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Species distribution, antifungal susceptibility and clonal relatedness of Candida isolates from patients in neonatal and pediatric intensive care units at a medical center in Turkey

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SUMMARY

The aim of this study was to assess species distribution, antifungal susceptibility and clonal relationships among *Candida* strains isolated from a group of pediatric/neonatal intensive care (PICU/NICU) patients that had a very high mortality rate (76%). The cases of 21 patients (19 with candidemia, 2 with *Candida* meningitides) treated over a 1-year period in a Turkish hospital PICU and NICU were retrospectively analyzed. Twenty-eight *Candida* isolates were detected from blood (20), cerebrospinal fluid (CSF) (2) and other specimens (6). *Candida* species were identified using the API ID 32C System. Susceptibility testing was done (all 28 isolates) for amphotericin B, fluconazole and itraconazole using the broth microdilution method. Arbitrarily primed polymerase chain reaction (AP-PCR) was used for molecular typing of the 3 most common ones; *C. albicans* (15), *C. parapsilosis* (6), and *C. pelliculosa* (4). Electrophoretic karyotyping (EK) was done to check clonal identity obtained by AP-PCR. Of the 20 blood isolates, 8 (40%) were *C. albicans*, 12 (60%) were non-albicans *Candida*, and one of the 2 CSF isolates was *C. albicans*. The overall species distribution was as follows: 15 *C. albicans* isolates, 6 *C. parapsilosis* isolates, 4 *C. pelliculosa* isolates, 2 *C. famata* isolates and 1 *C. tropicalis* isolate. Amphotericin B had the best antifungal activity with a MIC_{90} of 0.125 µg/ml, and the rates of susceptibility to fluconazole and itraconazole were 93% and 82%, respectively. AP-PCR revealed 11 genotypes (4 were identical pairs, 7 were distinct) among the 15 *C. albicans* isolates, 2 genotypes (5 were classified in the same type) among the 6 *C. parapsilosis* isolates, and 4 separate genotypes for the 4 *C. pelliculosa* isolates. Karyotyping results correlated well with the AP-PCR findings. As indicated in the previous research, our results confirmed that non-albicans *Candida* species have become more frequently causative agents for invasive fungal infections in the ICU. Transmission of *C. albicans* and *C. pelliculosa* was relatively low, but transmission of *C. parapsilosis* was high, suggesting that more effective control and very strict treatment protocols are needed for patients having high mortality and invasive fungal infection in ICU.

KEY WORDS: Bloodstream infections, *Candida* spp., Neonatal intensive care unit, Arbitrarily primed polymerase chain reaction, Electrophoretic karyotyping, Antifungal susceptibility testing

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INTRODUCTION

Over the past 20 years, there has been a significant increase in *Candida* spp. infections worldwide, particularly in patients admitted to inten-

sive care units (Vrioni and Matsiota-Bernard 2001; San Miguel *et al.*, 2005; Avila-Aguero *et al.*, 2005). Fungal bloodstream infections are considered to be life-threatening; they prolong hospital stays and are associated with high morbidity and mortality (Verduyn Lunel *et al.*, 1999). *C. albicans* is still the *Candida* species most commonly isolated from neonatal patients with invasive candidiasis, but there has been a significant rise in isolation rates for other species in recent years (Roilides *et al.*, 2004).

Prophylaxis with azole agents may increase the risk of infection with resistant *Candida* spp. (Zaoutis *et al.* 2005). Determining a pathogen's antifungal susceptibility is an important step in effective treatment (Pfaller and Diekema 2004). However, it takes considerable time to isolate and identify organisms from patients with invasive fungal infections, and to determine resistance profiles. Delayed initiation of appropriate antifungal therapy can raise the risk of mortality and morbidity in infants, and also increases the likelihood of cross-contamination (Avila-Aguero *et al.*, 2005).

