ORIGINAL ARTICLE

Invasive fungal infections in Neonatal Intensive Care Units of Southern Italy: a multicentre regional active surveillance (AURORA Project)

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Key words

Fungal infection • NICU • Surveillance • Incidence

Summary

Introduction. *During the past years invasive fungal infections (IFIs) have become an increasingly important problem in infants hospitalized in the Neonatal Intensive Care Unit (NICU). Candida species is the third most-common agent of late-onset infections in critically ill neonates, with an estimated incidence of 2.6-10% in very low birth weight and 5.5-20% in extremely low birth weight infants.*

The aim of this observational study is to evaluate the epidemiology of IFIs among infants admitted to NICUs of one Italian region by a multicenter surveillance (Aurora Project).

Methods. *The IFIs surveillance was carried out prospectively in Apulia (Southern Italy) between February 2007 and August 2008. This report focuses on the results from 6 enrolled NICUs.*

Results. *Twenty-one neonates developed IFIs: the overall incidence was 1.3% and crude mortality was 23.8%. Infants weighing ≤ 1500g (4.3%) showed a significantly higher incidence than those ≥ 2500g (0.2%). C.parapsilosis (61.9%) was the most frequent isolated species. The main potential risk factors were having a central venous catheter placed, length of stay in NICU > 7 days and total parenteral nutrition for > 5 days. The (1,3)-ß-D glucan (BDG), mannan antigens and anti-Candida antibodies' evaluation was performed in 7 neonates. All neonates were positive to the BDG; the mannan antigen result was positive in 5 newborns, the anti-mannan antibodies were always negative. All isolates were amphotericin B and fluconazole-susceptible.*

Discussion. *This first prospective study on neonatal fungal infection in one Italian region gives evidence of a preponderance of non-albicans Candida spp and indicates potential utility of BDG as an adjunct diagnostic test.*

Introduction

During the past two decades invasive fungal infections (IFIs) have become an increasingly important problem in preterm infants hospitalized in the Neonatal Intensive Care Unit (NICU). *Candida* spp is the third most-common agent of late-onset infections in critically ill neonates, especially in very low birth weight (1001-1500 g, VLBW) and extremely low birth weight (≤ 1000 g, ELBW) infants [1, 2], with an incidence ranging from 2.6 to 10% among VLBW [3-5], and from 5.5 to 20% among ELBW [2, 6, 7].

Although *C.albicans* is the most frequent aetiological agent, infections due to *C.* non-*albicans* have increased in frequency in recent years [8-10]. In many NICUs *C.parapsilosis* is the main pathogen causing clusters and nosocomial outbreaks [11-13]. Numerous risk factors for invasive candidiasis (IC) have been identified in NICU patients: some are related to host factors (immunodeficiency resulting from decreased numbers of neutrophils and T cells, immature skin structure, *Candida* colonization), others to medical care (central venous catheters, total parenteral nutrition, mechanical ventilation, broad spectrum antibiotic therapy, use of 3rd generation cephalosporin, administration of H2-blockers) [7, 14-17]. The mortality rate is high (25-60%) [17] and it is related to the difficulty to make an early diagnosis because of reduced sensitivity of diagnostic tests, non-specific clinical signs and to inadequate and / or delayed treatments [18, 19].

The present paper originates from the results of an active surveillance of IFIs carried out in the Southern Italy (Apulia region) throughout a 18-month period, focusing on the characteristics of neonatal IFIs, as incidence, aetiology and resistance to common antifungal drugs, and on the role of the additional biomarker-diagnostic test.

