Molecular epidemiology of *Candida albicans* colonization and fungemia in very low birthweight infants

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OBJECTIVE: This study investigated the relationship between colonization and fungemia. **DESIGN:** This was a prospective study involving surveillance cultures of the nares, base of umbilicus, point of entry of umbilical catheter and parenteral fluids. Blood cultures were done when sepsis was suspected. All *Candida albicans* isolates were typed using restriction enzyme analysis of DNA. **SETTING:** Patients were from the neonatal intensive care unit of a tertiary care hospital. **POPULATION STUDIED:** Twenty-nine very low birthweight infants. **MAIN RESULTS:** Eleven babies were colonized with *C albicans* and five of these babies developed fungemia, including five of seven who were colonized at the point of entry of the umbilical catheter. Three different strains of *C albicans* caused fungemia. In four of the five patients, initial catheter entry site isolates were identical to the subsequent blood isolates. Occasionally, infants were colonized with more than one strain of *C albicans*. **CONCLUSIONS:** Preceding colonization with *C albicans* and, in particular, colonization at the site of entry of umbilical vascular catheters are risk factors for subsequent development of *C albicans* fungemia. Fungemic and colonizing isolates are usually identical to one another by DNA typing.

Key Words: Candida albicans, *Colonization, DNA typing, Fungemia, Molecular epidemiology, Neonates*

Épidémiologie moléculaire de la colonisation de *Candida albicans* et fongémie chez des nourrissons de très petit poids de naissance

OBJECTIF: Cette étude s'est penchée sur le rapport entre colonisation et fongémie. **MODÈLE:** Cette étude prospective a nécessité la mise en culture de prélèvements au niveau des narines, de la base de l'ombilic, du point d'entrée du cathéter ombilical et des liquides parentéraux. Des hémocultures ont été effectuées quand l'infection était soupçonnée. Tous les isolats *Candida albicans* ont été typés à l'aide d'une analyse de l'ADN par l'enzyme de restriction. **CONTEXTE:** Patients de l'unité de soins intensifs pour prématurés d'un hôpital de soins tertiaires. **POPULATION ÉTUDIÉE:** Vingt-neuf nourrissons de très faible poids de naissance. **PRINCIPAUX RÉSULTATS:** Chez onze bébés, on a décelé la présence de *C albicans* et cinq d'entre eux ont développé une fongémie, y compris cinq sur les sept chez qui le prélèvement avait été fait au point d'entrée du cathéter ombilical. Trois souches différentes de *C albicans* ont causé la fongémie. Certains des nourrissons étaient porteurs de plus d'une souche de *C albicans*. **CONCLUSIONS:** La colonisation préalable avec *C albicans* et en particulier, la colonisation au point d'entrée du cathéter vasculaire ombilical constitue un facteur de risque à l'égard du développement subséquent d'une fongémie à *C albicans*. Les isolats du sang et de la colonisation sont généralement identiques lors du typage de l'ADN.

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CANDIDA ALBICANS SEPSIS CAUSES SIGNIFICANT MORBIDITY and mortality in low birthweight infants (1-3). Despite the recognized importance and increasing number of reports of candida infections in neonatal intensive care patients, the source of the organism and the relationship between colonization and infection are not clear.

Multiple methods of typing *C albicans* have been described in the literature (4,5). Phenotypic methods are not very useful in comparing strains of *C albicans*. Discriminatory power is low and results are not readily reproducible in different laboratories. Moreover, phenotypes are not stable over time (6). Therefore, with the advent of molecular biology, genotypic methods have been applied to the study of *C albicans*. The simplest genotypic methods of typing *C albicans* involve analyzing the banding pattern produced by DNA digested with restriction endonucleases and electrophoresed on agarose gels (7-9). These techniques have proven useful in epidemiological investigations. Methods employing DNA probes have also been described (6,10-14).

We report the results of a prospective study on colonization of very low birthweight (VLBW) infants by *C albicans* in a neonatal intensive care unit. The strains of *C albicans* isolated were analyzed by restriction enzyme fingerprinting. Surveillance culture isolates from patients at different body sites and subsequent bloodstream isolates were compared in order to determine whether a patient is colonized with more than one strain, and whether the colonizing organism heralds a candidemia involving the same strain.

