SPECIES DISTRIBUTION AND ANTIFUNGAL SUSCEPTIBILITY PROFILE OF CANDIDA SPECIES ISOLATED FROM BLOOD STREAM INFECTIONS

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ABSTRACT: **BACKGROUND:** Candidemia remains as one of the major cause of morbidity and mortality in health care setting. Over the last two decades, the proportion of blood stream infection (BSI) due to non-albicans Candida (NAC) species has increased. Several NAC spp. are inherently resistant to commonly used antifungal drugs. The increased isolation rates of NAC spp. and a gradual shift in the antifungal susceptibility profile underlines the need of early and accurate diagnosis of infecting Candida spp. along with antifungal susceptibility testing for selecting the most appropriate antifungal agent for therapy. **AIM:** The Aim of the present study was to investigate the distribution pattern of Candida spp. isolated from candidemia patients and evaluate its antifungal susceptibility pattern. **SETTING AND DESIGN:** The present study was conducted in the department of Microbiology for a period of six years (January 2006 to December 2011) which included, 194 Candida spp. isolated from the cases of candidemia. **MATERIALS AND METHODS:** Candida isolates were speciated by conventional techniques and HiCandida identification kit. Antifungal susceptibility test was performed using two disc diffusion methods. Clinical details and risk factors were recorded and analyzed. **RESULTS AND CONCLUSIONS:** Isolation of NAC spp. was significantly higher than Candida albicans. The most important risk factor associated with candidemia was intensive care unit stay, followed by diabetes and HIV infection. Azole resistance was more in NAC species as compared to C. albicans**.** The early and accurate diagnosis of infecting Candida spp. along with antifungal susceptibility testing plays a pivotal role in preventing morbidity and mortality associated with Candida BSI. Disc diffusion technique for antifungal susceptibility using glucose methylene blue Mueller- Hinton (GM-MH) agar was found to be simple, cost effective and sufficiently accurate for the routine testing of antifungal susceptibility of Candida spp. in resource constrained microbiology laboratories.

KEY WORDS: Candidemia, Candida albicans, NAC species, antifungal resistance

INTRODUCTION: Candida blood stream infection (BSI) has become a major problem in tertiary care hospital worldwide [1]. Despite some improvement in fungal BSI diagnosis during recent years, diagnosis of candidemia remains difficult [2]. Candidemia has been associated with many risk factors like long- term hospitalization, antibiotic therapy, use of intravascular catheters, and underlying diseases like diabetes and malignancy. Although Candida albicans continues to be

the most common cause of Candidal BSI, the epidemiology of species causing candidemia is changing. Recent longitudinal studies have detected an increase proportion of BSI by non-albicans Candida (NAC) species [3, 4].

Importantly many NAC spp. have decreased susceptibility to antifungal agents. Specifically C. krusei and C. glabrata demonstrate decreased susceptibility to fluconazole [5]. The changing epidemiology of Candida BSI has generated concern about the emergence of azole drug resistance and its clinical relevance.

Clinicians now depend on identification of Candida to the species level in order to optimize the selection of antifungal agents allowing them to provide the best possible patient care [6]. Therefore there is a need for continuous surveillance to monitor trends in incidence, species distribution and antifungal drug susceptibility profiles of Candida BSI.

The Clinical and Laboratory Standards Institute (CLSI) (previously National Committee for Clinical Laboratory Standards (NCCLS)) after collaborative efforts with various laboratories has recommended an in-vitro standardized macro broth dilution antifungal susceptibility testing technique for yeasts [7]. CLSI has recommended alternative techniques like ATB fungus, API, Vitek and disc diffusion to address issues in different laboratories. These methodologies give reproducible results.[8] Disc diffusion procedure appears to be generally acceptable as a simple, in house standardized procedure for antifungal susceptibility of yeasts [9].

The present study was carried out in a rural tertiary care hospital with an aim to determine the distribution of Candida spp. isolated from the cases of candidemia and to compare the efficacy of yeast nitrogen base with glucose (YNBG) media and glucose methylene blue Mueller- Hinton (GM-MH) agar for the antifungal susceptibility testing of Candida isolates.

MATERIALS AND METHODS: The present study is part of a PhD thesis and was approved by the Institutional Ethics Committee (Registration No.FN.32/2010). A total 194 Candida spp. isolated from the blood of patients was included in the study.