Methods

Study design

A prospective multicenter surveillance (*Aurora Project*) was carried out in Apulia for a 18 month period (February 2007 - August 2008). The Apulia region is located in Southern Italy, has about 4 million inhabitants, with 38,000 births/year and a mean of 850,000 hospital admissions/year. The aim of this surveillance was to verify the incidence of IFIs among patients admitted to general ICUs, haematology wards and NICUs of this region, together with the causative pathogens, potential predisposing factors, treatment characteristics, as well as to determine the antifungal susceptibility patterns of the isolated yeasts. In this present paper, we report the data concerning the neonatal subjects.
All six participating NICUs followed the Italian Society of Neonatology's guidelines and recommendations promoting the use of breast milk, both expressed or banked, in VLBW neonates, as well as written common policies concerning the use of antibiotics and probiotics.
According to the protocol, for each IFIs episode participating hospitals had to identify and store the fungal isolates and to complete an electronic report form about clinical data (age, sex, birth weight, underlying disease), microbiological diagnosis (techniques used to isolate and identify the etiological agents, valuation of circulating antigens and antibodies), risk or predisposing factors (vascular lines, total parenteral nutrition, endotracheal intubation, previous *Candida* colonization), therapeutic approach and outcome 30 days after diagnosis.
All reports and isolates were sent to the Coordinating Center (CC, Laboratory of Mycology - Department of Biomedical Sciences and Human Oncology, University of Bari "Aldo Moro") for data analysis, species confirmation and antifungal susceptibility testing.

Case definitions and inclusion criteria

IFI cases were defined using the criteria of the international guidelines [20, 21] and the recommendations of the Italian Neonatology Society's Fungal Infections Task Force [22].
A microbiologically documented fungal infection was defined through a positive culture from sterile sites (blood, cerebrospinal fluid and urine collected by suprapubic sterile puncture or sterile bladder catheterization, with growth of > 10 000 CFU/mL) of an infant with clinical signs of infection (apnea, elevated or depressed leukocyte count, increased C-reactive protein levels, abdominal distension, thrombocytopenia).
An episode of IFI was considered new infection in the same infant if it occurred at least 15 days after the first isolation.
Candidemia was defined as blood culture drawn from either an intravascular catheter or a peripheral vein, positive for *Candida* spp. Duration of candidemia was defined by the number of days between the first and the last blood cultures yielding the same *Candida* species. Persistent candidemia was defined as the isolation of the same *Candida* species from blood cultures for at least 5 consecutive days despite therapy. Disseminated infection was defined as positive cultures from more than one normally sterile body fluid or site.
In the present study, all possible fungal infections cases and superficial mycoses were excluded from the analysis.

Laboratory procedures

Detection and determination of different isolates were performed by each laboratory according to their standard protocols. The CC sub-cultured all the isolates on antimicrobial agent-free Sabouraud dextrose agar plates (bioMérieux, Marcy l'Etoile-France) and CHROMagar *Candida* Medium (Becton Dickinson, Germany) to ensure viability and purity. The yeasts identification was confirmed by germ tube production and sugar assimilation profiles obtained using the API ID32C and VITEK System (bioMérieux, Marcy l'Etoile-France). Differentiation of the two closely related species *Candida albicans* and *Candida dubliniensis* was made by different carbohydrate assimilation and ability to grow at 45°C.
Mannan antigen was measured using a commercial sandwich immunoassay, Platelia *Candida* Ag (BioRad, Marnes La Coquette, France), while anti-mannan antibodies were assessed using a two-stage indirect immunoassay, Platelia *Candida* Ab/Ac/Ak kit, (Bio-Rad, Marnes La Coquette, France). Both tests were performed according to the instructions of the manufacturer. The presence of (1,3)-ß-D-glucan (BDG) was measured by a colorimetric assay, Fungitell (Associates of Cape Cod Inc., E. Falmouth, MA, USA).
The *in vitro* antifungal susceptibility test was performed using the broth microdilution method in accordance with the CLSI M27-A3 recommendations [23]. The antifungal drugs tested were amphotericin B (AmB), anidulafungin (AND), caspofungin (CS), fluconazole (FL), itraconazole (IT), voriconazole (VO), posaconazole (POS). CLSI breakpoints for susceptibility were used for AND, CS, FL, VO, while a provisional susceptibility breakpoint of ≤ 1 μg/ml was used for AmB and POS [24]. *C.krusei* ATCC 6258 and *C.parapsilosis* ATCC 22019 were used as control strains in all tests.