Molecular typing can provide significant information on nosocomial transmission and helps differentiate endogenous infections from exogenous infections (Boccia *et al.*, 2002). Today, it is possible to determine the source and transmission route of infections, and to establish the transmission dynamics in a hospital, population or country. Randomly amplified polymorphic DNA analysis, karyotyping, and pulsed-field gel electrophoresis (PFGE) are the molecular methods commonly used for typing yeasts (Vrioni and Matsiota-Bernard 2001; Roilides *et al.*, 2003).

Having information on causative organisms, antifungal susceptibility, and clonal relationships among *Candida* strains that cause significant mortality in children is a considerable advantage when attempting to treat and control these infections. Our 850-bed university hospital serves approximately 250,000 patients per year. Outbreaks caused by various bacteria (*Klebsiella* spp. (Ayan *et al.*, 2003b), *Acinetobacter baumannii* (Ayan *et al.*, 2003a), *Chryseobacterium meningosepticum* (Gungor *et al.*, 2003), *Stenotrophomonas maltophilia* (Gulcan *et al.*, 2004)) have occurred in the pediatric ward and molecular epidemiological studies showed that clonal identity was not rare among the nosocomial isolates in our hospital. However no molecular epidemiological study of *Candida* isolates has been done in this medical center to date. The aim of this study was to investigate the species distribution, antifungal susceptibility, and clonal relatedness of *Candida* isolates obtained from a group of pediatric/neonatal intensive care patients that had a very high death rate.

MATERIALS AND METHODS

Patients

The subjects were patients treated in our hospital's pediatric intensive care unit (PICU) and neonatal intensive care unit (NICU) between February 2003 and February 2004. The total capacity of these units is 49 beds. During the study period, 309 and 182 patients were admitted to the PICU and NICU, respectively. Only those who had candidemia or had *Candida* species isolated from sterile body sites were included in the study (21 patients total). The subjects with candidemia also had other samples (in addition to blood) collected for analysis of colonization. For each of the 21 cases, we retrospectively assessed patient demographic characteristics, administration of parenteral nutrition, antibiotics used, invasive procedures performed, and length of stay in the NICU or PICU.

Mycological studies

Candida species were identified using the germ tube test and the carbohydrate assimilation profile API ID 32C System (bioMerieux, France). Susceptibility testing was performed for amphotericin B (Sigma), fluconazole (Pfizer) and itraconazole (Eczacibasi) using the broth microdilution method. Minimum inhibitory concentrations (MICs) were determined according to Clinical and Laboratory Standards Institute (CLSI) M27-A2 criteria (CLSI 2002). *Candida crusei* ATCC 6258 was used as the control strain.

Molecular typing

Molecular typing was done for the 3 most common species identified: *C. albicans* (15 isolates), *C. parapsilosis* (6 isolates) and *C. pelliculosa* (4 isolates). Initially, these 25 isolates were subjected to arbitrarily primed polymerase chain reaction analysis (AP-PCR). Electrophoretic kary-

otyping (EK) was used to confirm type when AP-PCR results identified the strains as "same type" or "subtype".

AP-PCR analysis

Genomic DNA was extracted from each of the 25 isolates using QIAamp DNA mini kits (Qiagen, USA). Using the method optimized previously in our laboratory (Ayan *et al.*, 2003b), PCR amplification was performed in a 50-mL master mix containing approximately 100 ng of template DNA, 1X amplification buffer, 0.4 mM dNTP mix, 4 mM MgCl 2, 2.5 U Taq DNA polymerase, and 100 pmol M13 (5'-GAGGGTGGCGGTTCT-3') primer. The reaction mixture was amplified in a Thermal Cycler (MJ Research PTC-200, USA) using the following program: 2 cycles of 5 minutes at 94°C, 5 minutes at 40°C, and 5 minutes at 72°C, and then 40 cycles of 1 minute at 94°C, 1 minute at 40°C, and 2 minutes at 72°C. Amplification products were electrophoresed in 2% agarose gel and then stained, observed and photographed under UV illumination.

Analysis of AP-PCR band profiles was done using the GelCompar version 4.0 software package (Applied Maths, Kourtrai, Belgium). If the Dice similarity coefficient value was below 90%, isolates were identified as different genotypes. If the value was between 90% and 95%, isolates were identified as subtypes of the same strain. If the value was between 96% and 100%, isolates were identified as same genotype.

