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An Epidemiological Study of Blood Culture Isolates of Coagulase-Negative Staphylococci Demonstrating Hospital-Acquired Infection

J. P. BURNIE,* M. NADERI-NASAB, K. W. LOUDON, AND R. C. MATTHEWS

Department of Medical Microbiology, Manchester Royal Infirmary, Manchester M13 9WL, United Kingdom

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We applied pulsed-field gel electrophoresis (PFGE) after *Sma*I digestion and random amplification of polymorphic DNA (RAPD) analysis with nine oligonucleotide primers to 146 blood culture isolates of *Staphylococcus epidermidis* and 25 blood culture isolates of *Staphylococcus haemolyticus*. These were obtained over a 12-month period from patients on the neonatal and hematology units of the Central Manchester Health Care Trust. PFGE demonstrated two clusters of isolates of *S. epidermidis* (type A and type B) on the neonatal ward and a single cluster (type C) on the hematology unit. Type A was represented by 10 indistinguishable isolates from nine patients, type B was represented by 20 isolates from 14 patients, and type C was represented by 26 isolates from 10 patients. Type A isolates were resistant to chloramphenicol and type C isolates were resistant to ciprofloxacin, mirroring current antibiotic usage. There was no evidence of cross infection due to *S. haemolyticus*. RAPD analysis, on the basis of a single band difference, produced 58 types of *S. epidermidis* and 12 types of *S. haemolyticus* with primer 8 (ATG TAA GCT CCT GGG GAT TCA C; 5′ to 3′) and 54 types of *S. epidermidis* and 10 types of *S. haemolyticus* with primer 9 (AAG TAA GTG ACT GGG GTG AGC G; 5′ to 3′). Combining the results confirmed cross infection. Types A, B, and C were concurrently isolated from the hands of the staff of the appropriate unit. Partial control was achieved by withdrawing ciprofloxacin use in the case of the hematology unit and improving hand hygiene in both units.

Coagulase-negative staphylococci (CoNS) are now recognized as the most frequently isolated organisms from blood cultures (12), where they account for one-fourth of nosocomial bloodstream infections (30). The EPIC Study in Europe gave a prevalence of 44.9% in intensive care units, which was rising and which was associated with an increased use of intravascular catheters (36). The crude mortality rate in uncontrolled studies has ranged from 18.5 to 57% (24). *Staphylococcus epidermidis* has been the dominant species, but *Staphylococcus haemolyticus* has been shown to be clinically important (8, 26) and, on occasion, resistant to teicoplanin and vancomycin (8, 26, 33, 39). The presence of epidemic strains has repeatedly been described (10, 11, 17, 22, 23, 27, 29), which has raised the issue of how much infection due to CoNS is hospital acquired and, hence, potentially preventable. The high prevalence of this infection on both the neonatal and the hematology units of our hospital led us to look for cross infection by typing the blood culture isolates from these patients. The staff were also examined to see if they acted as a reservoir.

Classical typing methods such as biotyping, phage typing, and antibiotic susceptibility pattern analysis used separately or in combination have only been of limited success (3). There has thus been a move to molecular biology-based methods. These have included plasmid DNA typing, restriction endonuclease fingerprinting, ribotyping, pulsed-field gel electrophoresis (PFGE), and random amplification of polymorphic DNA (RAPD) analysis. Plasmid DNA typing has been unsatisfactory because some isolates contain only one plasmid and plasmid content may be unstable (29).

Restriction endonuclease fingerprinting is technically demanding and, for *S. haemolyticus*, displays little variation (3). In addition, the comparison between strains is limited by the variability in intensity and resolution of the bands from lane to lane, especially in the regions where large fragments comigrated or where small fragments resolve poorly (22). The level of discrimination by ribotyping was low, and there is a need to radiolabel the probe (2, 40).

PFGE, after restriction with the rarely cutting enzyme *Sma*I, has been shown to be able to both determine the species of the staphylococcus and type *S. epidermidis*, *S. haemolyticus*, *Staphylococcus lugdunensis*, and *Staphylococcus intermedius* strains (7, 9, 14, 18–20, 28, 34, 35). It is thus potentially the method of choice for typing CoNS. RAPD is a newly described typing method in which single short primers with arbitrary nucleotide sequences are used in a PCR to amplify genomic DNA. The profiles obtained after electrophoretic separation of the amplification products successfully fingerprinted both *Staphylococcus aureus* (32, 38) and CoNS (4).

