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Nosocomial Spread of a *Staphylococcus capitis* Strain with Heteroresistance to Vancomycin in a Neonatal Intensive Care Unit

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A premature infant in a neonatal intensive care unit (NICU) developed a bloodstream infection caused by coagulase-negative staphylococci (CoNS) sensitive to vancomycin. The infection persisted for 3 weeks, despite therapy with vancomycin and replacement of all intravenous catheters. The neonate died due to necrotizing enterocolitis which developed during the ongoing sepsis. We screened this strain and 216 other strains of CoNS from cultures of blood obtained from neonates between 1997 and 2000 for heteroresistance to vancomycin. Forty-eight isolates, including the strain that caused ongoing sepsis, proved heteroresistant. All isolates were identified as *Staphylococcus capitis* and were identical, just as their resistant stable subcolonies were, when they were genetically fingerprinted by amplified-fragment length polymorphism analysis. The heteroresistant phenotype of this endemic strain was confirmed by population analysis. We conclude that heteroresistance to vancomycin occurs in *S. capitis* and might be the cause of therapeutic failures in NICUs. Moreover, heteroresistant strains can become endemic in such units.

During the last two decades, coagulase-negative staphylococci (CoNS) have emerged as the leading cause of bloodstream infections and septicemia in neonatal intensive care units (NICUs) (11, 12). Although bloodstream infections caused by CoNS do not cause excess mortality, they lead to increased rates of morbidity and longer hospital stays (7). Various independent risk factors have been identified for bloodstream infections caused by CoNS (6, 13, 21). In an ongoing surveillance project, we found that in the NICU at the VU University Medical Center more than 50% of bloodstream infections are caused by CoNS. Because the majority of these microorganisms are resistant to β-lactam antibiotics, the treatment of choice in the NICU at the VU University Medical Center is vancomycin.

Over the past 5 years, the reduced sensitivity of methicillin-resistant *Staphylococcus aureus* to vancomycin and the possible implications of this reduced sensitivity for therapy have generated much concern (8, 17, 33). In Japan, a homogeneously vancomycin-resistant (MIC, >8 mg/liter) *S. aureus* strain emerged from an endemic methicillin-resistant *S. aureus* strain which was heteroresistant to vancomycin (17). Heteroresistance is defined as the presence of $>10^{-6}$ stable cell subpopulations of a strain that is apparently susceptible to vancomycin but for which the vancomycin MIC for the subpopulation of cells is greater than or equal to 8 mg/liter (17).

Decreasing susceptibility to vancomycin has also been described for CoNS (10, 30, 32, 37). Although CoNS are less pathogenic than *S. aureus*, they are important causes of opportunistic infections in hospitalized patients. However, the true clinical significance of the heteroresistant phenotype remains to be elucidated. Against staphylococci with heteroresistance to vancomycin, the effect of therapy with a combination of vancomycin and β-lactam antibiotics can be either antagonistic or synergistic (1, 16, 19, 37).

Routine laboratory techniques, such as the disk diffusion method and the E-test, fail to detect heteroresistance to vancomycin in staphylococci (3). This can best be detected by population analysis, which is not suitable for routine purposes (15). Recently, a convenient disk agar screening method for the detection of heteroresistance to vancomycin was developed at the Department of Bacteriology of Juntendo University (18). Wong et al. (37) used this method to study the clinical significance of vancomycin-heteroresistant staphylococci.

A case of ongoing sepsis caused by CoNS in a neonate who did not respond to vancomycin therapy prompted the present study. The aim of the study was to investigate whether heteroresistance to vancomycin might have played a role in this case of therapeutic failure. Furthermore, we screened more than 200 strains of CoNS from cultures of blood from neonates in the VU University Medical Center NICU for the presence of heteroresistance. Heteroresistant strains were genetically typed to study their relatedness.