The culture was considered true candidemia only when Candida spp. was isolated from at least two blood culture samples or from a clinically significant single blood culture sample among hospitalized patients [10]. Patient's demographic features such as age, sex, ward, date of admission, underlying illness, various associated risk factors like presence of urinary catheter, respiratory ventilation, central line insertion, duration of antibiotic therapy and antifungal prophylaxis if any, were recorded and analyzed.

SPECIES IDENTIFICATION: Speciation of Candida isolates was done by conventional techniques and colony colour on Chrom agar [11] Hicandida identification kit (Himedia Laboratories Pvt. Ltd Mumbai, India) was used for the identification of isolates which could not be identified by conventional techniques.

ANTIFUNGAL AGENTS: The antifungal agents used were amphotericin B (10 µg), fluconazole (25 µg), and itraconazole (10µg). Antifungal discs were procured from Himedia Laboratories Pvt. Ltd Mumbai, India.

ANTIFUNGAL SUSCEPTIBILITY TESTS:

Antifungal susceptibility tests were performed using 2 disc diffusion methods.

1. Yeast Nitrogen base with glucose (YNBG) media.
2. Glucose methylene blue Mueller- Hinton (GM-MH) agar.

ANTIFUNGAL SUSCEPTIBITY TESTING USING YNBG MEDIA:[9] YNBG media was prepared using yeast nitrogen base 10g and glucose 10g, dissolved in 100ml of distilled water and filter sterilized. Susceptibility test against azoles was performed with addition of 1.5% L-asparagine to YNBG media. YNBG media mixed with 2% sterile Difco agar (Difco, USA) was poured into 9cm diameter Petri dishes. Inoculum size of 10^6 Candida cells/ml was inoculated in one half with the control strain (C. kefyr Y/16). The cell density was spectrophotometrically adjusted to 0.5 McFarland standard. Inoculation was done by swabbing from the edge of the plate to the centre using a sterile swab. Discs were placed in the centre of the control and test organism with the help of sterile forceps. After incubation of the plate at 37^0C for 48 hours the diameter of inhibition was read.

The result of the disc diffusion method was interpreted according to the following criteria: test strain was considered sensitive when the zone diameter was ≥ 80% of the zone diameter of control strain; intermediate when the zone diameter was < 80% but there was visible zone of the inhibition; resistant, when there was no zone of inhibition.

2. BY USING GM-MH AGAR: Mueller-Hinton (MH) agar was solidified after addition of 2% glucose and 0.5ug of methylene blue. Inoculum was prepared by picking five distinct colonies of approximately 1mm from 24 hours old culture grown on Sabouraud's dextrose agar (SDA). Colonies were suspended in 5ml of sterile 0.85% saline. This suspension was vortexed to adjust the turbidity yielding $1x10^6$-$5x10^6$ cells/ml and streaked on the entire surface of GM-MH agar. The antifungal disc was placed 24mm apart from each other. The plates were incubated at 37^oC for 24 hours. If insufficient growth was observed after 24 hours the plates were read after 48 hours. Zone diameters were interpreted as per the approved CLSI /NCCLS (M44-A) guidelines [12]. The quality control test was performed by using C. parapsilosis (ATCC 22019), C. krusei (ATCC 6258), and C. albicans (ATCC 90028).

RESULTS: Between January 2006 to December 2011, out of 4984 blood culture samples processed in Department of Microbiology, Candida spp. were isolated from the blood culture of 194 patients. Male predominance was noted in our study (n=126/194) 64.9%. Candidemia was common in more than 50 years age group in males (n=58/126) 46.03%, whereas in females it was with 0-10 years age group (n=22/68) 32.35% (Figure.1). The most important risk factor associated with candidemia was ICU stay (49\194) 25.25%, followed by diabetes (40\194) 20.61% and HIV infection (38\194) 19.58% (Figure.2). In this study predominant isolates were NAC spp. C. albicans was isolated from 78 (40.2%) cases. Among the NAC species, C. tropicalis, C. glabrata and C. krusei were the major isolates. (Figure.3). All 194 isolates recovered were tested for antifungal susceptibility by using YNGB medium and GM-MH agar. Their pattern of resistance is summarized in Table 1. NAC spp. showed more resistance to antifungal agents as compared to C. albicans.

