Original Article

Neonatal Candidaemia in Tertiary Care Hospitals in Dhaka- A Comparison of Different Carbohydrate Assimilation Methods and Chromagar Technique for Speciation

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Abstract

Background: Nosocomial candidiasis are becoming increasingly important worldwide. Candida is a major causative agent of health care associated bloodstream infections, and lately non-albican Candida species are increasingly isolated from blood samples. Some of the Candida species have intrinsic and acquired resistance to the limited arsenal of antifungals; therefore early speciation is essential for the timely initiation of effective antifungal therapy.

Methods: A hospital based cross-sectional study was conducted to evaluate the performance of different carbohydrate assimilation tests and commercially available HiCrome Candida Differential Media (CHROMagar) for the identification of Candida in the four tertiary level hospitals in Dhaka.

Results: A total of 58 yeasts samples was included in this study. Non-albicans Candida accounted for 100% of the isolates of which *C. tropicalis* was the predominant species (81.03%) followed by *C. parapsilosis* (12.07%), *C. auris* (5.17%) and *C. dubliniensis* (1.72%). Swab auxanographic technique and microtitre plate based miniaturized CHO assimilation methods were equally effective in identification of Candida sp. in comparison to CHO impregnated YNB plate method (98.28% and 100% vs 89.66%).

Conclusion: By using Chromogenic agar 75.86% yeasts were identified but it could not give the conclusive differentiating color between the species of *C. parapsilosis* and *C. auris*.

Keywords: Neonatal candidaemia, carbohydrate assimilation methods, chromagar technique.

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Introduction

The rate of mycotic infections has dramatically increased over the last few years. Fungi once thought to be non-pathogenic or less virulent are nowadays considered as the major cause of morbidity and mortality not only in immune-compromised and critically ill patients, but also in immunocompetent hosts.[1] Globally, invasive candidiasis has been reported as a leading cause of morbidity and mortality in neonatal intensive care units (NICU). This is the third commonest cause of late onset sepsis in NICU patients and responsible for 9-13% of bloodstream infections (BSI) in neonates.[2]

In the recent decades *C. albicans* were the most commonly isolated organism but lately non albicans Candida have emerged as a potential pathogen, eminently *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. glabrata*.[3,4] This paradigm shift has caused a major clinical

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impact because of reduced sensitivity of these non-albican yeasts to antifungal agents.[5] Recently identified and emerging *C. auris* has caused invasive infections globally and has been embroiled in difficult to control hospital outbreaks. More than 90% and 35% of the isolates are resistant to fluconazole and amphotericin B respectively and thereby challenges clinicians with limited antifungal depot and questions regarding optimal care.[6] There is need for speciation as it helps to detect strains that may be inherently resistant to some of the antifungal agents or have acquired resistance during treatment.[7-9]

Candida speciation is conventionally performed by culture on Sabouraud Dexrose Agar (SDA), wet film, germ tube test, sugar assimilation and sugar fermentation assays. Newer techniques include CHROM agar, API systems, Vitek 2 ID system and molecular methods.[10-12] However, for a developing country like Bangladesh these newer methods are not cost effective. Carbohydrate (CHO) assimilation test was first introduced by Wickerham and Burton in 1948 and is probably the most commonly used of all method for yeast identification.[13] It involves the use of carbohydrate free yeast nitrogen base (YNB) agar and observing any growth on carbohydrate containing media after an adequate incubation period. The test is said to be positive when there is presence of growth of the media and also by an alteration in the colour of an indicator. Chromogenic agar is a selective and differential medium, including chromogenic substrates that react with enzymes released by target organisms to produce colonies of varying colours.

Diagnostic microbiology laboratories in Bangladesh provide a range of services in bacteriology but their services in mycology are still limited to direct microscopic examination and culture of clinical specimens. In the perspective of the above discussions, the present study was designed to identify the isolated Candida at species level by using multiple standard methods to find out the most convenient method for regular practice in microbiology laboratory.