PATIENTS AND METHODS

Patient selection and follow-up: Entry criteria and follow-up have been described previously (15). Entry criteria comprised: birthweight less than 1250 g; admission to the neonatal intensive care unit at the Oregon Health Sciences University and placement of an umbilical catheter within 24 h after birth; and first surveillance cultures performed within 48 h after admission. Studied patients were admitted September 29, 1983 through December 3, 1983 and March 7, 1984 through April 10, 1984. Surveillance cultures were done twice weekly. Surveillance culture sites included the nares, base of umbilicus, point of entry of the umbilical catheter and parenteral fluids being given (other than blood and blood products). Oral, stool and rectal cultures were not done because of funding constraints. Initially umbilical catheter entry site care consisted of cleansing with 70% alcohol followed by the application of polymyxin-neomycin-bacitracin ointment thrice daily. After January 15, 1984 10% povidine-iodine diluted 1:5 with sterile water applied once daily was used.

Blood cultures were obtained from a peripheral vein when sepsis was suspected on a clinical basis. Likewise, umbilical catheter tips were cultured when the physician considered that results would yield clinically relevant information. Physicians were not aware of results of surveillance cultures. All neonates were followed until umbilical catheter removal or transfer from the unit. One neonate became candidemic after his umbilical catheter was removed, and he was included in the study. Culture techniques were as described previously (15).

TABLE 1
Characteristics of, and care provided to, the 29 studied very low birthweight neonates

Factor	Mean (range)
Gestational age (weeks)	27 (24-33)
Birthweight (grams)	882 (400-1180)
Umbilical vessel catheterization (days)*	16 (9-32)
Mechanical ventilation (days)*	15 (2-32)
Parenteral nutrition (days)*	14 (1-32)
Parenteral antibiotics (days)*	9 (2-16)

**Days represent those during umbilical vessel catheterization and before the first episode of bacteremia or fungemia, if applicable*

Isolate identification and analysis: Candida were identified by appearance of colonies on 5% sheep blood agar, Gram stain morphology, and production of pseudohyphae on corn meal agar containing 1% Tween 80 (16). Speciation was done with the API 20C Clinical Yeast System (Analytab Products, New York) (17). Isolates were stored in skim milk at –70°C.

The method of DNA analysis described earlier (8) was modified to facilitate the use of 1.5 mL Eppendorf tubes. One loopful of organisms was suspended in 1.0 mL of phosphate buffered saline (PBS). Supernatant was discarded after 5 mins centrifugation. The pellet was suspended in 1.0 mL of 1.3 M sorbitol and 0.1 M potassium phosphate (pH 7.5) with 6.2 µL beta-mercaptoethanol and 1 mg lyticase (dissolved in 50 µL PBS). This was incubated for 45 mins at 30°C and then centrifuged 5 mins. The pellet was washed twice with 1 M sorbitol and 0.1 M EDTA (pH 7.5) and then suspended in 0.5 mL of 0.15 M sodium chloride and 0.1 M EDTA (pH 8.0). Proteinase K was added to a final concentration of 1.2 mg/mL. This was incubated for 15 mins at 60°C followed by 60 mins at 37°C. Sodium dodecyl sulphate was added to a final concentration of 1% and the solution was incubated at 60°C for 15 mins. The sample was extracted twice with phenol:chloroform:isoamyl-alcohol (50:50:1) and twice with chloroform:isoamyl alcohol (24:1). The DNA was ethanol precipitated and the pellet dissolved in 0.5 mL of 1 x standard saline citrate. RNase was added (25 µg/mL) and the solution incubated at 37°C for 60 mins. Extraction, precipitation and drying were repeated and the pellet was resuspended in 50 µL water. Restriction digests were performed using *Eco*R1 under the conditions recommended by the manufacturer. Samples were electrophoresed on 0.6% agarose gels and stained with ethidium bromide. Lambda phage DNA digested with *Hind*III was used as a molecular weight standard.

