Assessment of Similarity among Coagulase-Negative Staphylococci from Sequential Blood Cultures of Neonates and Children by Pulsed-Field Gel Electrophoresis

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One of the criteria used to determine the clinical importance of coagulase-negative staphylococci (CoNS) is isolation of the bacteria from sequential blood cultures. Pulsed-field gel electrophoresis was used to characterize sequential blood isolates of CoNS collected within a 7-day period from neonates and children with bacteremia. Of 18 episodes among neonates, 6 (33%) involved unrelated strains of CoNS. All unrelated strains were from neonates who received antimicrobial therapy after the first culture and who had a second culture \( \geq 36 \) h later. Among older children, 5 (19%) of the 27 episodes of presumed central venous (CV) catheter-related sepsis involved unrelated isolates. All of the unrelated isolates were from patients who had blood samples obtained through CV catheters only. Thus, even repeated isolation of CoNS from blood cultures may represent contamination if samples are drawn through CV catheters only or if second samples are obtained \( > 1 \) day after appropriate antimicrobial therapy.

Coagulase-negative staphylococci (CoNS) are the leading cause of nosocomial bacteremia in most pediatric hospitals [1]. CoNS bacteremia among neonates in intensive care nurseries is attributed to the improved survival of very-low-birth-weight premature infants requiring prolonged hospitalization and placement of intravascular devices for sustenance [2–5]. Other pediatric patients at high risk for developing sepsis due to CoNS are those requiring prolonged intravascular access for chemotherapy or parenteral nutrition because of serious underlying diseases [6–9].

Diagnosis of CoNS sepsis is made by isolating the microorganism from blood cultures. However, CoNS are normal skin flora and, as such, are also the most common contaminating microorganism found in blood cultures [10–12]. Therefore, it is often difficult to determine if CoNS isolated from blood cultures of neonates and other children with central venous (CV) catheters are skin contaminants or the true cause of sepsis [13–16]. One criterion used to determine the clinical importance of CoNS is their isolation from sequential blood cultures [4, 10, 14, 17, 18]. Implicit in the use of this criterion, however, is the assumption that the isolates are the same. For CoNS, pulsed-field gel electrophoresis (PFGE), a molecular typing technique that allows separation of DNA fragments, has been shown to be superior to conventional typing methods, such as speciation, serotyping, bacteriophage typing, and antibiograms, and to molecular techniques, such as plasmid profile analysis and multilocus enzyme electrophoresis (MLEE) [19–23]. Therefore, we used PFGE to genetically characterize sequential isolates of CoNS from blood samples obtained from neonates and older children with suspected CV catheter-related sepsis.

**Patients and Methods**

*Patients.* Neonates in the intensive care nursery with a clinical diagnosis of nosocomial bacteremia (bacteremia occurring \( \geq 72 \) h after admission) and older children with suspected CV catheter-related sepsis due to community- or hospital-acquired CoNS were prospectively identified from May 1994 to May 1995. Only those patients with \( > 2 \) blood culture sets positive for CoNS within a 7-day period were selected for further study. Relevant clinical and demographic data from these patients were also obtained. For neonates, this included recording the presence of apnea, bradycardia, hypothermia, hypoglycemia, or other signs suggestive of sepsis. To be included in the study, older children had to have a temperature \( > 38^\circ C \) at or shortly before blood was obtained for the first culture. In addition, the peripheral white blood cell count, the presence of central or peripheral arterial or venous catheters, and antibiotic therapy, if any, were recorded. Surveillance blood cultures were excluded from this study.

*PFGE.* PFGE is a molecular technique that allows the separation of chromosomal DNA fragments ranging in size from a few hundred to \( > 10 \) million bp by the use of rare cutting restriction enzymes and the application of multidirectional electrical pulses through an agarose matrix. The technique is not affected by exchange or loss of extrachromosomal genetic material [19]. Isolates for PFGE were subcultured onto trypticase soy agar with 5% sheep blood and incubated at 35°C in 5% CO\(_2\) for 48–72 h so that
differences in colonial morphology of CoNS could be detected. A single colony of each distinct morphologic type was then grown in 3 mL of tryptose soy broth overnight at 37°C on a shaker. An 80-μL aliquot of the broth suspension was spun down, and the intact bacterial cells were embedded in small agarose plugs. The samples were lysed with lysostaphin and lysozyme and then deproteinized in situ using a reagent kit (GenPath Group 1; Bio-Rad Laboratories, Hercules, CA). Restriction enzyme digestion was done using Smal, and slices of the plugs with embedded DNA were loaded into the wells of a 1% molecular biology-grade agarose gel (Bio-Rad) and analyzed by PFGE using the reagent kit instrument settings for staphylococci as described by the manufacturer. The gel was stained with ethidium bromide and photographed under UV light.