Materials and Methods

This hospital based cross sectional study was carried out to evaluate the performance of different carbohydrate assimilation tests and commercially available HiCrome Candida Differential Media (CHROMagar) for the identification of Candida in the four tertiary level hospitals from March, 2018 to December, 2018. Informed written consent obtained from patient's guardian. A total of 146 peripheral venous blood samples from neonates with suspected septicemia admitted in the NICUs of four tertiary care hospitals- Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM); Bangabandhu Sheikh Mujib Medical University (BSMMU); Dhaka Medical College Hospital (DMCH) and Green Life Medical College Hospital (GMCH) were collected aseptically in lytic tubes. Candidemia was diagnosed by isolation of Candida species from at least two blood culture samples or at least one positive blood culture containing pure growth of Candida species with supportive clinical features. Lytic tubes were vortexed, blood was drawn in a sterile disposable syringe and then inoculated into Blood agar and MacConkey agar and incubated aerobically at 37°C. Isolated yeast colonies were examined by wet film and Gram staining and then inoculated into Sabourauds Dextrose agar media. For initial speciation, Germ tube test was done and the positives identified were either *C. albicans* or *C. dubliniensis*. Candida species were further identified by carbohydrate assimilation test, modified enrichment broth growth assay and subculture in chromogenic agar media.

Assimilation was done by 3 techniques: swab auxanographic technique, microtitre plate based miniaturized carbohydrate assimilation test and CHO impregnated yeast nitrogen base plate method. Yeast nitrogen base agar, Bromocresol purple and 11 carbohydrates namely glucose, sucrose, maltose, galactose, lactose, cellobiose, raffinose, rhamnose, arabinose, xylose and mannitol were used in all the 3 methods. In swab auxanographic method, carbohydrate was incorporated in individual discs and in the mictotitre plate and CHO impregnated YNB plate methods, the carbohydrates were incorporated in the media. Growth in the media correlated with pH change turning the bromocresol purple to yellow. Assimilation of different carbohydrates by different species of Candida is shown in Figure 1.

Swab auxanographic technique

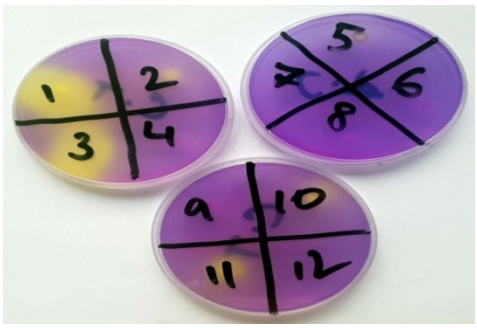
The YNB agar plates with bromocresol purple were streaked for confluent growth with a swab saturated in the dense suspension of Candida isolate equivalent to a McFarland Standard No. 4 (approx. cell density $12x10^8$ CFU/ml) and after 15 minutes carbohydrate discs were applied to the surface of the agar in the four quadrants and pressed down firmly with a flamed forcep. Three agar plates were used for each yeast isolates. The carbohydrate disks were prepared in the laboratory by soaking filter paper in 10% solution of each carbohydrate. The plates were incubated for 24 to 48 hours at 37°C and observed for the formation of a yellow zone around the discs indicating utilization of the respective carbohydrates.[14]

Carbohydrate impregnated YNB plate method

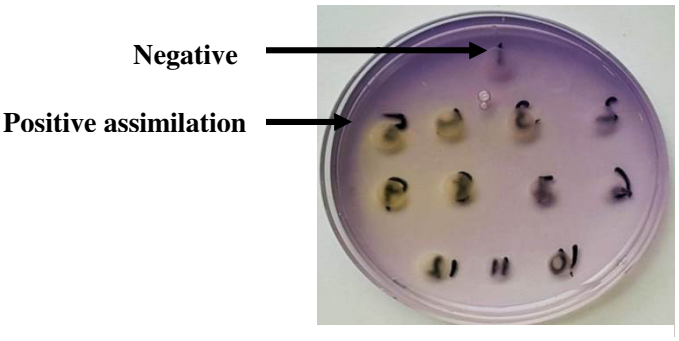
Individual carbohydrate and bromocresol purple containing YNB agar media was prepared and stored at 2-8°C until further use. The carbohydrate containing medium along with the control medium was inoculated with 5 µl of the yeast inoculum ($12x10^8$ cells) calibrated with Mc Farland Standard No. 4, incubated at 37° C for 24 and 48 hours and observed for growth. About 12 different yeast isolates can be tested simultaneously in each plate. Well circumscribed opaque yeast growth with a yellow hue was indicative of assimilation of the test carbohydrate. Translucent to opalescent growth with the outline of initial inoculum alone was indicative of lack of assimilation.[15]