Statistical Analysis

Data were entered into a database (Microsoft Access 2003). Statistical analysis was performed with the free software R (version 2.10.0). Categorical variables are expressed as proportions or percentages, and numerical data are expressed as the median ± SD and range.

Results

During the 18-month study period, 1597 infants were admitted to the 6 participating NICUs, of whom 248 were VLBW and 171 ELBW. Among all enrolled infants, 21 developed IFIs with an overall incidence of 1.3% (Tab. I). The incidence of IFIs was inversely proportional to birth weight: 4.7%, 4% and 0.2% of infants weighing ≤ 1000, ≤ 1500, ≤ 2500 g respectively.

Tab. I. IFI incidence in 6 NICUs included in the surveillance.

NICUs	No. of beds	No. admitted	No. (%) IFIs	Incidence/100 admitted
A	6	240	7 (33.4)	2.9
B	10	220	4 (19.0)	1.8
C	8	280	3 (14.3)	1.1
D	8	331	3 (14.3)	0.9
E	4	259	2 (9.5)	0.8
F	8	267	2 (9.5)	0.7
Total	**44**	**1597**	**21 (100)**	**1.3**

Bloodstream infection was the most frequent IFI (95.2%): 12 episodes were caused by *C.parapsilosis* (60%), 7 by *C.albicans* (35%) and 1 by *C.glabrata* (5%). Moreover, 1 *C.parapsilosis* disseminated infection was diagnosed (4.8%).

The demographic characteristics and underlying diseases are shown in Table II. The overall male/female ratio was 12/9. The median age at diagnosis was 39.7 ± 40.6 days (range, 5-150) and 2 (9.5%) neonates were ≤ 7 days old when IFI occurred. Ten neonates (47.6%) were VLBW, 8 (38.1%) ELBW and only 3 (14.3%) infants were low birth weight (1501-2500g). The most common underlying disease was respiratory distress syndrome (57.1%).

Tab. II. Demographic characteristics and underlying diseases of 21 screened patients.

Characteristic	Patient	
	No.	%
Sex		
Male	12	57.1
Female	9	42.9
Age (days) - mean ± SD	39.7 ± 40.6 [range 5-150]	
Birth weight (g)		
≤ 1000 (ELBW)	8	38.1
1001-1500 (VLBW)	10	47.6
1501-2500 (LBW)	3	14.3
Underlying disease		
Respiratory distress syndrome	12	57.1
Necrotizing enterocolitis	3	14.3
Short bowel syndrome	2	9.5
Esophagel atresia	1	4.8
Ectodermal dysplasia	1	4.8
Bronchopulmonary dysplasia	1	4.8
Cerebral hemorrhage	1	4.8

The presence of a central venous catheter *in situ* for > 4 days was the most frequent predisposing factor for IFI (100%), followed by a length of stay > 7 days (90.5%), receiving total parenteral nutrition for > 5 days (90.5%), a birth weight ≤ 1500 g (85.7%), gestational age < 32 weeks (71.4%), mechanical ventilation (61.9%), previous *Candida* spp colonization in more than two sites (47.6%), multiple antibiotic therapy ≥ 5 days (47.6%) (Tab. III).

The Central Venous Catheter (CVC) was removed in 20 episodes (95.2%): in 7 patients (35%) at the time of blood sampling, in 13 (65%) 5.1 ± 4.9 days (range, 1-20) after the onset of candidemia. In 19 (95%) cases, catheter tip culture was performed, of which 17 (89.5%) were positive for the same fungal species found in blood culture.

The mean duration of IC was 3.5 ± 5.1 days (range 1-20). Persistent candidemia was found in 5 (23.8%) neonates (range, 5-20 days). Mixed infections were documented in 2 (9.5%) infants: *C.parapsilosis* associated with *Pseudomonas aeruginosa* and *C.parapsilosis* associated with *Chryseobacterium meningosepticum*.

