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Candidemia-induced pediatric sepsis and its association with free radicals, nitric oxide, and cytokine level in host☆,☆☆

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ABSTRACT

Candida species has become the seventh most frequent causal microorganisms of nosocomial sepsis. Prematurity and low birth weights are strongly associated with the development of neonatal nosocomial bloodstream infections. *Candida albicans* has been the species most often associated with neonatal infections, but recently, there has been a changing pattern in the isolates recovered from neonates with invasive candidiasis, which poses resistance to the existing class of azoles such as fluconazole antifungals along with cross resistance to newer triazoles, which results in a therapeutic challenge in invasive fungal infections causing high incidence of mortality.

Candida species was isolated from blood of neonates and children younger than 15 years admitted to hospital and susceptible for *Candida*-induced sepsis. Polymerase chain reaction–based identification and confirmation of individual *Candida* species were done using DNA sequencing. Antibiotic susceptibility assay and resistance pattern for fluconazole, voriconazole, and amphotericin were done for all the isolates. Furthermore, the change in free radical, cytokine release, and nitric oxide synthase expression and nitric oxide release from polymorphonuclear leukocytes isolated from control and pediatric sepsis cases were also performed.

The present study probably for the first time reports the change in increasing incidence of nonalbicans *Candida*-induced sepsis in neonates and children admitted to the intensive care unit of hospital, and current antibiotics load posing resistance for antifungal treatment strategy and provide serious threats in future treatment. The increase in free radicals in polymorphonuclear leukocytes and increase in expression of nitric oxide synthase expression and nitric oxide release in *Candida*-infected pediatric sepsis cases underlie the role of host factor in dissemination and invasiveness of infection from exogenous sources and pathogenesis of systemic inflammation during sepsis.

1. Introduction

The incidence and prevalence of invasive fungal infections have increased since the 1980s, especially in the large population of immunocompromised patients and/or those hospitalized with serious underlying diseases [1,2]. *Candida* species are responsible for up to 78% of the cases of nosocomial fungal infections and represent the major cause of bloodstream infections [3]. Invasive fungal infections represent a leading cause of sepsis in very-low-birth-weight (VLBW) infants and result in high rates of morbidity and mortality due to exposure to invasive procedure that predisposes to nosocomial infection [4]. Among many risk factors responsible for development of invasive fungal infection, previous mucosal and skin colonization is of primary importance [4]. Colonization with *Candida* in neonates is secondary to either maternal transmission or to nosocomial acquisition in the neonatal intensive care unit (NICU) [2]. Furthermore, risk factors such as use of central venous catheters, longer use of broad spectrum antibiotics, and use of parenteral nutrition contribute as well. Over the last decade, nonalbicans *Candida* species are emerging as causative pathogens for systemic *Candida* infections in children [5]. Systematic fungal infection is associated with high severity, substantial morbidity, and high rates of neurodevelopment impairment [6]. *Candida*-attributable mortality ranges from 25% to 55%, and systematic fungal infection is associated with neurodevelopmental impairment in 57% of survivors at 18 months of life [7]. Currently, *Candida* species has become the fourth most frequent causal microorganisms of nosocomial sepsis [8]. *Candida albicans* has been the species most often associated with neonatal infections [9], but recently, there has been a changing pattern in the isolates recovered from neonates with invasive candidiasis. Although *C* albicans remains

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the most frequently isolated species in many centers, infections due to nonalbicans *Candida* species have increased in frequency in recent years [10]. In particular, non–*C albicans*, such as *Candida parapsilosis*, *Candida tropicalis*, *Candida glabrata*, and *Candida krusei*, are now approaching. *C albicans* is the most frequent cause of candidemia in some institutions. The frequency of non–*C albicans* recovery is influenced by the patient population studied, the therapeutic regimens used, and the antibiotics or other supportive care erasures used in specific institutions [11]. The increased isolation rates of nonalbicans *Candida* species and a gradual shift in the antifungal susceptibility profile, especially against azole antifungal agents, have underlined the need to monitor laboratory data for possible emergence of resistance and to select the most appropriate antifungal agent for therapy [2,10,12]. The majority of nosocomially acquired candidemia is due to *C glabrata*, which is often responsible for severe and rapidly progressive bloodstream infections. Many reports, however, have documented the emergence of *C albicans*, *C tropicalis*, *C krusei*, and *C parapsilosis* as important nosocomial pathogens, with *C glabrata* identified as the predominant species causing bloodstream infections in premature newborns [13]. Unlike other *Candida* species, *C glabrata* causes nosocomial candidemia without prior colonization of other sites, suggesting that this yeast can gain access to the bloodstream directly from exogenous locations [14]. Infections are associated with the use of central venous catheters, the use of parenteral nutrition, and the transmission by the hands of the health professionals [15]. Most of the nonalbicans *Candida* species (eg, *C glabrata*, *C krusei*, and *C tropicalis*) harbor resistance to the existing class of azoles such as fluconazole antifungals along with cross resistance to newer triazoles, which results in a therapeutic challenge in invasive fungal infections [2,16]. Fluconazole and amphotericin B are the main therapy for serious fungal infections for more than 35 years. Infusion-related side effects and dose-limiting nephrotoxicity associated with its use prompted continuous search for equally effective but less toxic alternatives. Azoles are safe and effective agents for the treatment of oropharyngeal candidiasis and have gradually replaced amphotericin B. However, resistance to azoles is becoming common. Neonatal sepsis is a highly inflammatory disease that results in septic shock due to uncontrolled activation of inflammatory response to pathogen [17]. The immune system of neonates is poorly developed, and invasion of pathogen results in activation of immune response, mainly macrophages, dendritic cell, and polymorphonuclear leukocytes (PMNs). The release of cytokines and free radical at the site of infection is critical as host defense against pathogen. The release of preformed mediators from granules of PMNs and proinflammatory mediators during infection is well reported [18]. The factors responsible for systemic inflammatory response in neonates after blood stream infection are unclear.

