NAC species was resistant to miconazole and nystatin.

Table-1: *Rate of isolation of NAC species and bacteria from different category of study population*

Category of study population	Number of cases	Positive for		
		Bacteria n (%)	NAC *species* n (%)	Total n (%)
All cases	146	33 (22.6)	58 (39.7)	91 (62.3)
Male	99	19 (19.2)	49 (49.5)	68 (68.7)
Female	47	14 (29.8)	9 (19.2)	23 (48.9)
Term	21	1 (4.8)	4 (19.1)	5 (23.8)
Preterm	125	32 (25.6)	54 (43.2)	86 (68.8)
NBW	19	1 (5.3)	5 (26.3)	6 (31.6)
LBW	79	12 (15.2)	37 (46.8)	49 (62.0)
VLBW	48	20 (41.7)	16 (33.3)	36 (75.0)

Note: NBW – normal birth weight, LBW – low birth weight, VLBW – very low birth weight.

Table-2: *Types of NAC species isolated from study population (n=58)*

Candida species	Number (%)
C. tropicalis	47 (81.0)
C. parapsilosis	7 (12.1)
C. auris	3 (5.2)
C. dubliniensis	1(1.7)

Table-4 shows the resistance pattern of isolated NAC species to fluconazole, amphotericin B and anidulafungin by MIC method. Among the 47 *C. tropicalis*, 44 (93.6%) were resistant to fluconazole. *C. parapsilosis*, *C. auris* and *C. dubliniensis* were 100% resistant to fluconazole by both disc diffusion and MIC methods. All the *C. tropicalis*, *C. auris* and *C. dubliniensis* were sensitive to amphotericin B and anidulafungin. Out of 7 *C. parapsilosis*, 1

(14.3%) and 4 (57.1%) were found resistant to amphotericin B and anidulafungin respectively. The MIC results obtained by using RPMI 1640 and TSB as growth medium were concordant suggesting that TSB media could be a good alternative to expensive RPMI 1640.

Table-5 shows the MIC_{50} and MIC_{90} of fluconazole, amphotericin B and anidulafungin of isolated NAC species. MIC_{50} value of fluconazole for all the NAC isolates were in the resistant range. MIC_{50} of amphotericin B for *C. tropicalis*, *C. parapsilosis*, *C. auris* and *C. dubliniensis* were 0.5 µg/ml, 1 µg/ml, 1 µg/ml and 0.25 µg/ml respectively and these values were all within the sensitive range. Only MIC_{90} of amphotericin B for *C. parapsilosis* was 2 µg/ml which was in the resistant range. MIC_{50} of anidulafungin for *C. parapsilosis* was 4 µg/ml and was in the resistant range. Both MIC_{50} and MIC_{90} of

Table-3: *Antifungal susceptibility pattern of the isolated NAC species by disk diffusion method*

Organism	Number	Resistant n (%) to						
		Fluconazole	Voriconazole	Ketoconazole	Itraconazole	Miconazole	Clotrimazole	Nystatin
C. tropicalis	47	45 (95.7)	0	38 (80.9)	8 (17.0)	0	19 (40.4)	0
C. parapsilosis	7	7 (100)	1 (14.3)	7 (100)	0	0	6 (85.7)	0
C. auris	3	3 (100)	0	3 (100)	0	0	0	0
C. dubliniensis	1	1 (100)	0	1 (100)	0	0	0	0
Total	**58**	**56 (96.6)**	**1 (1.7)**	**49 (84.5)**	**8 (13.8)**	**0**	**25 (43.1)**	**0**

Table-4: *Antifungal susceptibility pattern of isolated NAC species to fluconazole, amphotericin B and anidulafungin by MIC method*

Candida sp.	Number	Resistant to		
		Fluconazole n (%)	Amphotericin B n (%)	Anidulafungin n (%)
C. tropicalis	47	44 (93.6)	0	0
C. parapsilosis	7	7 (100)	1 (14.3)	4 (57.1)
C. auris	3	3 (100)	0	0
C. dubliniensis	1	1 (100)	0	0

Table-5: *MIC$_{50}$ and MIC$_{90}$ of fluconazole, amphotericin B and anidulafungin of isolated NAC species*

Candida sp	Number	Fluconazole		Amphotericin B		Anidulafungin	
		MIC$_{50}$	MIC$_{90}$	MIC$_{50}$	MIC$_{90}$	MIC$_{50}$	MIC$_{90}$
C. tropicalis	47	32	32	0.5	1	0.032	0.032
C. parapsilosis	7	16	32	1	2	4	4
C. auris	3	32	32	1	1	0.032	0.032
C. dubliniensis	1	16	16	0.25	0.25	0.008	0.008

anidulafungin for rest of the *Candida* species were within the sensitive range. The lowest drug concentration required to inhibit the growth of 50% of organisms (MIC$_{50}$) and minimum concentration required to kill 50% of viable organisms (MFC$_{50}$) of the antifungal drugs for all the *Candida* species were calculated and shown in Table-6. MFC$_{50}$ was 2 fold higher than MIC$_{50}$ for fluconazole, amphotericin B and anidulafungin for all the NAC species.