DISCUSSION: In the last twenty years, various factors like the AIDS epidemic, increase in the number immunosuppressive therapy recipients and the use of long term antibiotic therapy have altered the epidemiology of invasive mycoses in general and of candidemia in particular. Candida spp. is the fourth most common pathogens isolated from the blood of hospitalized patients[13]. More recently, NAC spp. has been recovered with increasing frequency. Linked with this is a recent increase in treatment failure of these infections to standard antifungal therapy, largely due to the emergence of drug resistance in fungi.

In our study the Candida spp. were isolated from 3.9% of total blood cultures processed in the Department of Microbiology. Lot of variation in the prevalence and incidence of candidemia have been reported from India. Kumar et al[14] from South India reported an incidence rate of 5.7% for candidemia among children with onco-haematological malignancies. Verma et al [15] reported an incidence rate of 1.61% whereas, Xess et al [16] found a prevalence rate of 6% for candidemia. A study by Sahni et al [17] from Maulana Azad Medical College, New Delhi, found an incidence rate of 6.9% for Candida spp.

The age and sex distribution of the patients in our study correlates with the observation of other researchers like Verma et al [15] and Hajjeh et al [18]. The importance of risk factors analysis cannot be over emphasized for infection like candidemia so that preventive measures and prophylactic therapy can be initiated for patients at risk. Many studies have established independent risk factors for candidemia on the basis of multivariate analyses. The ICU stay followed by diabetes and HIV infection was the major risk factors responsible for candidemia in our study. This was also an observation of other researchers like, Shivaprakasha et al [10], Verma et al [15]and Sandven et al [19]. This might be because of severely ill and immunocompromised patients being cared for in the unit with most of them being on life support systems. Wenzel and Gennings [20] and Shorr et al [21] have tried to develop risk assessment strategies and calculate "Candida scores" to predict the true risk of disease in patients admitted in ICU. Candida risk scores may help clinician to rule out candidemia and to identify the patients at high risk of developing Candida BSI in the hospital stay. Al- Attas et al [22] have reported high Candida spp. colonization in diabetic patients compared to control subjects. Isolates colonizing diabetic patients have also been found to show a greater degree of resistance to antifungal agents than strains isolated from control subjects.

Our study also underlines the importance of HIV infection as factor contributing to candidemia. In the United State the proportion of candidemia cases varied from 10% to 15% [23]. The contribution of HIV infection as a predisposing risk factor for candidemia is further emphasized by a report from Italy, where Candida spp. was the third most common cause of blood stream infections in HIV patients [24]. From India Chowta et al [25] also reported HIV as one of the major predisposing risk for candidemia. The duration of hospital stay, antibiotic prophylaxis and treatment, level of immunosuppression, presence of other opportunistic infection and other clinical types of candidiasis increases the risk of candidemia in HIV infected patients.

The emergence of new species Candida as potential pathogens is a reflection of the changing scenario in medicine since 1960s. More than 17 species of Candida have been implicated in human infections till date. However, the list of new species continues to grow. The use of automated identification system in addition to conventional methods and increase in the number of HIV infected patients can explain this fact. The incidence of BSI caused by NAC spp. was higher than C. albicans at our hospital. Among the NAC spp., C. tropicalis followed by C. glabrata and C. krusei predominately caused BSI. A number of international surveillance programs like ARTEMIS Antifungal Surveillance Study conducted in 127 health-care centres in 39 countries have documented increased prevalence of NAC species like C. tropicalis and C. parapsilosis [26].

Epidemiological studies from India reports C. tropicalis as aetiological agent in as many as 67-90% cases of candidemia. Shivprakasha et al [10] found C. tropicalis to be the most common isolate from candidemia patients. Other workers have also documented the similar observation [16]. The increased use of fluconazole has been determined to be the major reason for predominance of C. tropicalis over C. albicans.

C. glabrata has emerged as an important opportunistic pathogen worldwide. It is the second most common yeast isolated as part of normal flora and its role as a pathogen has only been recognized in the past few decades. Trick et al [27]reported a considerable increase in the incidence in the rate of isolation of C. glabrata from BSI patients. There is concern over an increase in azole resistance among strains of C. glabrata.

With the increased incidence of candidemia and the growing number of antifungal agents, laboratory aids to guide in the selection of antifungal therapy have gained greater attention. The standardized broth micro dilution method is expensive, laborious and cumbersome for use in clinical microbiology laboratories. Recently, a disc diffusion method has been approved by CLSI for antifungal susceptibility testing of yeast.