Microtitre plate based miniaturized carbohydrate assimilation test

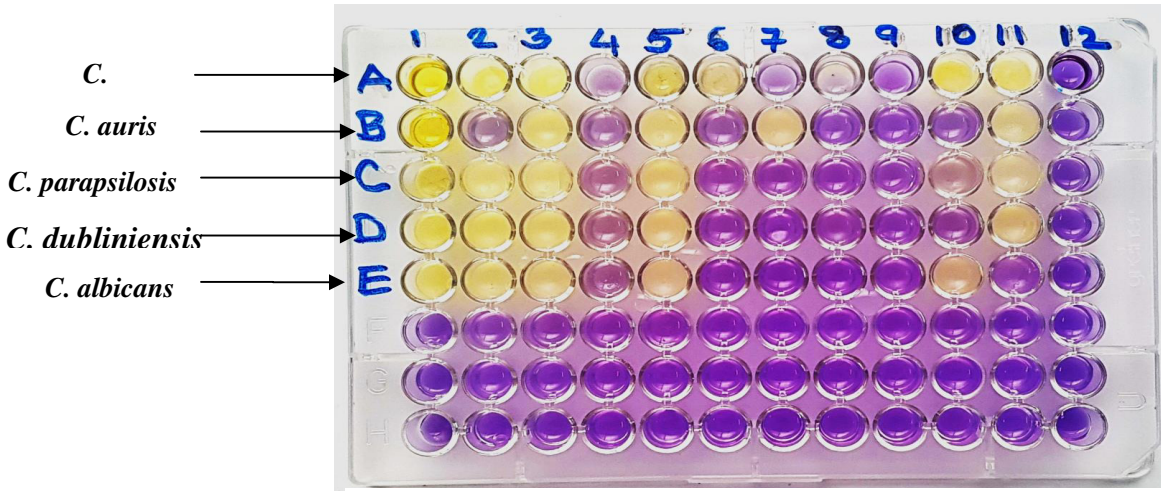
The carbohydrate assimilation test was devised in a U-shaped 96 well (8x12) microtitre plate. 167.5mg of YNB, 5g agar and 5mg bromocresol purple was added to 250 ml distilled water and autoclaved at 121°C for 15 minutes. 20 ml of the media was poured in each of the 11 sterile falcon tubes and labeled properly with the name of each carbohydrate. Then 2ml of 10% filter sterilized 11 carbohydrate solutions were added to each of the falcon tubes containing the media. The whole procedure was carried out in a water bath with the temperature set at 50°C. The wells of the vertical rows of the microtitre plate were utilized for testing assimilation reaction of a particular carbohydrate. 250ul of the media containing 1% of the test sugar were added to each well of the vertical row and labeled properly. The 12th row served as the control containing sugar free media only. The microtitre plates covered with lids were stored at 2-8°C until use.Yeast suspension in sterile distilled water equivalent to 0.5 McFarland standards was prepared and using a pipette 1µl of it was inoculated in each well of the horizontal column. Eight different yeast isolates can be tested using a single microtitre plate. The microtitre plate was covered with the lid and incubated at 37°C for 18 to 24 hours and development of a yellow colour was considered as a positive result.



Swab auxanographic technique



CHO impregnated YNB plate method



Microtitre plate based miniaturized CHO assimilation test

Figure 1: Carbohydrate (CHO) assimilation test by different methods

C. auris was further confirmed by modifed enrichment broth growth assay with salt, yeast nitrogen base broth.[16] *C. auris*, a local laboratory strain confirmed by McLab, California, USA served as a positive control and *C. tropicalis* confirmed by subculture on Chromogenic agar and carbohydrate assimilation test was used as a negative control. Each strain was grown in 3 replicate eppendorfs at 42°C for 72 hours. Only *C. auris* is halotolerant and can grow at 42°C so development of a yellow colour in the medium was considered as a positive result (Figure 1).

HiCrome™ Candida Differential Media was prepared according to the manufacturer's instruction (Hi-Media, India). Suspension of isolates was prepared in sterile distilled water (turbidity was adjusted to 2 McFarland standard) from primary culture in SDA and was inoculated onto the Chromogenic agar medium. The plates were incubated at 37°C for 48 hours. The sensitivity and specificity of chromogenic agar media considering carbohydrate assimilation test as gold standard[17,18] was calculated.

