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# Molecular Epidemiology of Coagulase-Negative Staphylococci Causing Sepsis in a Neonatal Intensive Care Unit over an 11-Year Period

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**Coagulase-negative staphylococci (CoNS) are the major causative microorganisms in neonatal nosocomial sepsis. Previous studies have shown that CoNS sepsis in the neonatal intensive care unit (NICU) is caused by predominant molecular types that are widely distributed among both neonates and staff. Some of these molecular types may persist in the NICU for years. The purpose of the present study was to determine the dynamic behavior of CoNS strains causing sepsis over a prolonged period of time by determining the molecular types of all blood isolates from septicemic infants over a period of 11 years (1991 to 2001). The results show that neonatal CoNS sepsis is increasingly caused by a few predominant molecular clusters. The most striking finding was that in recent years one molecular cluster emerged as the predominant cause of neonatal CoNS sepsis, responsible for no less than 31% (20 of 65) of blood isolates in 2001. Antibiotic resistance, particularly beta-lactam resistance, is probably an important selective force considering the high *mecA* gene carriage of CoNS blood isolates (70 to 92%). We conclude that neonatal CoNS sepsis is increasingly caused by a limited number of predominant molecular CoNS types and that antibiotic resistance is probably a major selective force.**

Coagulase-negative staphylococci (CoNS) are the major causative microorganisms in neonatal nosocomial sepsis (8, 9). Several studies demonstrated that CoNS sepsis is caused by predominant molecular types which are widely distributed among both neonates and staff, suggesting cross-contamination (4, 12, 13). These predominant molecular types can persist in neonatal intensive care units (NICUs) for prolonged periods of time (13). The characteristics and virulence factors of these CoNS types and the mechanisms by which they are selected to persist are not clear. The capacity to develop antibiotic resistance and to colonize a wide variety of hosts may be key factors for selection and persistence of these CoNS types. The purpose of the present study was to investigate the dynamics of emergence and persistence of CoNS strains causing sepsis in the NICU by determining the molecular types and methicillin resistance of all CoNS blood isolates of neonates with sepsis from a period of 11 years.

## MATERIALS AND METHODS

**Patients.** All patients with sepsis due to CoNS during the odd years from 1991 to 2001 were included in the study. Criteria for definition of CoNS sepsis have been described previously (2, 5). One blood isolate was included in the study from each of 224 of the 252 neonates with CoNS sepsis.

**Bacterial isolates.** One sample of blood (1 to 2 ml) for culture was drawn from a peripheral vein. Samples were inoculated into pediatric blood culture bottles and incubated in an automated blood culture incubator. From 1991 to 1999, pediatric blood culture bottles and a Bactec incubator (NR 730; Becton Dickinson, Sparks, Md.) were used; from 1999 to 2001, pediatric blood culture bottles and an automated BacTAlert incubator (Organon Teknika Corporation, Durham, N.C.) were used. Blood culture isolates of CoNS were considered significant if results of blood culture bottles were positive within 24 to 48 h (6). CoNS blood isolates were subcultured on blood agar plates. Bacterial colonies were identified as CoNS by Gram staining, production of catalase, and absence of a coagulase gene. Identification and susceptibility testing were conducted with a Vitek automated determination and susceptibility testing system (bioMérieux SA, Marcy-l'Etoile, France). Subsequently, the blood isolates were stored at −80°C in glycerol-containing liquid medium. In addition, the methicillin resistance of blood isolates was determined by *mecA* gene PCR, as described previously (7).

**Genotyping.** Genotyping was performed by pulsed-field gel electrophoresis (PFGE), as described previously (11, 12). In short, a suspension of bacteria in NaCl-EDTA was mixed with a 2% agarose solution. Agarose plugs were incubated with lysostaphin. Spheroplasts were lysed by sodium dodecyl sulfate and proteinase K (Boehringer, Mannheim, Germany), washed six times for 20 min each time in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA, and stored at 4°C until further use. Prior to electrophoresis, DNA was digested overnight with the restriction enzyme *Sma*I (Boehringer). PFGE was carried out with 1% SeaKem GTG agarose gels (FMC Bioproducts) in 0.5× Tris-borate-EDTA at 14°C in a contour-clamped homogenous electric field mapper (Bio-Rad, Veenendaal, The Netherlands). The running time was 20 h. Gels were stained with ethidium bromide and photographed. Differences in banding patterns were documented by visual examination by two independent observers. In addition, the PFGE patterns were analyzed with Bionumerics software: genotypes with >70% similarity were arbitrarily assigned to one cluster (10). For these closely related CoNS strains, the terms "cluster" and "clone" were used interchangeably.