MATERIALS AND METHODS

Selection of isolates for screening for heteroresistance to vancomycin. At the Department of Medical Microbiology and Infection Control of the VU University Medical Center, significant strains isolated from blood cultures are routinely stored at −80°C for future reference. Isolates of CoNS from cultures of blood

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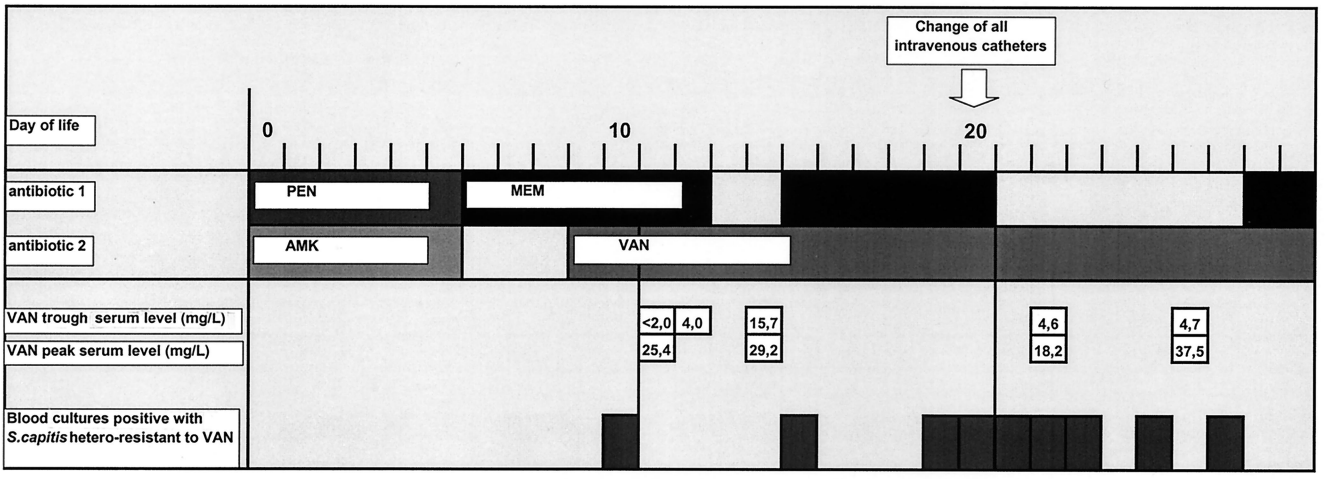

FIG. 1. Schematic overview of period of NICU admission of neonate A with ongoing sepsis caused by an *S. capitis* strain heteroresistant to vancomycin. For detailed information, see the Results. Normal values for vancomycin are as follows: trough level in serum, 5.0 to 10.0 mg/liter; peak level in serum, 20.0 to 40.0 mg/liter. Abbreviations: PEN, penicillin; AMK, amikacin; MEM, meropenem; VAN, vancomycin.

from neonates in the NICU were identified by scrutinizing laboratory records for the years from 1997 to 2000. If more than one culture of blood from a single patient was positive within 2 days and yielded two strains of CoNS with identical antibiotic profiles, only one of the two strains was selected for screening. A total of 217 isolates were studied.

Screening for heteroresistance. Screening for heteroresistance was performed essentially as described by Wong et al. (37). Brain heart infusion (BHI) agar plates with 4 mg of vancomycin per liter were prepared in-house. Isolates were cultured overnight at 37°C on sheep blood agar. A sterile swab was dipped into a suspension of the strain, adjusted to a turbidity equivalent to that of a 1 McFarland standard, and used to inoculate two BHI agar plates containing vancomycin. A 30-μg aztreonam disk was placed on the BHI plate within 5 min of inoculation. The plates were read after 48 h of incubation at 37°C; strains with a zone of enhanced growth around the aztreonam disk were regarded as candidates for vancomycin heteroresistance.

Testing of stabilities of vancomycin-resistant subcolonies. Five colonies from strains A, B, and C that grew on BHI agar containing 8 mg of vancomycin per liter were tested for the stability of resistance. These colonies were subcultured five times on antibiotic-free sheep blood agar. Subsequently, resistance to vancomycin was tested by plating 40 μl of a suspension with a turbidity equivalent to that of a 2 McFarland standard on a BHI agar plate containing 0, 2, 4, 8, or 16 mg of vancomycin per liter.

Population analysis. Population analysis was performed with strains A, B, and C and five subcolonies (subcolonies A1 to A5) of strain A after five passages on antibiotic-free agar (15). Furthermore, *S. capitis* strains isolated from neonates in 1999 (strain D) and 2000 (strain E) were investigated. For this purpose strains were cultured overnight in BHI broth. Then, 50 μl of the bacterial suspension was transferred to prewarmed BHI broth and incubated at 37°C until the optical density at 578 nm was approximately 0.7. After the optical density was adjusted to 0.3 (approximately 10^8 CFU/ml), 10-fold dilutions of cell suspensions were made with 0.9% sodium chloride. Fifty microliters of each of these dilutions was used to inoculate BHI agar plates containing 0, 1, 2, 3, 4, 5, 6, 7, or 8 mg of vancomycin per liter or 0, 0.5, 1, 2, 4, 8, 16, or 32 mg of teicoplanin per liter with a spreader. The plates were incubated at 37°C for 48 h and the colonies were counted.