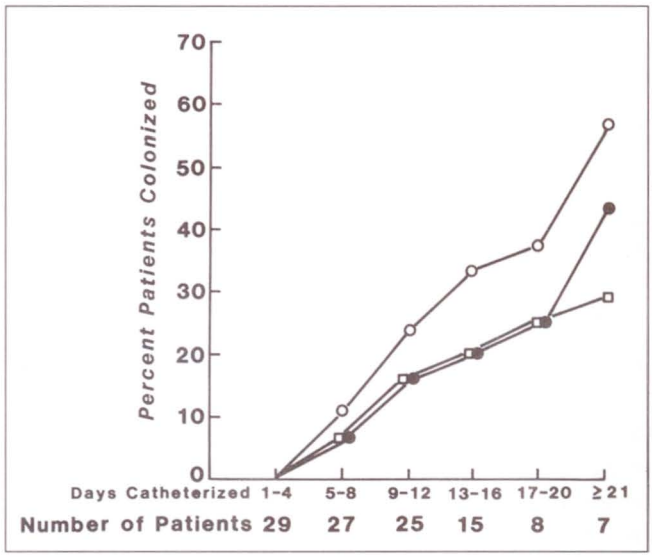

Figure 1) *Colonization with* Candida albicans *during umbilical catheterization. ○ Nares; □ Base of umbilical cord; ● Umbilical catheter entry site*

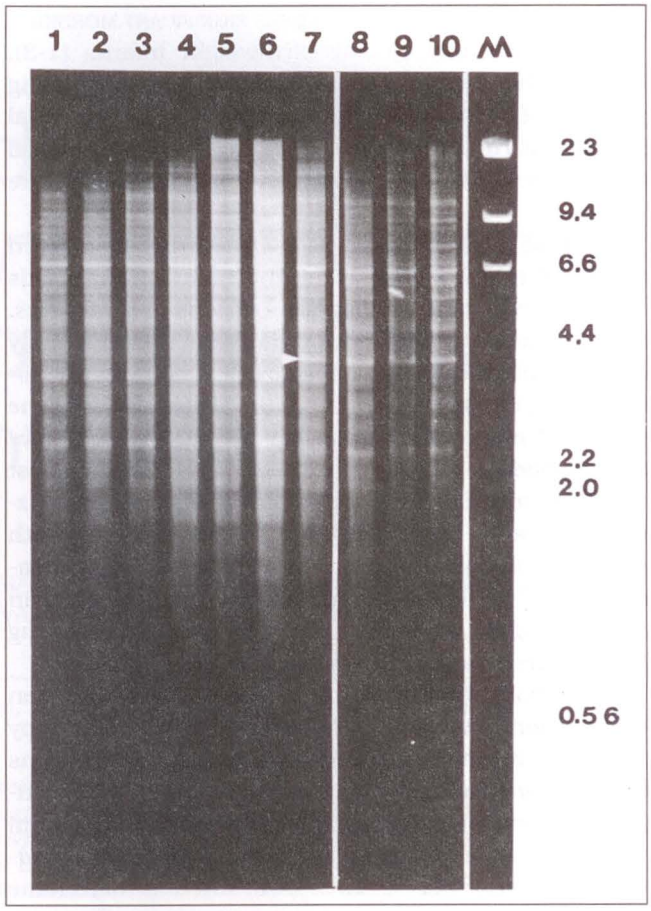

Figure 2) Eco*R1 digests of DNA from* Candida albicans *analyzed by agarose gel electrophoresis. Size markers are indicated on the right (kilobase pairs). Lanes 1 to 7 contain DNA from patient 2. Six of these samples show the same pattern (designated pattern B) with predominant bands at 6.5, 3.8 and 2.6 kb, but the sample in lane 7 has an extra band at 4.2 kb (arrowhead) and has been designated pattern C. Lanes 8 to 10 contain DNA from patient 1. Predominant bands are at 6.5, 4.2 and 2.6 kb (designated pattern A)*

RESULTS

Study patients: Thirty-four VLBW neonates were admitted during the study entry intervals. Five were excluded from analysis because surveillance cultures were not obtained within 48 h of admission (one patient), admission occurred later than 24 h following birth (one patient), or death occurred before a second set of surveillance cultures was obtained (three patients). None of the last three patients had candidemia or colonization with candida. The 29 patients studied had characteristics and supportive care that are summarized in Table 1. All were placed on mechanical ventilation, had umbilical artery catheters, received parenteral nutrition, and were administered parenteral antibiotics. Nineteen (66%) had umbilical vein catheters in addition to umbilical artery catheters. Results of bacterial cultures are described elsewhere (15).