Restriction patterns of different isolates from each patient were compared. The isolates were considered indistinguishable if there were no chromosomal band differences, related if they differed by one to three bands, and unrelated if they differed by four or more bands [19, 24].

Species identification. Isolates were identified and antimicrobial susceptibilities were determined by use of the MicroScan WalkAway-40 System and the Pos Combo type 6 Dried Panel (Dade MicroScan, West Sacramento, CA).

Results

During the 13-month study period, 45 episodes of CoNS bacteremia with ≥2 positive blood cultures per episode were identified. Of these episodes, 18 were in 17 neonates in the intensive care nursery, and 27 were suspected CV catheter-related infections in 25 children seen in the oncology clinics or admitted to the pediatric wards or the Pediatric Intensive Care Unit.

Neonates. The median age of neonates at the time of bacteremia was 14 days (range, 5–86). Of the 18 episodes of bacteremia, 12 (67%) occurred while CV or percutaneously inserted central (PIC) catheters were present. A total of 45 cultures (2.5/episode) were positive for CoNS from these patients. There were 7 episodes with ≥2 positive cultures. Of the 45 cultures, 10 (22%) had ≥1 genotype of CoNS. Therefore, a total of 55 isolates were available for analysis. Of these, 45 (82%) were Staphylococcus epidermidis, 2 (4%) Staphylococcus haemolyticus, 1 (2%) Staphylococcus saprophyticus, and 7 (13%) Staphylococcus species that could not be identified further.

Of the 45 cultures, blood samples for 13 (29%) were drawn through CV or PIC catheters. The remaining 32 (71%) were drawn either by peripheral venipuncture or arterial puncture. In 9 of the 18 episodes, all samples were obtained by a peripheral arterial puncture or venipuncture, 5 (28%) were central/peripheral samples, and 4 (22%) were central/central samples. The median time between sequential blood cultures was 36 h (mean, 30.9; range, 0–68). In 11 (61%) of the 18 episodes, sequential blood samples were obtained ≥36 h apart.

PFGE restriction patterns showed that sequential CoNS isolates from 9 of the 18 episodes were indistinguishable, 3 (17%) were related, and 6 (33%) were unrelated (figure 1). Of the 7 episodes in which there were ≥3 sequential isolates, all were similar (indistinguishable or related) in 2 episodes, most were similar in 2 episodes, and all were unrelated in 3 episodes. CoNS isolates were unrelated in 4 (44%) of 9 peripherally drawn blood samples, 1 of 5 central/peripheral samples, and 1 of 4 central/central samples. Six of the 9 patients with only peripherally drawn samples did not have central catheters, 2 had PIC catheters, and 1 had a Broviac catheter. Overall, sequential isolates from only 12 (67%) of the 18 episodes were similar. Of 7 blood samples obtained 0 to 1 day apart, all were similar. Of samples drawn >1 day apart, however, only 5 (45%) of 11 were similar (P < .05 by Fisher’s exact test).

In 14 of the 18 neonatal episodes, empiric antibiotic therapy with vancomycin was started shortly after obtaining the first blood sample. Subsequent isolates in 6 (43%) of these 14 episodes were unrelated by PFGE analysis. In the remaining 4 episodes, at least 2 blood samples were drawn within minutes of each other before initiation of antibiotic therapy, and all of these had similar (indistinguishable or related) PFGE patterns. Vancomycin was prescribed for 1–4 weeks in 16 of 18 episodes, since the treating physician deemed the isolates clinically important without knowledge of our PFGE results. One patient with related isolates did well clinically after removal of a CV catheter only, and the other neonate with documented influenza infection had unrelated isolates judged to be contaminants.
Children (non-neonates) with CV catheters. Of the 27 episodes of suspected CV catheter–related sepsis due to CoNS, 21 (78%) were in children with cancer, 4 (15%) in children with congenital heart disease, and 2 (7%) in children with congenital deficiencies of the immune system. A total of 67 (2.5/episode) cultures for these patients were positive for CoNS; none had >1 genotype of CoNS. Of these isolates, 62 (93%) were S. epidermidis, 3 (4%) Staphylococcus hominis, and 2 (3%) S. haemolyticus.

Of the 67 blood samples for culture, 43 (64%) were obtained through CV catheters and 24 (36%) were obtained by peripheral venipuncture. In 16 (59%) of the 27 episodes, all samples were obtained through CV catheters; the remaining 11 (41%) were central/peripheral samples. Of the 16 episodes in which blood was obtained through CV catheters only, there were 8 episodes in which blood samples from different lumens of the same catheter were sent to the microbiology laboratory as independently obtained samples with the color of the lumen specified on the label as the site of the sample. Seven of these 8 episodes were in patients with underlying cancer. At least 2 blood samples were obtained before initiation of antibiotic therapy in 11 (41%) of 27 episodes.