Identification of heteroresistant strains of CoNS. Identification of heteroresistant strains was performed with the API Staph system (Biomérieux Inc., Marcy l'Etoile, France).

Genetic fingerprinting of heteroresistant strains. Genotypic fingerprints were obtained by amplified-fragment length polymorphism (AFLP) analysis. DNA was isolated as described previously (2). Purified DNA was aliquoted and stored at −20°C. All procedures relating to the preparation of templates for AFLP analysis were performed essentially as described by Koeleman et al. (22). Briefly, total cellular DNA (50 ng) was digested with the restriction enzymes *Eco*RI and *Mse*I, and restriction half-site-specific double-stranded oligonucleotide adaptors were ligated to the restriction fragments. Restriction and ligation were carried out simultaneously (3 h, 37°C). Amplification was performed with a combination of primers (primers *Eco*-A and *Mse*-C) with one selective base. Primer *Eco*-A was fluorescently labeled with Texas red (Isogen Bioscience BV, Maarssen, The Netherlands). Amplification was performed in a Gene Amp PCR system 9700 thermal cycler (Perkin-Elmer) for 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 65 to 56°C, and DNA molecule extension for 1 min at 72°C. In the first 12 cycles the annealing temperature was lowered 0.7°C per cycle. Fluorescent amplified fragments were separated by polyacrylamide gel electrophoresis (Rapid Gel-XL-6; Amersham Life Science, Cleveland, Ohio) according to the instructions of the manufacturer in a Vistra 725 automated DNA sequencer (Amersham Life Science). The gel images were processed with Gel Compar software (version 4.0; Applied Maths, Kortrijk, Belgium). Levels of similarity between fingerprints were calculated with the Pearson product moment correlation (r), and grouping was obtained by the unweighted pair group method with average linkages.

All heteroresistant strains and five vancomycin-resistant subcolonies from strains A, B, and C were investigated by AFLP analysis. For comparison, several different (reference) species of staphylococci were included (28). Reproducibility was tested by repeat typing.

RESULTS

Descriptions of the clinical courses for neonates from which strains A, B, and C were isolated. A summary of the clinical course for the neonate from which strain A was isolated is presented in Fig. 1.

(i) Strain A. Strain A was isolated in September 1998 from a neonate of 28 weeks of gestation (birth weight, 640 g) who was admitted to the VU University Medical Center NICU because of respiratory insufficiency. The neonate was treated with penicillin and amikacin for 6 days. On day 7 of life, the neonate's clinical situation deteriorated and therapy was switched to meropenem. Blood cultures grew CoNS resistant to oxacillin but sensitive to vancomycin, as determined by the disk diffusion method. On day 9, treatment with vancomycin (20 mg/kg of body weight/day as one dose) was started. On day 16 of life the neonate's clinical condition once again deteriorated; meropenem was added to vancomycin. Blood cultures were again positive for CoNS. The vancomycin MIC for this strain was 3 mg/liter, as determined by the E-test. The central venous catheter was removed, and peripheral venous and arterial catheters were changed on day 20, but blood cultures remained positive for CoNS. Ultrasound investigations were

TABLE 1. Results of screening of strains of CoNS from 217 positive cultures of blood from 163 neonates from the NICU obtained between 1997 and 2000

Year	Blood cultures positive for CoNS				Neonates for which cultures were positive for CoNS	
	Total no.	No. (%) with heteroresistant CoNS	No. (%) with heteroresistant *S. capitis*	Mean (minimum-maximum) diam (cm) of enhanced growth around aztreonam disk on screening plate	Total no.	No. (%) infected with heteroresistant *S. capitis*
1997	58	0 (0)			38	0
1998	49	14 (29)	14	1.8 (1.5–2.1)	36	11 (31)
1999	59	14 (24)	14	2.0 (1.5–2.9)	42	13 (31)
2000	51	20 (39)	20	2.3 (1.9–3.0)	47	18 (36)

repeatedly negative for thrombi in the heart or elsewhere. On a few occasions during the septic episode, the trough levels of vancomycin in serum were too low (normal trough levels, 5 to 10 mg/liter), but the peak levels in serum were always good (normal peak levels, 20 to 40 mg/liter). The neonate developed necrotizing enterocolitis on day 27 and died on day 29. On autopsy, no intravascular thrombus was found. Postmortem cultures of intraperitoneal and pleural fluid grew *Klebsiella pneumoniae.*