Colonization with *C albicans* and candidemia: None of the patients was colonized with *C albicans* at any site when cultured during the first four days of umbilical catheterization. Thereafter, colonization of patients remaining catheterized increased over time at all three sites (Figure 1). By 21 or more days of catheterization, patients were colonized at the nares (57%), the base of the umbilical cord (29%), and the catheter entry site (43%). Once the patients became colonized at any site with *C albicans*, persistent colonization was the rule. This was evident at the nares (84%), base of the umbilical cord (55%), and at the catheter entry site (89%). Eleven of the 29 babies (55%) had *C albicans* isolated from at least one surveillance culture, with seven of these having positive cultures from the umbilical catheter entry site.

Eighteen babies did not have positive surveillance culture for *C albicans* and did not develop fungemia with this organism. Of the 11 with positive surveillance cultures, five (45%) developed positive blood cultures for *C albicans*. These latter babies ranged in age from nine to 52 days at the time of the first positive blood culture. Four of the five affected babies still had umbilical artery catheters in place at the time of candidemia. In each case, *C albicans* had been isolated from the umbilical catheter entry site before the occurrence of candidemia. Umbilical catheter tip cultures were positive in two of these four infants (one infant did not have tip cultures done and one had a negative tip culture). The fifth affected infant had his umbilical artery catheter replaced by an indwelling long line because of persistently positive blood cultures for *Staphylococcus epidermidis* before developing fungemia. *C albicans* had grown from the umbilical catheter entry site before catheter removal.

None of the parenteral fluid cultures were positive for yeast.

DNA analysis of candida isolates from blood and surveillance cultures: All 36 isolates from the five babies who developed candidemia were subjected to DNA analysis. Multiple bands were visible on ethidium bromide stained gels. The most prominent bands (6.5, 4.2, 3.8 and 2.6 kb) probably represent repeat sequences (12). Figure 2 shows a gel with samples from two of the five candidemic patients (patient 1 and patient 2). This demonstrates three different patterns which can be recognized by the DNA restriction fragment characteristics. All 36 isolates manifested one of these three patterns.

Table 2 categorizes the colonization patterns of the five candidemic patients based on restriction endonuclease profiles of the tested isolates. Pattern A described a restriction enzyme profile with prominent DNA fragments of 6.5, 4.2 and 2.6 kb; pattern B with fragments of 6.5, 4.2, 3.8 and 2.6 kb; and pattern C with fragments of 6.5, 3.8 and 2.6 kb.

Three samples were analyzed from patient 1; all showed the same profile, pattern A. Nine samples were analyzed from patient 2. Eight had the same profile as one another (but different from that seen in patient 1) which was labelled pattern B. The ninth sample revealed a third pattern, designated pattern C. This sample was obtained from the nares, although two previous samples from the nares were pattern B. Seven samples were analyzed from patient 3; all had the same profile as that seen in patient 1, pattern A. All eight samples analyzed from patient 4 were pattern A. Nine samples were analyzed from patient 5. All were the same as those seen in patients 1, 3 and 4 (pattern A) with the exception of the blood culture specimen. This sample was the same as the discrepant strain from patient 2, representing pattern C. Interestingly, the umbilical artery catheter was removed two days after the candidemia was documented, and the tip yielded *C albicans* with DNA pattern A.

TABLE 2
Patterns obtained after *Eco*R1 digestion of *Candida albicans* from various sites over time from neonatal intensive care patients who developed candidemia

	Number of samples per site					
Patient	Nares	UB	UCJ	BC	CT	Pattern
1		1	1	1		All A
2	3	1	3	2		All B except 1 of 3 from nares which was C
3	4	1	1	1		All A
4	1	2	3	1	1	All A
5	2	4	1	1	1	All A except BC which was C

BC Blood culture; CT Umbilical catheter tip; UB Base of umbilicus; UCJ Umbilicus-catheter junction. Examples of these patterns are shown in Figure 2