## RESULTS

**Genotyping and distribution of genotype clusters in time.** Molecular typing was performed for 199 of the total of 224 stored CoNS isolates (25 isolates from 1991, 23 isolates from 1993, 35 isolates from 1995, 34 isolates from 1997, 42 isolates from 1999, and 65 isolates from 2001). Twenty-five isolates, randomly distributed over the years, could not be typed. Evaluation of the banding patterns revealed 10 major clusters of 6 isolates or more with >80% homology, comprising 117 isolates. Among these 10 clusters, there were 4 clusters of at least 10 isolates (clusters A, B, C, and D), comprising 77 isolates. Figure 1 shows the dendrogram of all PFGE-typed CoNS

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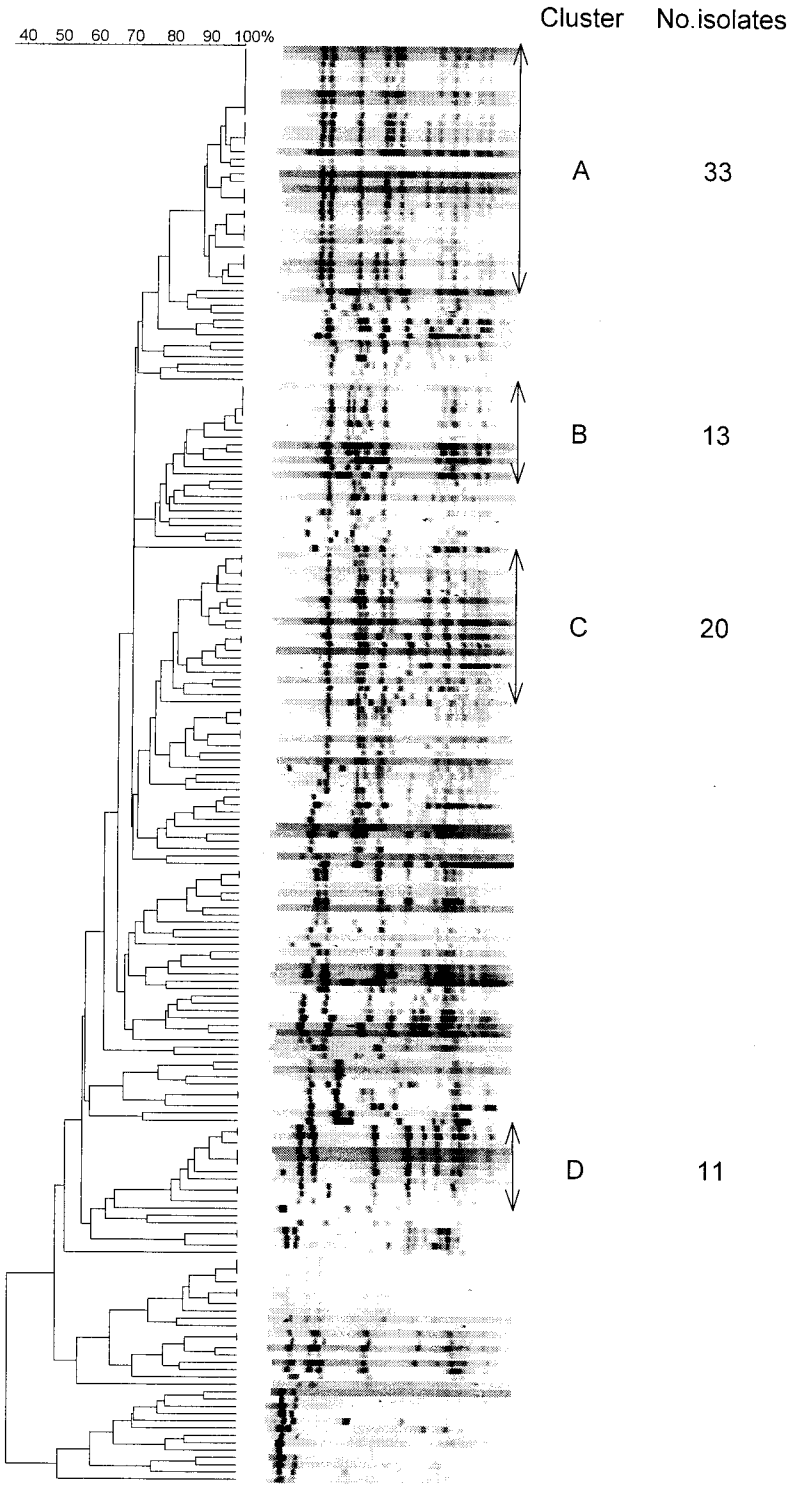