(ii) Strains B and C. Strains B and C were isolated from neonates admitted to the VU University Medical Center NICU during the same period that neonate A was admitted. Neonate B had a septic episode caused by CoNS in the second week of life. He was treated with vancomycin for 7 days and recovered. Neonate C twice experienced sepsis caused by a strain of CoNS over an interval of 1 week during the second half of August and was treated with vancomycin for 6 and 8 days, respectively, with good results. In these neonates, trough and peak levels of vancomycin in serum were also suboptimal on some occasions. Vancomycin and meropenem were also administered to these two patients simultaneously for a few days: for 4 days in neonate B and for 10 days in neonate C.

Screening for heteroresistance. We screened 217 strains of CoNS from positive cultures of blood from 163 neonates admitted to the VU University Medical Center NICU between 1997 and 2000, including the strains that caused sepsis in neonates A to C described above (Table 1). Among the strains isolated in 1997, none was positive by the screening test. For the period from 1998 to 2000, about one-third of the bloodstream infections were caused by heteroresistant strains of CoNS that showed enhanced growth around the aztreonam disk on the screening plate. Over these years, we observed a significant increase in the diameter of enhanced growth around the aztreonam disk (Table 1); the meaning of this observation is unclear. Two isolates showed confluent growth on the screening plate but did not show enhanced growth around the aztreonam disk.

Population analysis. Strains A to E possessed heterogeneous growth curves by population analysis with vancomycin and teicoplanin (Fig. 2). The growth curves for strains A1 to A5 did not differ from those for strains A to E (data not shown). Strains A to E fulfilled the definition of vancomycin heteroresistance, having subpopulations resistant to 4 mg of vancomycin per liter at frequencies of 2.8×10^{-5}, 1.8×10^{-4}, 3.0×10^{-5}, 6.5×10^{-5}, and 3.4×10^{-5}, respectively (17).

Stability of vancomycin-resistant subcolonies. The heteroresistant phenotype was stable in all subcolonies tested. After five passages on antibiotic-free agar plates, the subcolonies still grew on a BHI agar plate containing 8 mg of vancomycin per liter.

Identification of heteroresistant strains of CoNS. All 48 strains positive by screening, including the three confirmed heteroresistant strains, were identified as *S. capitis* (Table 1). The two strains with confluent growth on the screening plate were identified as *S. haemolyticus*.

Genetic fingerprinting of heteroresistant strains. AFLP analysis showed a good reproducibility and discriminatory power when the region from 50 to 500 bp was tested (Fig. 3). On the basis of the results of tests with various *Staphylococcus* species, a cutoff value of 90% for identical strains was determined. Strains A, B, and C and the five stable vancomycin-

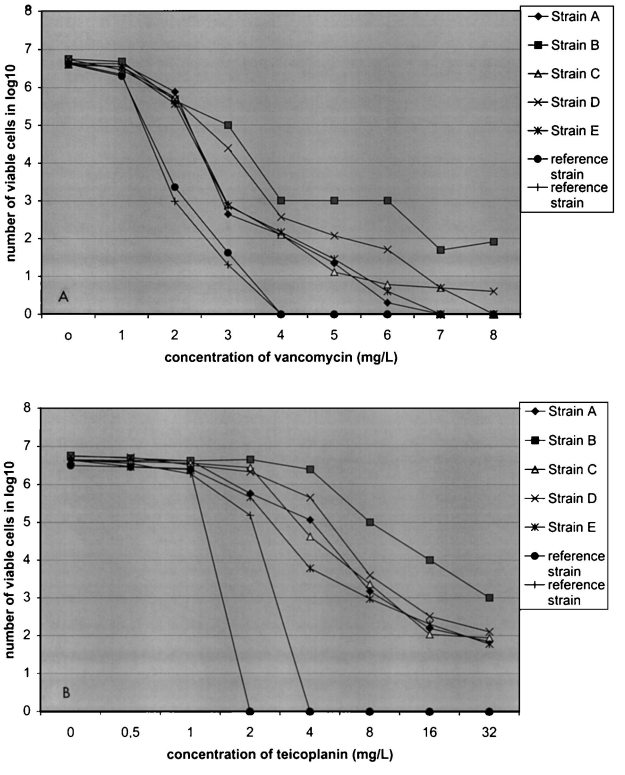

FIG. 2. Population analysis of five clinical isolates (strains A to E) of *S. capitis* that were positive by screening for inducible heteroresistance; (for descriptions of the strains, see Materials and Methods) and two reference strains of *S. capitis*. Profiles for vancomycin (A) and teicoplanin (B) are shown.