In the present study we performed disc diffusion method for antifungal susceptibility testing of Candida isolates. In our study Fluconazole resistance was noted in 19.07% of Candida isolates, 11.9% showed resistance to itraconazole and 4.63% of Candida isolates showed resistance to amphotericin B. In India, there is lack of multicentric studies regarding antifungal susceptibility pattern. Goel et al [28] and Capoor et al [29] reported less incidence of resistance to fluconazole. On the other hand, workers like Kumar et al [14], Kothari et al [30] and Gupta et al [31] reported high incidence of resistance to fluconazole.

The resistance to fluconazole is of great concern because it is the most common azole used for treatment of disseminated candidiasis including candidemia. It is available in both intravenous and oral formulation with high bioavailability and is more cost effective than other antifungal agents. Although Amphotericin B is effective against most strains of Candida species, it is not the first choice for the treatment of candidemia because of nephrotoxicity associated with it. Itraconazole is used for treatment of mucosal candidiasis [32]. Studies regarding its role in treatment of candidemia are less. In a study by Kothari et al [30] 24% of Candida isolates were resistant to itraconazole.

In the present investigation, resistance to antifungal agents was observed more in NAC spp. as compared to C. albicans. Fluconazole resistance was high in C. tropicalis and C. glabrata. Itraconazole resistance was more in C. tropicalis. Amphotericin B resistance was higher in C. glabrata isolates. Other researchers have also documented high antifungal resistance among NAC spp as compared to C. albicans [1, 3].

We have compared the efficacy of YNBG medium and GM-MH agar for the antifungal susceptibility testing of Candida isolate. Trailing phenomena around the zone margin were infrequent and minimal on the GM-MH agar. Zone edges with this method were frequently definite and clear, facilitating the measurement of zone sizes and minimizing subjectivity in zone size measurements. The occurrence of the macrocolonies near the center of the clear zone was also less with this method. The methylene blue in this medium stained the Candida colony facilitating the identification. Our study showed that there is less variation in the result of GM-MH agar and YNBG medium. Therefore GM-MH agar can be recommended as simple, cost effective and sufficiently accurate medium for the routine testing of antifungal susceptibility of Candida spp.

To conclude, the spectrum of Candida BSI has shifted dramatically from C. albicans to NAC spp. Hence, it is essential that an early and accurate diagnosis be made of infecting species of Candida, since each species varies markedly in susceptibility to the currently used antifungal drugs. It is imperative that antifungal susceptibility testing be carried out routinely in the laboratory. This will aid the clinician in timely institution of the appropriate and accurate

antifungal drug to be used and will restrict the empirical use of antifungal agents, as being commonly done today.

Figure 1. Age and sex distribution of candidemia patients.

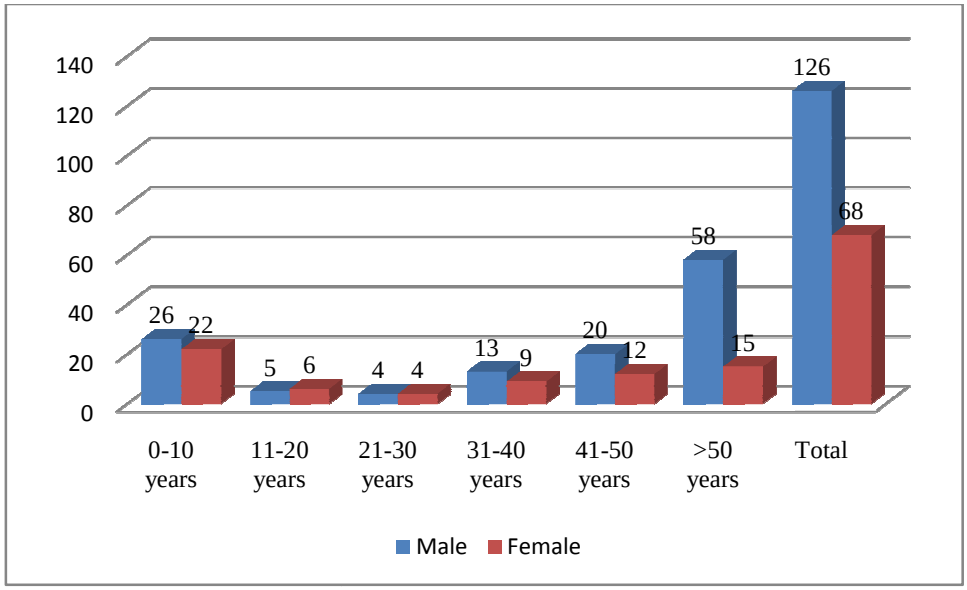

Figure 2. Risk factors predisposing Candidemia.