The BDG, mannan antigens and anti-mannan antibodies detection was performed in only 7 babies suspected to have candidiasis. All neonates were positive according to the BDG test (> 80 pg/ml). The mannan antigen resulted positive in 5 newborns (> 0.5 ng/ml) while the anti-mannan antibodies were always negative (< 5 UA/ml).

Before candidemia was detected, one preterm birth (< 24 weeks) had received antifungal prophylaxis with FL (3

Tab. III. Potential predisposing factors of 21 IFI cases by *Candida albicans* or non *albicans Candida* spp.

Predisposing factors	All *Candida* spp (n = 21)	*C. albicans* (n = 7)	*C.* non-*albicans* (n = 14)
Central venous catheter	21 (100)*	7 (100)	14 (100)
Length of stay >7 days	19 (90.5)	6 (85.7)	13 (92.8)
Total parenteral nutrition	19 (90.5)	5 (71.4)	14 (100)
Birth weight ≤1500 g	18 (85.7)	5 (71.4)	13 (92.8)
Gestational age < 32 weeks	15 (71.4)	6 (85.7)	9 (64.3)
Mechanical ventilation	13 (61.9)	5 (71.4)	8 (57.1)
Candida colonization	10 (47.6)	5 (71.4)	5 (35.7)
Antibiotic therapy	10 (47.6)	3 (42.8)	7 (50.0)
Previous surgery	1 (4.8)	–	1 (7.1)

*Numbers in parentheses, percent

mg/kg/day) and 6 (28.6%) an empirical treatment with FL (6 mg/kg/day).
After diagnosis, all the patients received antifungal therapy: 15 (71.4%) were treated with liposomal AmB (1-5 mg/kg/day), 5 (23.8%) with FL (6 mg/kg/day). Only in one case (4.8%) FL therapy for 4 days (6 mg/kg/day) was later switched to liposomal AmB (3 mg/kg/day) because of clinical complications. Median length of treatment was 17 days (range, 7-38 days).
Overall mortality was 23.8%: 4 patients died by *C.parapsilosis* and 1 by *C.glabrata*. Death occurred after 11 ± 4.1 days (range, 7-16) from the onset of infection; it was observed in newborns with a birth weight ≤ 1500 g and a gestional age < 32 weeks.
Antifungal susceptibility tests were carried out on all 21 *Candida* spp isolates (Tab. IV). All yeasts were susceptible to tested antifungal drugs, except 4 *C.parapsilosis* that resulted resistant to IT (MIC 1 μg/ml) and 1 *C.glabrata* that was dose-dependent susceptible to FL (MIC 16 μg/ml) and resistant to IT (MIC 4 μg/ml).