Electrophoretic karyotyping

EK analysis was performed using a contour-clamped homogeneous electric field system (CHEF DR-II, BioRad, Richmond, USA) as described previously (Lopez-Ribot *et al.*, 2000) with minor modifications. Yeast cells were grown on Sabouraud agar plates for 48 hours at 35°C. Colonies were suspended in 2 mL SE buffer (75 mM NaCl, 25 mM EDTA [pH:8.6]) and the optical density was adjusted so that it was approximately equivalent to McFarland's Standard No. 5.0. The cells were washed and re-suspended in 2 mL SE buffer.

Low-melting-point agarose (1.6%) (Gibco, UK) was prepared in 125 mM EDTA (pH 7.5) and mixed with an equal volume of yeast cell suspension at 37°C. Then 50 mL of Lyticase (2000 U/mL, Sigma) was added. The mixture of cells, low-melting-point agarose, and Lyticase was distributed into plug molds and allowed to solidify. The solidified plugs were then placed in 5 mL of 0.5 mM EDTA (pH 9.0) including 7.5% β-mercaptoethanol, and were incubated overnight in a 37°C water bath.

The plugs were washed once with 5 mL of 50 mM EDTA (pH 7.5), transferred into an ESP solution (0.5 M EDTA [pH 9.5], 1% sarcosine and proteinase K [1 mg/mL]), and incubated overnight in a 50°C water bath. DNA fragments were separated in a 1.0% pulse-field certified agarose gel (Bio-Rad Laboratories, Nazareth, Belgium) run in 0.5X Tris-borate-EDTA buffer (44.5 mM Tris, 44.5 mM Boric Acid, 1 mM EDTA [pH:8.0]). The electrophoresis conditions were 12°C at 5.5 V/cm^2 for 36 hours. The initial and final switch times were 90 seconds and 360 seconds, respectively. Each gel was stained with ethidium bromide (5 mg/mL) and photographed under UV light.

RESULTS

The 21 patients with *Candida* infections comprised 4.3% of the 491 total who were admitted to our PICU and NICU during the 1-year study period. Nineteen of the subjects had candidemia and 2 had *Candida* meningitidis. Sixteen were PICU patients and 5 were NICU patients. Six of the patients were girls and 15 were boys. Age group analysis revealed that 7 patients were between 0 and 2 months old, 5 were between 3 and 11 months, 6 were between 1 and 5 years, and 3 were between 6 and 12 years.

The mean ICU stay for the 21 patients was 31 days. Prior to infection, all 21 of the patients had received broad-spectrum cephalosporins or a carbapenem, an aminoglycoside, or a glycopeptide. Fourteen (67%) of the patients had undergone mechanical ventilation, 7 (33%) had received total parenteral nutrition, and 5 (24%) had had a central venous catheter placed. Seventeen (81%) of the patients had underlying diseases: renal failure in 5 cases, neurological disorders (hypoxic encephalopathy, cerebral palsy, myotonic dystrophy) in 5 cases, and 1 case each of intracranial hemorrhage, Down syndrome, multiple congenital abnormalities, aplastic anemia, medulloblastoma, Hirschsprung's disease, and organophosphate poisoning.

In total, 16 of the infections were treated with amphotericin B and 3 were treated with fluconazole. One patient died before therapy could be administered, and medical records were unavailable in 1 case. Sixteen (76%) of the 21 patients died.

A total of 28 *Candida* isolates were detected in the 21 patients. Twenty were from blood (8 *C. albicans*, 6 *C. parapsilosis*, 4 *C. pelliculosa*, 2 *C. famata*,), 2 were from urine (both *C. albicans*), 1 was from a central venous catheter (*C. albicans*), 2 were from tracheal aspirate (both *C. albicans*), 2 were from cerebrospinal fluid (1 *C. albicans*, 1 *C. tropicalis*), and 1 was from dialysis fluid (*C. albicans*). The overall species distribution was as follows: 15 *C. albicans* isolates, 6 *C. parapsilosis* isolates, 4 *C. pelliculosa* isolates, 2 *C. famata* isolates and 1 *C. tropicalis* isolate.