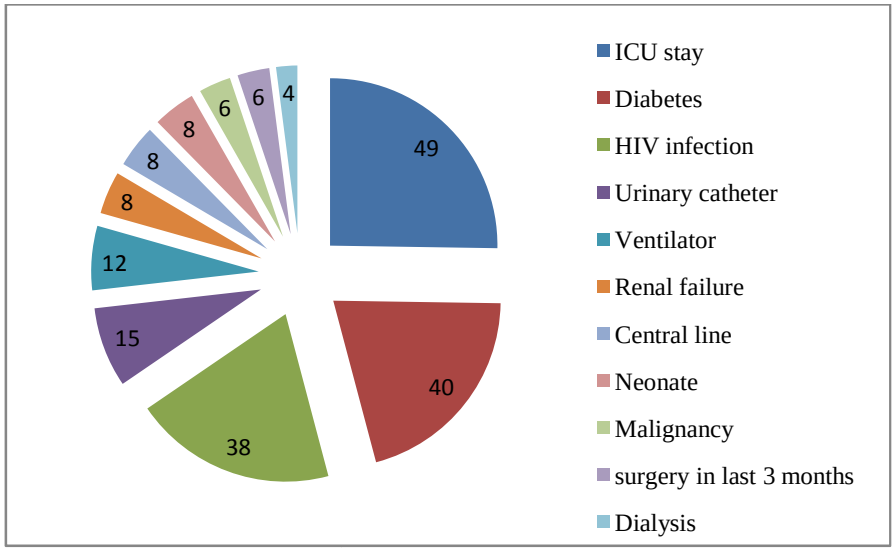

Figure 3. Species distribution of Candida isolates obtained from candidemia patients.

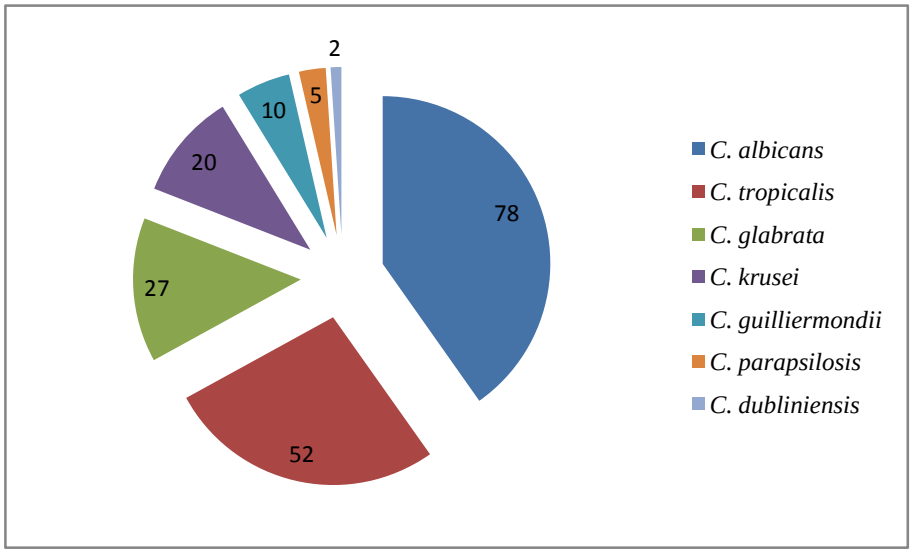

Table 1 Comparison of antifungal resistance pattern of Candida isolates using YNBG medium and GM-MH agar.

Isolate	Total	Amphotericin B		Fluconazole		Itraconazole	
		YNBG	GM-MH	YNBG	GM-MH	YNBG	GM-MH
C. albicans	78	02	02	07	07	04	03
C. tropicalis	52	02	02	19	19	20	21
C. glabrata	27	05	04	08	08	05	05
C. krusei	20	-	-	02	02	02	02
C. guilliermondii	10	-	-	01	01	01	01
C. parapsilosis	05	-	-	-	-	-	-
C. dubliniensis	02	-	-	-	-	-	-

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