Table-6: *MIC$_{50}$ versus MFC$_{50}$ of fluconazole, amphotericin B and anidulafungin for all NAC species*

Antifungal agents	MIC$_{50}$ µg/ml	MFC$_{50}$ µg/ml
Fluconazole	32	64
Amphotericin B	0.5	1
Anidulafungin	0.032	0.064

Discussion

The present study has for the first time demonstrated the distribution of different non-albicans *Candida* species responsible for sepsis among neonates admitted in the NICUs of different hospitals in Dhaka city. In the current study 39.7% neonates were culture positive for NAC species. ARTEMIS Antifungal Surveillance study conducted between June 1997 and December 2007 in 41 countries reported a declining trend in isolation of *C. albicans* from 70.9% to 65.9% [24]. A similar trend of emergence of NAC species in bloodstream infection has also been documented in a number of studies [25-31].

In this study, prematurity, LBW, VLBW, antibiotic prophylaxis and extended hospital stay could be the important reasons for high isolation of NAC species. Long-term use of broad-spectrum antibiotics in routine empiric therapy also contributes to an overgrowth of opportunistic *Candida* by reducing the competitive pressure imparted by normal bacterial flora [32]. Also, increased use of azole antifungal agents, particularly fluconazole, leads to an increase in the distribution of NAC species and a decrease in *C. albicans* [33].

In our study, we also observed higher rate of fungal septicemia in neonates born to diabetic mothers than that of non-diabetic mothers (66.7% versus 22.5%; p<0.05)). Pregnant women with diabetes are at high risk of infection with *Candida* because the raised sugar promotes overgrowth of *Candida* and thus the neonates of the diabetic mothers are prone to be colonized with *Candida* from birth [34].

In the present study, among NAC species, *C. tropicalis* was the most common species (81.0%). Various other studies have also reported *C. tropicalis* to be the most common isolate [3,30,31]. Pressure of fluconazole prophylaxis could be the reason behind this high rate of isolation of *C. tropicalis* in this study.

The isolated NAC species were 98.3% sensitive to voriconazole and this finding is similar to the results published by other studies [35,36]. In the present study, resistant rate to fluconazole by the *Candida* isolates was 87.9% by disk diffusion and 94.8% by MIC method. All the C*. parapsilosis* and *C. auris* were resistant to fluconazole while *C. tropicalis* isolates were 93.6% resistant. These findings were in agreement with Pandita et al. and Yadav et al [37,38]. All the *C. tropicalis*, *C. auris* and *C. dubliniensis* were sensitive to amphotericin B and anidulafungin. Out of 7, only 1 (14.3%) *C. parapsilosis* was found resistant to amphotericin B and 4 (57.1%) were resistant to anidulafungin. Therefore, it appeared that amphotericin B and anidulafungin could be used against NAC species when the organisms become

resistant to other antifungal agents. There are only few literatures available regarding the use of echinocandin particularly anidulafungin in the pediatric ICU. Anidulafungin might be used as an alternative drug in neonates particularly when the local *Candida* strains are resistant to azoles. However, since the first introduction of echinocandins, these antifungal agents have exhibited higher minimum inhibitory concentrations against *C. parapsilosis*. Compared to other species of *Candida*, *C. parapsilosis* demonstrates higher *in vitro* MICs to echinocandin, and treatment failures with these antifungal agents have been reported for *C. parapsilosis* infections [39,40].

Antifungal susceptibility was done by disk-diffusion and MIC broth microdilution methods. RPMI 1640 is the proposed medium for carrying out micro broth dilution by EUCAST and NCCLs (M27-A2). However, RPMI 1640 is very expensive and not easily available in many laboratories. So, MIC of fluconazole and amphotericin B for *Candida* species were done using both TSB and RPMI 1640 medium and the results were compared. The MIC results obtained by both the media were concordant suggesting that TSB broth media could be a good alternative to RPMI 1640.

In our study, we observed the minimum fungicidal concentrations (MFC) of fluconazole, amphotericin B and anidulafungin as 2 fold higher than the MIC. Very little is known regarding the role of differences between MIC and MFC in treatment failure, and further studies are required.

The present study provided evidence of colossal burden of NAC species as an important cause of neonatal sepsis in our NICUs. The isolated NAC species were found highly resistant to widely used fluconazole whereas amphotericin B, voriconazole and anidulafungin were the most effective agents. The results of our study could be used as a template for the establishment of local guidelines for the effective treatment and prevention of neonatal candidemia.

Competing interest
The authors declared no competing interests.

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