Antifungal susceptibility results for all tested drugs were showed on Table, as MIC90 and MIC50 values. Amphotericin B was most effective drug against the 28 isolates with a MIC90 of 0.125 µg/mL (Table 1). Twenty-six isolates (93%) were susceptible to fluconazole and 23 (82%) were susceptible to itraconazole. Four of the 15 total *C. albicans* isolates were resistant to itraconazole and 2 were resistant to fluconazole. One of the 4 *C. pelliculosa* isolates was resistant to itraconazole.

AP-PCR typing of the 15 *C. albicans* isolates yielded 11 genotypes (profile difference 73.3%). Of these 11 AP-PCR types, 4 (Figure 1a: lines 1 and 3, lines 5 and 6, lines 8 and 9, and lines 12 and 13) were identical pairs (each a cluster) and 7 were distinct. Of the 8 total *C. albicans* isolates from blood (8 patients), 2 were in the same cluster and the other 6 were unrelated. Two patients had *C. albicans* isolates from both blood and tracheal aspirate, and each of these pairs had identical AP-PCR profiles. One patient had *C. albicans* isolates from blood and dialysis fluid, and this pair was also identical types.

Since, for each isolate, the AP-PCR method revealed sufficient numbers of bands (≥ 10 bands) to correctly type each isolate, EK was not necessary for the 7 *C. albicans* isolates that had unique AP-PCR profiles. EK was carried out for the other 8 *C. albicans* isolates that were classified in clusters by AP-PCR, and the results were in exact concordance with respective PCR typing results (Figures 1a and 1b: lines 1 and 3, lines 5 and 6, lines 8 and 9, lines 12 and 13). For the 6 *C. parapsilosis* isolates (all from blood), AP-PCR revealed 2 genotypes and EK revealed 3 genotypes. Both typing procedures identified 4 of the *C. parapsilosis* isolates as identical (Figures 1c and 1d: lines 17-20). For the 4 *C. pelliculosa* isolates (all from blood), both AP-PCR and EK identified 4 distinct genotype profiles (Figures 1c and 1d: lines 22-25).

Comparisons of genotyping results with epidemiological data taken from patient records suggested that *C. albicans* isolates from blood and tracheal aspirates of the same patients were more likely to be related. Six of the 8 *C. albicans* isolates that comprised the 4 clusters were detected within a two-week period. One of the clusters comprised 2 strains from 2 different patients. Each of the other 3 clusters was a pair of isolates from the same patient. The typing results for the *C. parapsilosis* isolates were strongly correlated with the epidemiological data. As noted, both typing methods revealed that all 4 *C. parapsilosis* isolates were identical, and all these were isolated from the PICU within 6 days. In contrast, the 4 *C. pelliculosa* isolates were distinct, and these organisms were isolated from the PICU and NICU with intervals of longer than 1 month between isolations.

TABLE 1 - *In vitro susceptibilities of the Candida isolates to three antifungal agents.*

Species (No of isolates tested)	*Antifungal agents*	*MIC (µg/mL)*		
		Range	*50*	*90*
C. albicans (15)	Amphotericin B	0.03 to 0.125	0.03	0.125
	Itraconazole	0.03 to 32	0.03	8.0
	Fluconazole	0.03 to >64	1.0	8.0
Other *Candida spp.* (13)	Amphotericin B	0.03 to 0.25	0.03	0.125
	Itraconazole	0.03 to >32	0.03	0.5
	Fluconazole	0.06 to 8	2.0	8.0