FIG. 1. Dendrogram of gel image obtained after PFGE of DNA macrorestriction fragments derived from CoNS blood isolates from neonates with sepsis. The four largest clusters comprising ≥10 isolates with >80% homology are indicated (A, B, C, and D).

blood isolates. Cluster A was the largest cluster, comprising 33 isolates with 90% homology. Figure 2 shows the distribution of these four largest clusters over the 11-year period. CoNS isolates from cluster B (13 isolates) were found in 1991 (3 isolates), 1993 (1 isolate), 1995 (6 isolates), 1997 (2 isolates), and 2001 (1 isolate). Isolates from cluster C (20 isolates) were found since 1993: 3 isolates in 1993, 4 isolates in 1995, 6 isolates in 1997, 2 isolates in 1999, and 5 isolates in 2001. Isolates from cluster D (11 isolates) were found in 1995 (2 isolates), 1999 (5 isolates), and 2001 (4 isolates). Isolates from

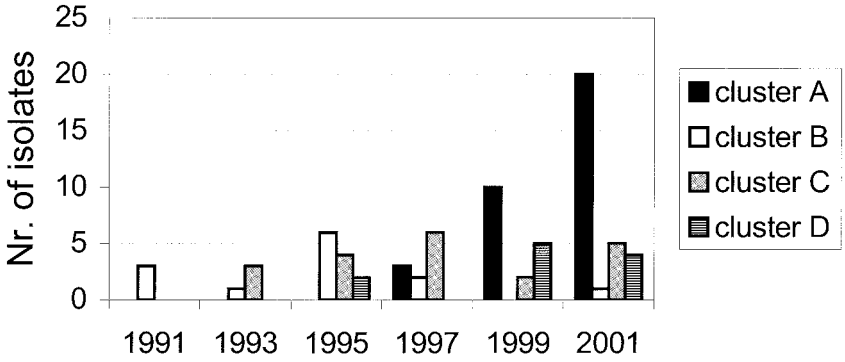


FIG. 2. Distribution over a period of 11 years of the four largest clusters of identical and closely related molecular types of CoNS blood isolates as determined by PFGE.

cluster A (33 isolates) appeared since 1997 (3 isolates) and increased substantially during the following years (1999 [10 isolates] and 2001 [20 isolates]). Thus, isolates from all four of the largest clusters were found in 2001, and these comprised 30 of the total of 65 blood isolates (46%). In addition, these data show that in 2001 no less than 20 of the total of 65 isolates (31%) belonged to one cluster, cluster A.

***mecA* gene carriage and distribution among genotype clusters.** *mecA* gene carriage of blood isolates from 1991 through 2001 was consistently high, varying from 70 to 92% without any clear increasing or decreasing trend (Fig. 3). The four largest clusters (A to D) showed a *mecA* gene carriage rate of 85 to 97% (Fig. 4). The other six smaller clusters of 6 isolates or more showed a *mecA* gene carriage rate of 75 to 100%.

## DISCUSSION

Nosocomial sepsis caused by CoNS continues to be an important cause of morbidity in NICUs (1, 2). It has been shown before that many clones of CoNS circulate in NICUs but that a small number of clones predominates (3–5). These clones circulate in the air and among the medical staff and the neonates. When comparing the molecular types of CoNS that caused sepsis, it was found that the blood isolates belonged to a very limited number of types (3). These clones may persist in a NICU for prolonged periods of time (4). In the present study, we have confirmed that a relatively small number of molecular types of CoNS persist in the NICU for many years, some for as long as 11 years (cluster B). The contribution of clusters A, B, C, and D as causative agents of neonatal sepsis increased during the 11 years from 3 isolates (from cluster B) of the total of 25 blood isolates (12%) in 1991 to 30 (20 isolates of cluster A, 1 isolate of cluster B, 5 isolates of cluster C, and 4 isolates of cluster D) of the total of 65 blood isolates (46%) in 2001. In addition, in 2001, 20 of the 30 (67%) CoNS blood isolates of the four most frequent types (A to D) belonged to a single cluster (cluster A). Moreover, this cluster comprised no less than 20 of 65 (31%) of all CoNS blood isolates from that year. This phenomenon suggests that cross-contamination indeed contributes importantly to the distribution and survival of certain clones in the NICU environment, as has been documented previously in a surveillance study during a much shorter period (3).