The present study was conducted to determine the molecular epidemiology and drug susceptibility of *Candida* isolates causing invasive candidiasis in pediatric cases leading to sepsis admitted to intensive care unit of Sir Sundar Lal hospital, Banaras Hindu University, Varanasi, India, for a period of 1 year. In the present experimental investigation, we have characterized the *Candida* species of NICU patients on the basis of media and its major predisposing factors with its species distribution by first-round PCR, and antifungal susceptibility (minimum inhibitory concentration [MIC]) of isolated *Candida* species has also been studied against different antifungal agents. Furthermore, release of free radicals, mainly superoxide dismutase (SOD), catalase, glutathione peroxidase, and nitric oxide, was estimated in PMNs. The release of proinflammatory cytokine tumor necrosis factor α (TNF-α), interleukin (IL) β, and anti-inflammatory cytokine (IL-10) was estimated in plasma of pediatric sepsis patients and controls. Furthermore, the expression of nitric oxide synthase gene was also performed in PMNs of pediatric sepsis patients and controls. The present study provides the epidemiological data of increasing nonalbicans *Candida*-induced sepsis in NICU and role of proinflammatory and anti-inflammatory and free radicals in the occurrence of systemic infection leading to pediatric sepsis.

2. Materials and methods

2.1. Materials

Sodium dihydrogen phosphate, di-sodium hydrogen phosphate, potassium chloride, EDTA, sucrose, potassium dichromate, acetic acid, sodium pyrophosphate, phenazine methosulphate, nitroblue tetrazolium salt, nicotenamide adenine dinucleotide reduced sodium salt, glutathione reduced, 1 chloro-2,4-dinitrobenzene, methanol, and hydrogen peroxide were procured from Sisco Research Laboratory, India. Sodium dodecyl sulfate, 2-thiobarbituric acid, 5,5-dithiobis(2-nitro-benzoic acid), trichloroacetic acid, and Trizol reagent were procured from Life Technologies (Banglore, India). Complementary DNA synthesis kit was procured from MBI Fermentas (Mumbai, India). Tumor necrosis factor α, IL-1β, and IL-10 kit were procured from Invitrogen Corporation (Banglore, India). The primers were synthesized from Eurofins Genomics Pvt, Ltd, Bangalore, India.

2.2. Sample collection

This study was carried out from June 2011 to May 2012. Neonates diagnosed with early onset sepsis (mean age, 2.4 days) and children younger than 15 years (mean age, 10.5 years) were enrolled in this study. Peripheral blood was collected from septic neonatal samples and children admitted to intensive care units with the approval of Institutional Ethical Committee of Banaras Hindu University, Varanasi, India, and informed consent was obtained from parent/guardian of all the subjects enrolled for this study. Normal samples include neonates and children of the same age, sex, and weight without any signs of infection. Septic samples were collected at the time of onset of clinical symptoms of sepsis and before initiation of any antibiotic therapy. Peripheral blood was obtained by vein puncture or drawn from a dwelling arterial or venous line. The blood was collected aseptically in precoated EDTA tubes. A total of 210 samples from both male and female neonates and children younger 15 years old were collected. The blood samples were centrifuged at 1500*g* for 15 minutes at 4°C. The plasma was separated and kept at −20°C for future use.

2.3. Isolation of PMNs

Polymorphonuclear leukocytes were isolated from the buffy coat by dextran sedimentation [19] and further purified with histopaque density gradient centrifugation at 700*g* for 30 minutes at 20°C. The PMN-rich layer was recovered at the interface of histopaque 1119/1077 and washed thrice with Hank's balanced salt solution (sodium chloride, 138 mmol/L; potassium chloride, 2.7 mmol/L; disodium hydrogen phosphate, 8.1 mmol/L; and potassium dihydrogen phosphate, 1.5 mmol/L) containing magnesium chloride of 0.6 mmol/L, calcium chloride of 1.0 mmol/L, and glucose of 10 mmol/L (pH 7.4). The viability of the cells was tested by Trypan blue exclusion test and was never less than 95%.

2.4. Isolation of Candida from blood

Candida species were collected into brain-heart infusion culture vials of Hi-media, Mumbai, India, from those younger than 15 years admitted to NICU with clinical suspicion of septicemia. Suspected individual bloods were cultured on Sabouraud's dextrose agar with chloramphenicol (0.05%) at 37°C. Preliminary identification was done as mentioned in Table 1.

2.5. Molecular phenotyping

DNA was isolated from *Candida* isolates, and PCR reaction was performed as mentioned elsewhere [20]. The total reaction volume