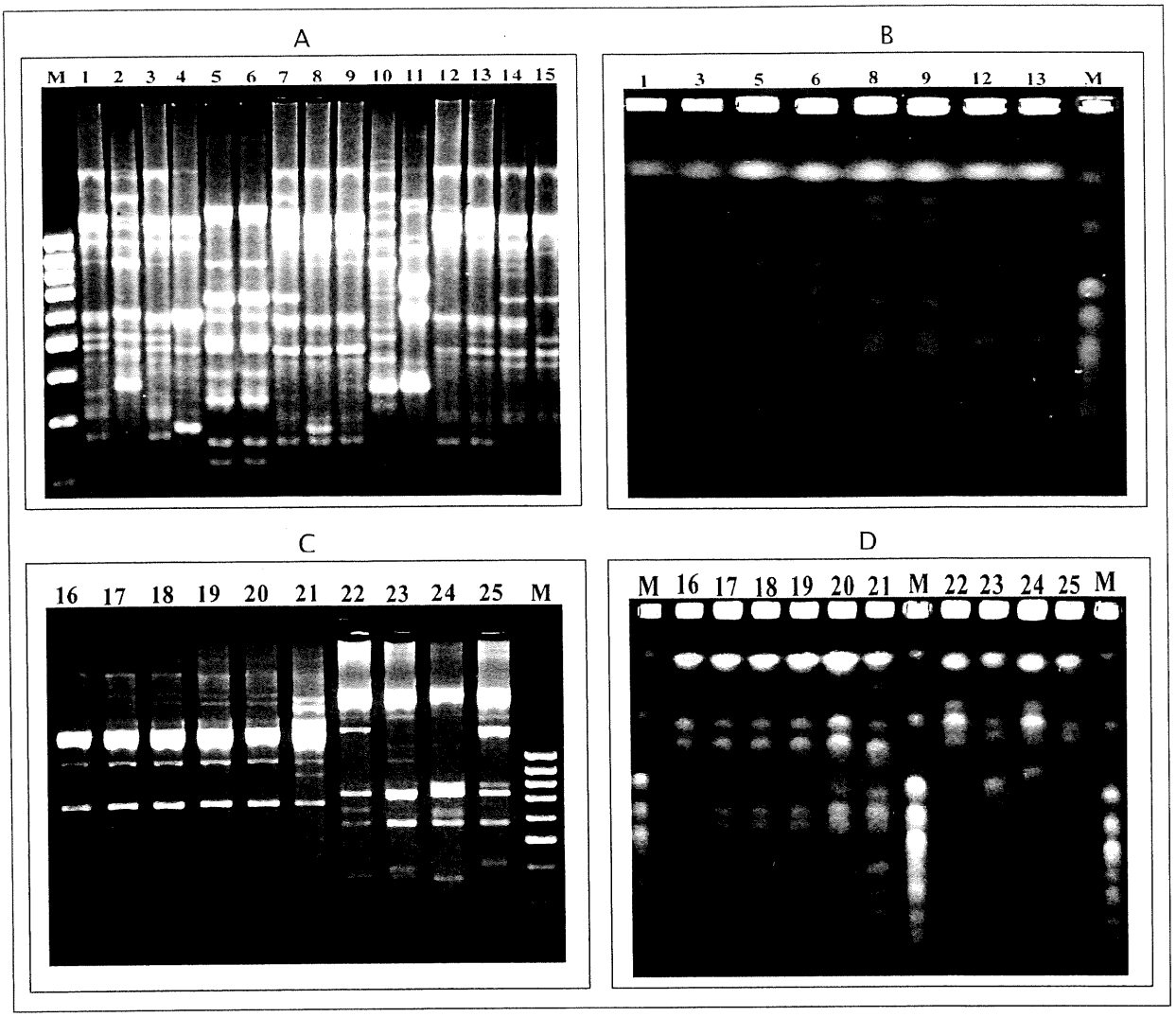


FIGURE 1 - ***A*** *(Top left): AP-PCR results for C. albicans. Lines 1-3: Patient 1 blood, urine and dialysis fluid isolates; Lines 4-6: Patient 2 urine, tracheal aspirate and blood isolates; Line 7: Patient 3 blood isolate; Line 8: Patient 4 blood isolate; Lines 9 and 10: Patient 5 blood and intravenous catheter isolates; Line 11: Patient 6 CSF isolate; Lines 12 and 13: Patient 7 blood and tracheal aspirate isolates; Line 14: Patient 8 blood isolate; Line 15: Patient 9 blood isolate.* ***B*** *(Top right): EK results for C. albicans. Lines 1 and 3: Patient 1 blood and dialysis fluid isolates; Lines 5 and 6: Patient 2 tracheal aspirate and blood isolates; Lines 8: Patient 4 blood isolate; Line 9: Patient 5 blood isolates; Lines 12 and 13: Patient 7 blood and tracheal aspirate isolates.* ***C*** *(Bottom left): AP-PCR results for C. parapsilosis (lines 16-21) and C. pelliculosa (lines 22-25). Line 16: Patient 10 blood isolate; Line 17: Patient 11 blood isolate; Line 18: Patient 12 blood isolate; Line 19: Patient 13 blood isolate; Line 20: Patient 14 blood isolate; Line 21: Patient 5 blood isolate; Line 22: Patient 15 blood isolate; Line 23: Patient 16 blood isolate; Line 24: Patient 17 blood isolate; Line 25: Patient 18 blood isolate.* ***D*** *(Bottom right): EK results for C. parapsilosis (lines 16-21) and C. pelliculosa (lines 22-25). Line 16: Patient 10 blood isolate; Line 17: Patient 11 blood isolate; Line 18: Patient 12 blood isolate; Line 19: Patient 13 blood isolate; Line 20: Patient 14 blood isolate; Line 21: Patient 5 blood isolate; Line 22: Patient 15 blood isolate; Line 23: Patient 16 blood isolate; Line 24: Patient 17 blood isolate; Line 25: Patient 18 blood isolate.*

DISCUSSION

As noted, rates of invasive *Candida* infection have increased significantly, and these infections are a special concern in PICUs and NICUs (Roilides *et al.*, 2004; Singhi *et al.*, 2004). *C. albicans* remains the most frequently isolated *Candida* species, but there has also been a notable rise in infections caused by non-albicans *Candida* species (Roilides *et al.* 2004; Roilides *et al.*, 2003;