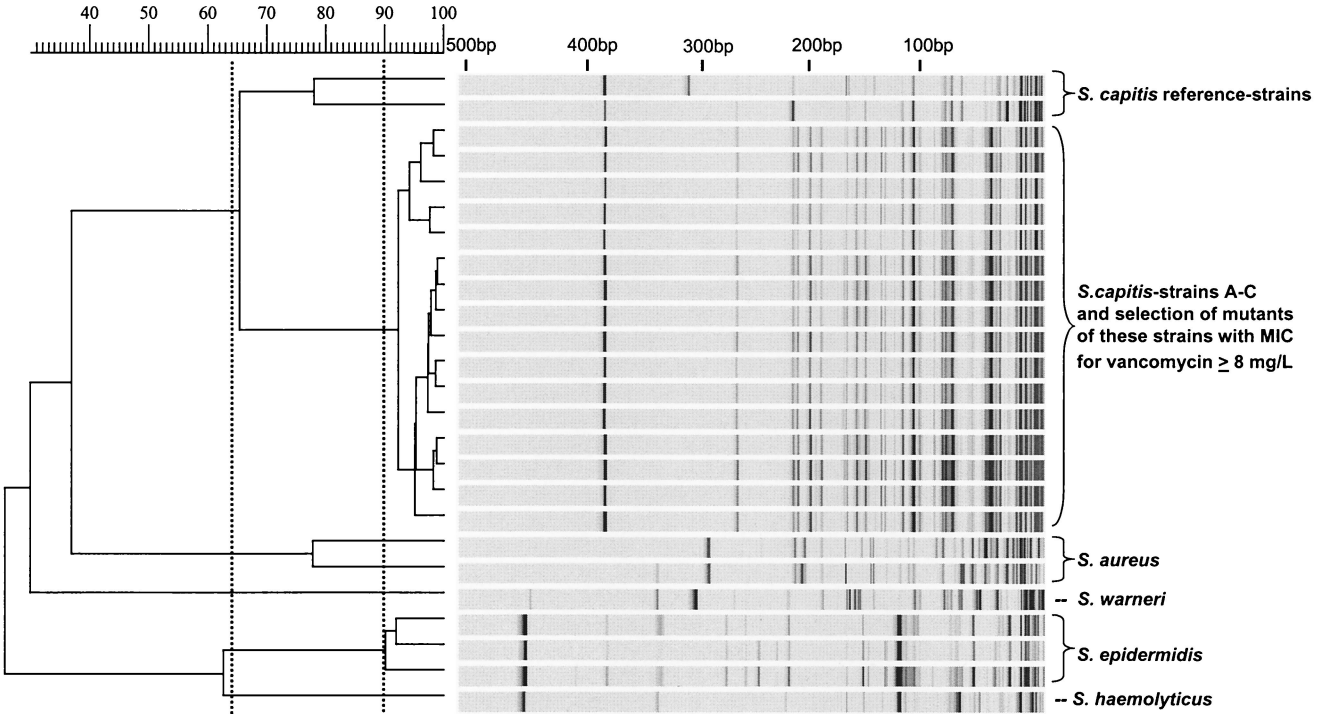

FIG. 3. Genetic fingerprinting by AFLP analysis (fragment length, 50 to 500 bp). The dendrogram on the left indicates the percent homologies between various strains of staphylococci. Levels of similarity between fingerprints were calculated with the Pearson product moment correlation (*r*), and groupings were obtained by the unweighted pair group method with average linkages. Homologies of >90% indicate that the strains are identical. Strains with from 65 to 90% homology are different strains of the same species. Strains A to C and a selection of stable vancomycin-resistant mutants of these strains (MICs, ≥8 mg/liter) are identical.

resistant subcolonies from each parent strain were identical (homologies, >90%) and different from the two *S. capitis* reference strains (Fig. 3). All 48 confirmed and presumptive heteroresistant *S. capitis* strains were identical (homologies, >90%) when the region from 50 to 250 bp was tested (Fig. 4). We concluded that clonal spread of a single heteroresistant *S. capitis* strain had occurred over a period of 3 years.