The median time between sequential blood cultures in children was 16.8 h (mean, 26.7; range, 0–143). The time interval between blood cultures was ≥36 h in only 8 (30%) of these episodes. PFGE restriction patterns showed that 22 (81%) of the 27 episodes had indistinguishable CoNS isolates, whereas 5 (19%) episodes had unrelated CoNS isolates (figure 1). Of the 16 central/central isolates, 5 (31%) were unrelated, whereas none of the 11 episodes in which blood was obtained through a CV catheter and by peripheral venipuncture had unrelated CoNS isolates. Eight of the 16 central/central isolates were in fact from blood samples drawn within minutes of each other through different lumens of the same catheter. Four of these 8 were unrelated. Two of the patients with unrelated isolates from different lumens had negative peripheral blood cultures, 1 had negative cultures from another central catheter, and 1 had a positive culture from one of the lumens drawn later in the day that was related to one lumen but unrelated to the other. Of the 4 patients with lumen cultures indistinguishable from one another, 2 had no other samples drawn, 1 had negative cultures from another central catheter, and 1 had a positive culture (not available for analysis) from another central catheter. The clinicians elected to treat 7 of these 8 patients with vancomycin and the other with nafcillin for 10–14 days because of the presence of fever in all 8, underlying malignancy in 7, and neutropenia in 6.

Comparison of typing methods. Overall, 11 (24%) of 45 episodes in pediatric patients involved unrelated isolates of CoNS by PFGE. Speciation by automated methods was helpful in showing differences between sequential isolates in only 3 (27%) of these 11 episodes because most of the isolates (88%) were identified as S. epidermidis. Antibiograms of individual isolates separated only 3 (27%) of 11 isolates on the basis of different susceptibility results for at least 2 of the 18 antibiotics tested. In one episode, however, the isolates were different by both speciation and antibiogram. Thus, overall, only 5 (45%) of 11 of the unrelated isolates could be differentiated on the basis of speciation or distinct antibiograms. Conversely, 3 (8%) of 36 episodes involved similar CoNS isolates by PFGE but different susceptibility results for two or more antibiotics tested.

Discussion

The judgment of whether or not a neonate in the intensive care nursery has CoNS sepsis depends heavily on the clinical impression of the treating physician. In practice, the neonatologist frequently relies on results of a single blood culture and most often elects to treat any isolate of CoNS as sepsis because of the nonspecific nature of the illness and the vast amount of literature documenting its pathogenicity in the preterm neonate [2–5, 15, 16, 25]. Multiple isolates of CoNS from these patients are almost always thought to represent sepsis. The issue of strain relatedness between sequential isolates of CoNS from neonates has not been well-studied. Tan et al. [18] evaluated multiple isolates of CoNS from 3 neonates and found those from 1 to be unrelated by MLEE. In the present study, 6 (33%) of 18 neonatal episodes involved unrelated isolates by PFGE, which can resolve DNA fragments that are not separated by other molecular methods, including MLEE.

Why should neonates have such a high rate of isolation of distinct strains of CoNS? Two plausible explanations are heavy colonization of neonates by CoNS soon after admission to the intensive care nursery [26–28] and technical difficulty in obtaining blood from these tiny patients. Maintaining strict asepsis is problematic when veins are barely palpable and the patient is moving; both problems result in a high risk of contamination of the blood sample by skin flora. That 22% (10 of 45) of the blood cultures for neonates in this study and none in older patients had >1 genotype of CoNS, and isolation of distinct strains is not conclusive proof of contamination.

One factor determining similarity between sequential isolates in neonates appears to be whether blood samples were obtained within a 1-day period or after a longer interval. There was only a 45% chance that samples drawn ≥36 h apart would yield similar isolates. This is not to suggest that CoNS isolated from the first culture did not represent true sepsis but rather that in the presence of adequate therapy for CoNS, there was a >40% (6 of 14) likelihood that a second culture growing CoNS would be different from the first. In practice, as in our study, physicians often obtain a single set of cultures for neonates suspected of sepsis because of the difficulty in obtaining blood from these tiny infants. A second blood sample is not drawn unless the laboratory reports the first as being positive. Adequate therapy
decreases the chance that an organism present earlier will persist in sufficient numbers to be detected. In other words, if a second blood sample is obtained >1 day after therapy and CoNS is isolated, it is more likely to be a skin contaminant than the organism initially cultured. The data become even more difficult to interpret if decisions such as whether a CV catheter needs to be removed or whether a patient has endocarditis are based on only 1 positive culture before starting antibiotic therapy. We suspect that many neonates with apparently persistent CoNS bacteremia despite therapy in fact have contaminated blood cultures.