Singhi *et al.*, 2004). In the SENTRY Antimicrobial Surveillance Study carried out between 1997 and 2000, *C. parapsilosis* was reported to be the most common non-albicans *Candida* species encountered in the pediatric age group (Pfaller *et al.*, 2002). The number of infections in our study was low, but we identified *C. parapsilosis* as the second most frequently isolated species after *C. albicans*. Prolonged hospitalization, central venous catheterization, previous antibiotic usage, hyperalimentation, surgical procedures, and administration of lipids and steroids are known risk factors for *Candida* infection in both adults and children (Roilides *et al.*, 2004; Leibovitz 2002; Feja *et al.*, 2005; Saiman L 1998). In accord with this, all 21 of our pediatric and neonatal patients with candidemia or *Candida* meningitidis had a history of antibiotic administration. In addition, 67% had received mechanical ventilation, 33% had received total parenteral nutrition, and 24% had had a central venous catheter inserted.

Despite antifungal therapy, reported rates of mortality related to candidemia are high, ranging from 60% to 78% (Barchiesi *et al.*, 2004; Morgan *et al.*, 2005). Research indicates that admission to ICU, delayed initiation of antifungal therapy, and insufficient therapy or treatment failure all increase the risk of death in patients with candidemia (Morgan *et al.*, 2005). The mortality rate for our 21 cases was 76% and risk factors such as multiple invasive procedures, underlying disease, and prolonged ICU stay may have influenced this rate.

Amphotericin B is the drug of choice for treating neonatal candidiasis, and azoles can be good alternatives (Roilides *et al.*, 2004; Linder *et al.*, 2003). A study by the International Fungal Surveillance Participant Group revealed little alteration in the fluconazole susceptibility of *Candida* isolates from bloodstream infections over a 10-year period, and showed that 90% of the isolates were susceptible to fluconazole (Pfaller and Diekema 2004). In the SENTRY study, *C. albicans* and *C. parapsilosis* were reported to be commonly isolated from blood cultures taken from pediatric patients aged ≤1 year and 2-15 years, and these species were more susceptible to azoles and amphotericin B than *C. glabrata* (Pfaller *et al.*, 2002). Another study revealed that many less common species of *Candida* bloodstream isolates exhibit decreased susceptibility to systemic antifungal drugs such as amphotericin B, fluconazole and flucytosine (Pfaller *et al.*, 2004). In our study, amphotericin B was very effective against all 28 *Candida* isolates (MIC range 0.03-0.125 µg/mL) and resistance to azoles varied.