*mecA* gene carriage of CoNS blood isolates was consistently high during 1999 and 2001 (Fig. 3). *mecA* gene carriage among the four most frequent clusters was highest in isolates of cluster A (97%), the cluster which predominated during 1999 and 2001. This suggests that antibiotic resistance may not only have been an important initial driving force in the selection of certain CoNS types which cause sepsis but probably continues to be a major selective force. In addition to selection by antibiotic resistance, other determinants, such as colonizing factors, biomaterial adhesion factors and the production of biofilm by

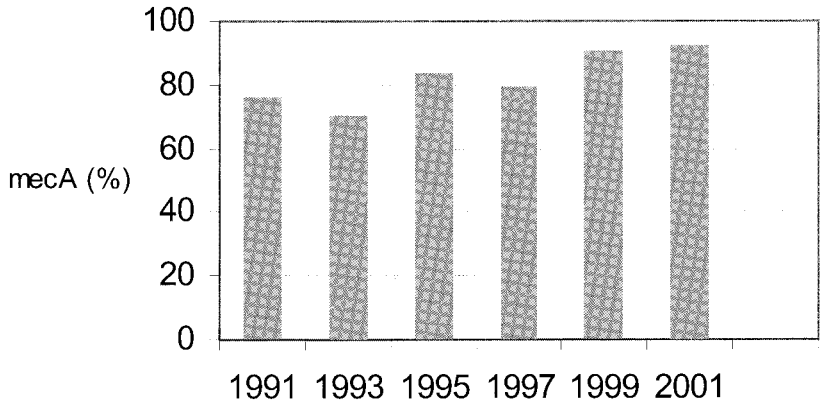


FIG. 3. *mecA* gene carriage shown as the percentage of CoNS blood isolates from 1991 to 2001.

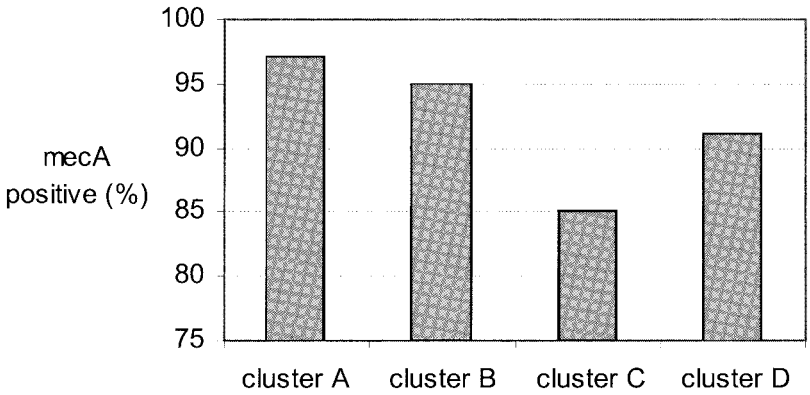


FIG. 4. *mecA* gene carriage (% positive) of CoNS blood isolates of the four largest clusters (A to D) of CoNS blood isolates.

CoNS, or resistance to opsonophagocytosis, may contribute to selection. A recent study in a NICU provided evidence that the quantity of biofilm produced by CoNS strains may be associated with the ability to cause infection in neonates (1). However, there were no significant differences between CoNS colonizing and infecting strains in the presence of the *ica* operon, the gene cluster encoding biofilm production (3). Therefore, the role of biofilm production, adhesion factors, and other putative selection forces mentioned above remains elusive at the present time and warrants further investigation. Nevertheless, the predominance of a few clusters is a testimony to the successful adaptation of these clusters to the NICU environment and probably the result of multiple selection forces.

In conclusion, neonatal nosocomial sepsis is caused by a limited number of clusters of molecular types of CoNS which are able to persist in the NICU for many years. In recent years, the increasing contribution of a single molecular clone causing sepsis was observed, an intriguing phenomenon which may provide clues for factors that determine survival and persistence of such clones in the NICU environment. Initially, these genotypes may have been selected primarily by antibiotic resistance, and it is likely that antibiotic resistance continues to be a major selective force. However, the contribution of other putative selective forces, notably biomaterial adhesion factors, biofilm production, and opsonophagocytic resistance to selection, and persistence of CoNS genotypes warrants further studies. These studies may provide more insight as to how these factors operate and determine survival and persistence. In addition, they may also aid in designing rational preventive measures. This is more relevant because, to date, efforts to prevent nosocomial CoNS sepsis in neonates have not been very successful and treating these infections results in a high antibiotic pressure in the NICU, leading not only to an increased risk of antibiotic resistance among CoNS and *Staphylococcus aureus* but also to the selection of antibiotic-resistant CoNS clones.

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