DISCUSSION

This study was initiated after the detection of a case of ongoing sepsis caused by CoNS in a premature neonate, as determined by several positive blood cultures. Treatment with vancomycin was not effective. The sepsis was caused by a strain of *S. capitis* which was heteroresistant to vancomycin. Screening of more than 200 isolates of CoNS from cultures of blood from neonates in the VU University Medical Center NICU showed that this *S. capitis* strain had been endemic in the unit since 1998 and that it was the causative agent of about one-third of all cases of bacteremia caused by CoNS in the VU University Medical Center NICU.

The heteroresistant phenotype of the isolate causing ongoing sepsis was not detected by methods routinely used in our laboratory, i.e., the disk diffusion method and the E-test (on Mueller-Hinton agar with an inoculum with a turbidity equivalent to that of a 0.5 McFarland standard). A recent study by Walsh et al. (36), which was published after our study was conducted, showed that a modified E-test (with BHI agar and an inoculum with a turbidity equivalent to that of a 2.0 McFarland standard) is the most sensitive and specific method for the detection of staphylococci with reduced susceptibilities to glycopeptides which can be used in the daily laboratory routine.

Persistent staphylococcal bacteremia in premature neonates that does not respond to vancomycin therapy, despite the susceptibility of the bacteria to vancomycin, has been described in a series of 10 neonates by Tan et al. (34). The isolates in that study were methicillin-resistant *S. aureus* ($n = 5$), methicillin-sensitive *S. aureus* ($n = 2$), and CoNS ($n = 3$; the isolates were not further identified). A detailed evaluation of some of the neonates to establish a focus of infection was negative, so heteroresistance may have played a role in the persistence of the staphylococcal bacteremia. Addition of intravenous rifampin to the antibiotic regimen was effective in sterilizing cultures of blood from these neonates.

Nosocomial transmission of endemic CoNS strains in a NICU, with the rate of endemicity being up to 45% among all blood culture isolates, has been described before (20, 24, 35). Little is known about why certain strains become endemic and others do not. Sepsis caused by CoNS is the most frequent nosocomial infection in the VU University Medical Center NICU. Vancomycin therapy is instituted only if an oxacillin-resistant strain of CoNS is isolated from a blood culture and the patient's clinical deterioration cannot otherwise be explained. The frequent use of vancomycin in the NICU might have given the heteroresistant strain described here a selective advantage over other nonheteroresistant strains (4). However,