Genotypic variations identified among tested isolates provide information on cross-contamination. In our study, molecular typing of the 15 *C. albicans* isolates yielded 11 genotypes (profile difference 73.3%) and the blood *C. albicans* isolates from 8 patients featured 6 separate types. This led us to conclude that rates of cross-contamination for *C. albicans* infections in our PICU and NICU are relatively low. Two of the blood isolates (each from a separate patient) were the same genotype (confirmed by AP-PCR and EK) and this suggests there is some cross-contamination among patients with *C. albicans* candidemia in these units.

Newborns acquire *C. albicans* infections either via vertical transmission (from the mother) or from environmental sources (cross-contamination). Reef *et al.*, found that *C. albicans* infections were transmitted to NICU patients predominantly via non-perinatal routes, and they identified cross-contamination via the hands of hospital staff and patients' family members as an important mode of transmission (Reef *et al.*, 1998). The same report revealed that various body sites of affected infants could be colonized with more than 1 *Candida* species, and that these colonizers could be the source of invasive disease. In 3 of our cases of *C. albicans* infection, the genotyping profiles for the blood isolates matched that of the isolate from the second sample (tracheal aspirate or dialysis fluid) collected from each patient. Thus, we believe that the candidemia could have been of endogenous origin in these patients. In other cases, the *C. albicans* strains isolated during sampling from different body sites of the same patient were of different molecular types, indicating colonization with more than 1 subtype of the same species responsible for the infection.

Rates of *C. parapsilosis* colonization in preterm infants are reportedly lower than rates for other *Candida* species and, unlike other *Candida* species, *C. parapsilosis* can cause nosocomial candidemia in the absence of colonization (Gagneur *et al.*, 2001; Shin *et al.*, 2001). Since *C. parapsilosis* is the species most frequently isolated from hands of hospital staff, *C. parapsilosis* is horizontally transmitted more frequently than other

Candida species. This explains the relatively high incidence of catheter-related *C. parapsilosis* infections (Bendel 2003; Hedderwick *et al.*, 2000; Barchiesi *et al.*, 2004). In our study, AP-PCR and EK identified 4 of the 6 total *C. parapsilosis* isolates as all the same genotype. These 4 isolates came from 4 patients who were all admitted to the PICU during the same week. In contrast, typing analysis of the 4 *C. pelliculosa* isolates indicated no transmission in the PICU and NICU.

In molecular typing studies, it is recommended that karyotyping or restriction fragment length polymorphism be performed on isolates that PCR-based methods identify as "same type." It is generally not considered necessary to conduct further analysis when PCR testing identifies an isolate as "different genotype" from others being tested (Durmaz *et al.*, 2003). Research indicates that genotyping results from PCR methods and EK are usually concordant (Huang *et al.*, 2004). In our molecular typing studies of 25 *Candida* isolates, we observed very good concordance between AP-PCR and karyotyping results. Thus, we conclude that AP-PCR is accurate and reliable for testing the relatedness of *Candida* strains.

In conclusion, in line with previous research, we found that non-albicans *Candida* species have become more frequently causative agents for invasive fungal infections in ICU. With respect to clonal relatedness of isolates, molecular typing showed very low-level transmission of *C. albicans* and *C. pelliculosa* strains among candidemia patients in our NICU and PICU, however the rate of *C. parapsilosis* transmission was high. Since cases of candidemia in our ICU have a very high mortality risk, stricter rules are needed to prevent transmission of *Candida* spp. infection and early presumptive antifungal therapy might be beneficial for patients in these units.

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