Discussion

To our knowledge, this is the first prospective multicenter survey presenting epidemiological data on neonatal IFIs from one Italian region. In fact several studies carried out in Italy are reviews of an individual hospital or regard specific patients population [25-28].
Consistent with earlier reports in the literature [5, 8, 29], the overall incidence is 1.3%, with an incidence significantly higher in neonates weighing ≤ 1500g (4.3%, 18/419) than in those ≥ 2500g (0.2%, 3/1178) ($p < 0.001$).
In our study, a variety of predisposing factors were identified. They are largely similar to those reported in previous studies [14-17], but it is difficult to assess to what these factors contributed to development of IFIs, because this survey was not carried out on a control population (no infected with fungi).
All infants had an intravascular catheter that was removed in 95.2% of the episodes: 89.5% of evaluated catheters were positive for same *Candida* species isolated from the blood, highlighting the catheter as possible source of candidemia. *C.parapsilosis* was isolated more frequently than *C.albicans* (61.9% vs 33.3%). The high incidence of *C.parapsilosis* infections may be correlated to its ability to form biofilms on catheters and to contaminate glucose-containing solutions (e.g. parenteral nutrition) [4, 30]. However, a possible transmission of *C.parapsilosis* from hands of healthcare workers to neonates has been suggested in cases of infection CVC-related, because it is a commensal of human skin (horizontal transmission) [4, 9, 11, 30, 31].
A previous colonization by *Candida* spp was demonstrated in 47.6% of episodes, mainly in urinary (100%), respiratory (87.5%) and intestinal (50%) system. Fungal colonization is very common among NICU patients not receiving antifungal prophylaxis [17]. Manzoni et al. [15, 32] showed that *Candida* colonization in multiple body sites is an important predictor of progression to IFI, underlining the need of systematic surveillance cultures in the preterm infants.
The search for mannan antigen by ELISA has good specificity for the diagnosis of IC in severely ill patients, although this technique requires frequent sampling due to the rapid clearance of this antigen from the blood. Recent studies have demonstrated a good diagnostic efficacy with the association of anti-mannan antibodies, but not in immunocompromised patients [33, 34]. Oliveri et al. [35] demonstrated that the sensitivity and specificity of mannan antigen test for IC in NICUs were 94.4% and 94.2% respectively, with a positive predictive value of 85% and a negative predictive value of 98%. Besides, during last years, the BDG detection as bio-markers fungus-specific seems to give a new diagnostic approach with encouraging results especially if it is valuated in tandem of mannan and anti-mannan antibodies, but its diagnostic performance is not well characterized [36, 37]. In our study, because of babies critical conditions, the mannan and BDG antigens detection was performed in only 7 infants: the mannan antigen resulted positive in 5 cases (4 with *C.albicans* and 1 with *C.glabrata* infection), and negative in 2 patients with *C.parapsilosis* infection, while BDG research was always positive. The low sensitivity of the mannan antigen test in patients with *C.parapsilosis* invasive infection is in agreement with what suggested by other Authors [35, 38]. Although our data refer a limited number of patients, we believe that the circulating antigens monitoring in NICU patients may be an useful contribution to the diagnosis of IC. In fact, the detection of BDG and mannan antigens, such as anti-

Tab. IV. *In vitro* antifungal susceptibilities (μg/ml) of 21 *Candida* spp isolates (CLSI test).

Drugs	*C. albicans* (n = 7)		*C. parapsilosis* (n = 13)		*C. glabrata* (n = 1)
	MIC_{90}	Range	MIC_{90}	Range	MIC
Anidulafungin	0.25	0.016-0.25	2	1-2	0.125
Caspofungin	1	0.06-1	2	2	2
Posaconazole	0.06	0.008-0.06	0.125	0.016-0.25	1
Voriconazole	0.008	0.008-0.016	0.03	0.008-0.03	1
Itraconazole	0.25	0.016-0.5	1	0.06-1	4
Fluconazole	0.25	0.125-0.25	2	0.25-8	16
Amphotericin B	0.5	0.125-0.5	1	0.06-1	1

mannan antibodies finding, singly or in combination, could improve the sensitivity and help eliminating false positive or negative results.

All our *C.albicans* strains were susceptible to the antifungal tested drugs. Similarly, *C.parapsilosis* isolates were susceptible, except 4 strains (30.8%) resistant to IT (MIC 1 µg/ml); the only *C.glabrata* strain was dose-dependent susceptible to FL (MIC 16 µg/ml) and resistant to IT (MIC 4 µg/ml). According to other Authors [8, 9], we have not found any significant resistance to AmB and FL, which are the antifungal drugs used for prophylaxis and treatment of IC in neonates [21]. The very low rate of azole resistance may be related to the treatment policy in use at our region: systemic antifungal prophylaxis and/or empiric therapy with FL were not usually employed; in fact their use was restricted only to few infants (1 and 6 respectively) in our study. We think the FL use as prophylaxis or starting therapy should be evaluated according to the epidemiology of the local NICU: if the incidence is low, it could be a better approach to use, as prophylaxis, probiotics [39] or lactoferrin [40] and save FL to treat documented infections. However, it is important to monitor susceptibility pattern of clinical isolates, particularly non-*albicans Candida* ones, to identify strains resistant to common antifungal drugs [41].

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