Results

Table 1 shows that out of 146 blood samples, 33(22.60%) Bacteria and 58(39.73%) Candida was isolated. Isolation rate of Candida was 63.74%(58 out of 91) among all isolated organisms. Table 2 shows that Non-albicans Candida accounted for 100% of the isolates of which *C. tropicalis* was the predominant species (81.03%) followed by *C. parapsilosis* (12.07%),

C. auris (5.17%) and *C. dubliniensis* (1.72%). Out of 58 isolated Candida, 75.86% were identified by chromogenic media, 98.28% by swab auxanographic technique, 100% by microtitre plate method and 89.66% by CHO impregnated YNB plate method (Table 3).

Table 1: Rate of isolation of microorganisms from study population (n=146)

Study population	Total cases	Blood culture positive for		
		Bacteria n(%)	Candida sp. n(%)	Total n(%)
Suspected EONS	44	8(18.18)	3(6.82)	11(25)
Suspected LONS	102	25(24.51)	55(53.92)	80(78.43)
	146	**33(22.60)**	**58(39.73)**	**91(62.33)**

Table 2: Distribution of Candida species isolated from blood sample cultures from NICUs of 4 hospitals (n=58)

Candida species	BIRDEM n(%)	BSMMU n(%)	DMCH n(%)	GMCH n(%)	Total n(%)
C. tropicalis	37 (84.09)	6(100)	2(40.00)	2(66.67)	47(81.03)
C. parapsilosis	4(9.09)	0(0)	2 (40.00)	1(33.33)	7(12.07)
C. auris	3(6.82)	0(0)	0(0)	0(0)	3(5.17)
C. dubliniensis	0(0)	0(0)	1(20.00)	0(0)	1(1.72)
	44(75.86)	**6(10.34)**	**5(8.62)**	**3(5.17)**	**58(100)**

Table 3: Identification of different species of Candida by different methods (n=58)

Species identified	Total cases	Chromogenic media n(%)	Swab auxanographic technique n(%)	Microtitre plate CHO assimilation n(%)	CHO impregnated YNB plate method n(%)
C. tropicalis	47	43(91.49)	47(100)	47(100)	41(70.69)
C. parapsilosis	7	Unidentified	6(85.71)	7(100)	7(100)
C. auris	3	Unidentified	3(100)	3(100)	3(100)
C. dubliniensis	1	1(100)	1(100)	1(100)	1(100)
	58	**44(75.86)**	**57(98.28)**	**58(100)**	**52(89.66)**

Table 3 shows in the chromogenic agar media, 43(91.49%) Candida were identified as *C. tropicalis* and 1(100%) was *C. dubliniensis*. But chromogenic agar could not give conclusive differentiating colour between species of *C. parapsilosis* and *C. auris*, further identification was done by carbohydrate assimilation test. Considering swab auxanographic technique of Carbohydrate assimilation test as the gold standard, the sensitivity and specificity of chromogenic agar media for *C. tropicalis* were 91.49% and 100% respectively. *C. dubliniensis* showed 100% specificity and 100% sensitivity in chromogenic media. The colour and colony characteristic of most of the Candida species in chromogenic agar media was similar as mentioned by the manufacturer in Table 4 and Figure 2.

Table 4: Color and colony characteristics of Candida species in chromogenic agar media

Species	Colony colour
C. tropicalis	Dark blue to metallic blue-purple
C. parapsilosis	Pink to pinkish purple
C. auris	Creamy beige colony with a purple hue
C. dubliniensis	Dark blue-green

Candida tropicalis *Candida parapsilosis* *Candida auris* *Candida dubliniensis*

Figure 2: *Candida* sp. identified in HiCrome Candida Differential media

Discussion

In this study, out of 58 Candida isolated from 146 blood cultures, all were non-albicans Candida sp. of which *C. tropicalis* was the predominant species (81.03%) followed by *C. parapsilosis* (12.07%), *C. auris* (5.17%) and *C. dubliniensis* (1.72%). Several studies have indicated that the increased use of azole agents, particularly fluconazole, has led to an increased distribution of non-albicans Candida and a decline in *C. albicans* with azole use may be responsible for changes in Candida etiology.[19] Since majority of the Candida isolates (75.86%) were from a hospital where fluconazole prophylaxis is practiced, from this study it is evident that antifungal prophylaxis is the leading cause of this epidemiological shift in Candida species.