DISCUSSION

Prior reports have demonstrated that VLBW infants with preceding long term umbilical vessel catheterization, mechanical ventilation, total parenteral nutrition and prolonged preceding antibiotic therapy are at particular risk for the development of fungemia and disseminated infections caused by *C albicans* (2,3,18). In this prospective study we evaluated the role of colonization in high risk neonates at specific surveillance sites which preceded *C albicans* fungemia. Surveillance and blood culture isolates from candidemic patients were typed by restriction endonuclease digests. Among 18 patients with negative surveillance cultures, none developed candidemia. Among the 11 colonized patients, five developed fungemia. This included five of seven with colonization at the umbilical catheter entry site, four of eight with colonization at the base of the umbilicus and three of eight with nasal colonization. These findings suggest that preceding colonization with *C albicans* is a potent risk factor for the eventual development of candidemia, as has been previously suggested (18,19). In addition, our data support the contention that colonization at the catheter entry site followed by extraluminal migration of the organism down the catheter and into the bloodstream is one source of fungemia and dissemination in this high risk patient population. This corresponds favourably with the most common pathogenesis for bacteremia and fungemia secondary to colonization and infection of other long term, nontunnelled central vascular access catheters (20). Where long term access is necessary for administration of parenteral fluids rather than for arterial sampling, the use of tunnelled access catheters rather than prolonged umbilical vessel catheterization offers a potential for decreasing the risk of catheter related bloodstream infections (21,22). The effectiveness of such an approach should be further explored in well designed randomized trials.

Restriction endonuclease typing of isolates using *Eco*R1 confirmed the epidemiological and surveillance culture findings in this study. In four of five instances, the preceding surveillance culture isolates were identical to the subsequent blood isolate(s) from each patient, suggesting that blood isolates usually mirror surveillance isolates. One patient did have a different organism by DNA type in the bloodstream from the type colonizing multiple sites preceding his fungemic event (patient 5). In previous studies patients have been shown occasionally to have more than one strain of *C albicans* at different body sites simultaneously. A study using genotyping found one of 13 adult patients to carry a discrepant strain (23). Differences between such strains may be pre-existing, or could arise due to mutations (12). There is some evidence that *C albicans* will assume a different phenotype when it changes from being a colonizing organism to a pathogen (23), but there is no evidence that genotype changes under these

circumstances. The fact that the discrepant strain in patient 5 resulted in candidemia could be interpreted to mean that although colonized with *C albicans* the patient became ill only after acquiring a second, possibly more virulent strain. This also raised the possibility that alternative pathogenic mechanisms do account for at least some fungemic episodes, such as heavy gastrointestinal load of organism followed by ingress into the bloodstream from the gastrointestinal tract (24).

Our study found that 45% of VLBW infants with *C albicans* colonization of the nares, base of umbilicus, or point of entry of the umbilical catheter eventually became fungemic with *C albicans*, with all fungemic babies having preceding colonization. Baley et al (19) found that only 8% of VLBW infants with fungal colonization of the mouth, groin, rectum, or of an endotracheal aspirate as detected by routine surveillance cultures eventually became fungemic, with all fungemic babies having preceding colonization. However, 10% of term babies have fungal colonization of the mouth or rectum (19), so it is perhaps not surprising that colonization at gastrointestinal sites is not a major predictor of fungemia. Nonetheless, a correlation has been demonstrated between the concentration of fungus in stool and the risk of fungemia in VLBW infants (25). The role of gastrointestinal fungi in fungemia could not be evaluated in the present study as oral, rectal, and stool cultures were not part of the surveillance process.

The present study again demonstrates the utility of DNA fingerprinting for investigating nosocomial candida infections. Typing using restriction endonuclease digestion with *Eco*R1 is very effective in terms of an ability to type all strains, reproducibility and reasonable ease of performance and interpretation (8,9). However, discriminatory powers of this typing tool for evaluation of strain transmission is limited since a variety of studies have shown that from 34 to 63% of epidemiologically unrelated isolates have the most common *Eco*R1 type (7,9, 26-28). It has recently been shown that the restriction endonucleases *Bgl*11 and *Hind*1 (29) or pulse-field gel electrophoresis (28,30) may allow further typing of *C albicans* beyond that demonstrated by *Eco*R1. The use of DNA probes may better differentiate between strains, but such probes may actually be too sensitive for practical purposes and show minor differences resulting from in vitro passage of the same strain (12). Computer based DNA analysis may offer increased discriminatory power with maintained typability and reproducibility but at a substantially increased time and cost of performance (31). Such tests may be most applicable to isolates that are identical by *Eco*R1 typing if horizontal transmission to multiple patients is in question.

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