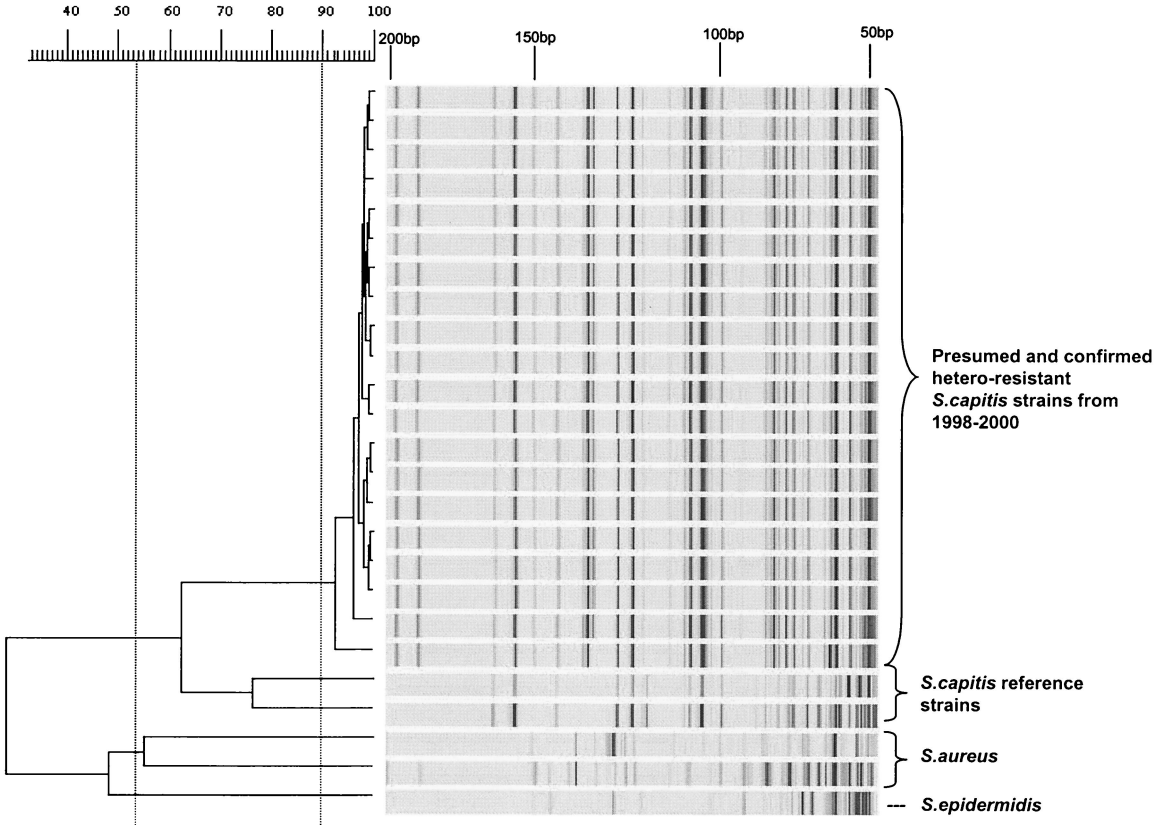

FIG. 4. Genetic fingerprinting by AFLP analysis (fragment length, 50 to 250 bp) of *S. capitis* strains heteroresistant to vancomycin, isolated from cultures of blood from neonates in the VU University Medical Center NICU between 1998 and 2000. The dendrogram on the left indicates the percent homology. Levels of similarity between fingerprints were calculated with the Pearson product moment correlation (*r*), and groupings were obtained by the unweighted pair group method with average linkages. Homologies of >90% indicate that the strains are identical. All clinical *S. capitis* isolates have homologies of >90%, indicating similarity.

in our screening we also identified two strains of *S. haemolyticus* which grew abundantly on the BHI agar plates with vancomycin. *S. haemolyticus* is known for its resistance to vancomycin (9, 29). Nosocomial spread of *S. haemolyticus* in NICUs has been described before (23, 26). The fact that in the VU University Medical Center NICU those strains did not become endemic indicates that other features of the endemic *S. capitis* strain may have facilitated its nosocomial spread (20).

Heteroresistance to vancomycin has been described in *S. aureus*, and it has been shown that it can be the predecessor of homoresistance (17). The mechanism of resistance to vancomycin in staphylococci is completely different from that in vancomycin-resistant enterococci. The *vanA*, *vanB*, and *vanC* genes have never been found in staphylococci, although experimentally the *vanA* gene has been transferred from enterococci to *S. aureus* (27). Although the precise genetic mechanism for vancomycin resistance in staphylococci awaits elucidation, it is considered that thickening of the cell wall peptidoglycan layer is responsible (14, 31). Vancomycin is captured in the thickened cell wall and is prevented from reaching its targets on the cell membrane.

Although the presence of subcolonies with reduced sensitivity to vancomycin may influence the clinical course of sepsis caused by CoNS (37), the true clinical significance of heteroresistant *S. capitis* must still be clarified. Neonate A died from necrotizing enterocolitis during ongoing sepsis caused by a heteroresistant *S. capitis* strain. The presence of necrotizing enterocolitis may explain why postmortem cultures of intraperitoneal and pleural fluid were positive for *K. pneumoniae*, which belongs to the normal intestinal flora. Although the cause of necrotizing enterocolitis is probably multifactorial and still unknown, infection-associated inflammatory mediators may play a role (25). Most of the other conceivable causes for the failure of vancomycin therapy in this neonate, such as colonization of intravascular catheters or intravascular thrombi, were excluded. The levels of vancomycin in serum were suboptimal on a few occasions; however, this was also the case for neonates B and C, who responded well to vancomycin therapy. It is well known that serum vancomycin concentrations in neonates cannot be reliably predicted (5). Finally, it has been described that β-lactam antibiotics may induce or enhance vancomycin resistance in staphylococci (1, 37). The combination of vancomycin and meropenem could have enhanced the level of vancomycin resistance in *S. capitis* and might have contributed to the therapeutic failure.

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