Similar pattern of non-albicans Candida in blood stream infection have also been documented in a number of Indian studies. Femitha et al reported 44.4% incidence of *C. glabrata* followed by 25% of *C. albicans*.[20] Rani et al reported 92% incidence of *C. tropicalis* and only 4% incidence of *C. albicans* while Gunjan et al reported 85.6% incidence of non-albicans species.[21,22] In the present study, among the 63.73% of candidal septicaemic cases, *C. tropicalis* was the most common isolate (81.03%). Various other studies have also reported *C. tropicalis* to be the most common isolate.[23-25] *C. auris*, an emerging fungus has become a global nosocomial concern. The first outbreak of *C.auris* was reported in Europe in 2016 and up till 2019 more than 30 countries across the world have documented its presence in clinical samples.[26]

Species identification was done by performing carbohydrate (CHO) assimilation test and using HiCrome Candida Differential media. Assimilation was done by 3 techniques: swab auxanographic technique, microtitre plate based miniaturized carbohydrate assimilation test and CHO impregnated yeast nitrogen base plate method. Out of 58 isolated Candida, 98.28% were identified by swab auxanographic technique, 100% by microtitre plate method and 89.66% by CHO impregnated YNB plate method. Bromocresol purple was added in all 3 methods and growth in the media correlated with pH change turning the bromocresol purple to

yellow. However, from the present study it was seen that omission of bromocresol purple in the CHO impregnated YNB plate method would have yielded better result. Twelve organisms were tested on 1 plate so diffusion of yellow colour gave false results. Microtitre method was easy to perform; rapid, easily interpretable and 8 Candida can be processed at a time. Only disadvantage was that it was time consuming and labourous process to prepare the microtitre wells for the test to be carried out later. Microtitre plate required incubation of 18-24 hours, while the other methods required 24-48 hours. Microtitre based miniaturized carbohydrate assimilation test and CHO impregnated YNB plate method have never been used in Bangladesh for identification of Candida sp. As far as our knowledge goes this microtitre based miniaturized carbohydrate assimilation test has not been tried elsewhere.

In chromogenic agar media, 43 (91.49%) species were identified as *C. tropicalis* and 1(100%) was *C. dubliniensis*. But chromogenic agar could not give the conclusive differentiating color between the species of *C. parapsilosis* and *C. auris*, further identification was done by carbohydrate assimilation test. *C. tropicalis* produced dark blue to metallic blue purple colonies, *C. parapsilosis* produced pink to pinkish purple colonies and *C. dubliniensis* produced dark blue-green colonies. Colonies of *C. auris* were creamy beige with a purple hue. Considering carbohydrate assimilation test as the gold standard, the sensitivity and specificity of chromogenic agar media for *C. tropicalis* were 91.49% and 100% respectively. Nigar et al in a study in Bangladesh reported the sensitivity and specificity of chromogenic agar media for *C. tropicalis* were 94.12% and 97.87%.[27] Baradkar et al showed 100% sensitivity and 100% specificity of chromogenic agar for *C. tropicalis.*[28] Willinger et al showed sensitivity of 66.1% and specificity of 99.8% on chromogenic agar.[29] *C. dubliniensis* showed 100% specificity and 100% sensitivity in chromogenic media which is similar to the finding of another study.[30]

C. auris was further confirmed by modifed enrichment broth growth assay with salt yeast nitrogen base broth. Only *C. auris* is halotolerant and can grow at 42°C so development of a yellow colour in the medium was considered as a positive result.

Conclusion

The alarming increase in the rate of non-albicans Candida demonstrated in the current study provides insight into the importance of speciation as it has a direct influence on the choice of empirical antifungal therapy. *Candida tropicalis* was the most common non-albicans Candida species isolated from blood. Both swab auxanographic and microtitre plate based methods can be used as a diagnostic tool in the laboratories for identification of species of Candida and will help for selection of appropriate antifungal agents. However, both the methods were labourous and time consuming, but in contrast species identification by chromogenic agar media was less time consuming and its efficacy was almost similar to the carbohydrate assimilation methods.

Ethical approval: Ethical approval was obtained from the Institutional Review Board (IRB) of the BIRDEM General Hospital, Dhaka 1000, Bangladesh. (Memo: BIRDEM/IRB/2018/133)

Author's Contributions: Jalil RA did the literature review & conceptualized the study; Jalil RA & Nurunnabi M performed statistical analyses; Jalil RA & Nurunnabi M prepared the first draft of the manuscript; and Jalil RA, Nurunnabi M, Jahan S &Islam KMS did the

critical review of the manuscript. All the authors approved the final manuscript.

Competing Interests: The authors declared no competing interest associated with this manuscript.

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