The method of collection of the blood sample did not appear to be a significant factor in predicting similarity between sequential isolates in neonates. The probability that sequential isolates would be unrelated was highest (44%) when both samples were drawn peripherally. In neonates, this could be due to poor aseptic technique in drawing blood or to interval antimicrobial therapy in all these patients. In addition, few of these patients had central catheters, the major risk factor for acquiring CoNS sepsis. Of note, 6 of the 18 episodes of suspected CoNS bacteremia occurred in neonates without CV or PIC catheters, and 4 of the 6 had similar isolates. Other investigators [4,5] have suggested that CoNS may cause invasive disease in neonates in the absence of central catheters. Therefore, most neonatologists treat these patients with antibiotics for 10–14 days. Whether these cases represent true bacteremia or contamination with distinct isolates needs further investigation.

Only 41% of older children with presumed catheter-related sepsis had central and peripheral blood samples obtained. All of these were indistinguishable, whereas 31% of the 16 episodes in which samples were obtained only through CV catheters were unrelated. This is in contrast to the data reported by Tan et al. [18], who found only 9% of their sequential isolates to be unrelated by MLEE. However, as mentioned, MLEE has lower discriminatory power than PFGE and may be able to resolve fewer differences among clinical isolates. In patients with blood samples drawn through different lumens of a multiple-lumen catheter, 50% of the strains were unrelated. The question of whether drawing blood for culture through CV catheters yields reliable results that correlate well with peripherally obtained blood samples has been addressed by several researchers [29–34] with reported sensitivity of between 57% and 96% and specificity between 80% and 98%. False-positive results (positive catheter culture with negative peripheral culture) have ranged from 4% to 28% [29–34], with the higher rates reported for catheters that had been in place for >4 days [33,34].

Most patients in our study had long-term indwelling catheters; therefore, catheter colonization would be a significant concern in these patients. That multiple blood samples obtained through these catheters grow CoNS does not prove systemic infection. In a review, Pizzo [35] states that obtaining blood samples from each catheter port and lumen and from a peripheral vein should be considered a general principle in the management of fever in patients with neutropenia. However, to the best of our knowledge, there are no data in the literature to suggest that obtaining blood samples through different lumens of the same catheter provides any more useful information than obtaining the same volume of blood from a single lumen. As we have shown, such cultures often grow different strains of CoNS, and in the absence of a peripheral sample for culture, they cannot give accurate information as to which, if any, of these isolates has invaded the bloodstream to cause systemic infection. Conversely, in 16 episodes in which both central and peripheral samples were obtained, only 1 neonate and no older children had unrelated isolates. Such results are highly indicative of true CoNS sepsis in these patients.

CoNS at most institutions, including ours, have become highly resistant to all commonly used antibiotics except vancomycin [21,23,36,37]. However, with the emergence of vancomycin-resistant enterococci, the use of vancomycin needs to be carefully monitored [38–40]. This is especially important with the threat of emergence of vancomycin-resistant *Staphylococcus aureus*, which has already been demonstrated experimentally [41]. One of the ways in which vancomycin use may be decreased would be to optimize collection of blood samples for culture so that contaminating CoNS are not treated with prolonged courses of vancomycin. Unfortunately, there is no easy and accurate method for typing CoNS [14,17,21,36]. PFGE, although an excellent technique to study differences among CoNS strains, is likely to remain a research and epidemiologic tool rather than to be integrated into routine clinical practice because it is immensely time and labor consuming. Therefore, clinicians must focus on improving blood collection techniques so that reliable cultures can be obtained.

In summary, a single blood culture that grows CoNS is uninterpretable in a patient at high risk for sepsis and from whom only 1 blood sample is drawn. Moreover, our data indicate that even repeated isolation of CoNS from blood cultures of both neonates and older children may represent contamination if blood samples are obtained several days apart or through CV catheters only. The most cost-effective approach to increasing the diagnostic utility of blood cultures is to improve the quality of specimen collection and not to request that laboratories spend additional time and effort on the analysis of inappropriately obtained samples. Also, as we have shown, speciation and antibiograms of CoNS are unreliable means of distinguishing isolates. The best approach to diagnosing CoNS bacteremia in patients at high risk for this infection is to obtain 2 blood samples for culture from different sites within a 1-day period. Sampling two different peripheral sites with strict attention to aseptic technique would be ideal but is not always feasible in pediatric patients. However, obtaining at least 1 peripheral sample every time blood is drawn through a central catheter should be considered the minimum standard of care. Such an approach would permit reliable interpretation of CoNS isolated from blood cultures: If both cultures were positive, it would be highly predictive of true bacteremia, whereas 1 positive culture would most likely represent contamination.
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References