The study described in this report thus examined the degree to which infection due to CoNS on the hematology and neonatal units was hospital acquired. This was ascertained by typing blood culture isolates by PFGE and RAPD analysis in order to see if there is a type that is endemic within each unit.

MATERIALS AND METHODS

Sources of isolates. Two hundred four isolates of CoNS were obtained from cultures of blood from either premature neonates or adult hematology patients suffering from leukemia between February 1993 and February 1994. The species of the isolates were determined by Gram staining, catalase and coagulase tests, sensitivity to 0.04 U of bacitracin, hemolysis, nitrate reduction, phosphatase and urease tests, novobiocin resistance, polymyxin B resistance, aerobic growth in thioglycolate broth, and aerobic acid production from fructose, lactose, mannitol, maltose, mannose, raffinose, sucrose, trehalose, and xylose (16).

Susceptibility testing. Susceptibility was measured by the agar diffusion method, as recommended by the National Committee for Clinical Laboratory Standards (25), with the following discs: penicillin, 10 μg; ampicillin, 10 μg; chloramphenicol, 30 μg; ciprofloxacin, 1 μg; erythromycin, 15 μg; fusidic acid, 10

* Corresponding author. Mailing address: Department of Medical Microbiology, 2nd Floor, Clinical Sciences Building, Manchester Royal Infirmary, Oxford Rd., Manchester M13 9WL, United Kingdom. Phone: 0161 276 8827. Fax: 0161 276 8826.

μg; rifampin, 5 μg; gentamicin, 10 μg; netilmicin, 30 μg; and vancomycin, 30 μg (Mast Diagnostic, Mast House, Bootle, United Kingdom). Methicillin susceptibility testing was performed with Mueller-Hinton agar supplemented with 4% NaCl (Merck Ltd., Poole, United Kingdom) and a 5-μg disk. The control strain was *S. aureus* ATCC 25923.

PFGE. DNA isolation was performed essentially as described previously (31). The cell density was adjusted to 3×10^8 CFU/ml. For each strain three agarose blocks containing genomic DNA were equilibrated at 25°C for 24 h in 300 ml of restriction enzyme buffer. Cleavage was performed with 30 U of *Sma*I (NBL Gene Sciences, Cramlington, United Kingdom), and the digestion was carried out overnight at 4°C. PFGE was performed with a CHEF-DRII System (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom) at room temperature with 0.9% agarose gels in Tris-borate-EDTA buffer. Electrophoresis was performed at 12°C with a constant voltage of 4 V cm^{-1} and a pulse time of 25 s for 40 h. Digestion patterns were visualized by staining with ethidium bromide (1 μg ml^{-1} in 300 ml of distilled water), washing in water, and photographing the result under UV light. *Bordetella pertussis* vaccine strain 3700 DNA was used as the molecular size marker (15).

RAPD analysis. Isolates were grown overnight at 37°C in Trypticase soy agar. A few colonies were suspended in 200 μl of lysis buffer consisting of 4 M guanidine isothiocyanate, 0.5% *N*-lauroyl sarcosine, 1 mM dithiothreitol, and 25 mM sodium citrate for 10 min. Nucleic acids were precipitated by adding 250 μl of isopropyl alcohol (−20°C). The samples were centrifuged at 12,000 rpm (MSE Micro Centaur) at 4°C for 10 min, and the pellet was washed in 500 μl of 70% ethanol for 10 min and dried. The DNA concentration was measured by spectrophotometry at 260 nm and was adjusted to 10 ng/μl in 20 mM Tris-HCl.

Primers and primer synthesis. Oligodeoxyribonucleotide primers were synthesized on an Applied Biosystems model 381-A DNA synthesizer and were used without further purification. DNA concentrations were determined by measuring the A_{260} on a Uvicam model PU 8270 spectrophotometer. The primer sequences (5′ to 3′) were as follows: primer 1, GCG CAC GG; primer 2, GCT GGT GG (5); primer 3, TTA TGT AAA AGG ACG GCC AGT (42); primer 4, GTA ACG CC (1); primer 5, TCA TGA TGC A; primer 6, TCA CGC TGC G (32); primer 7, TCA CGA TGC A (43); primer 8, ATG TAA GCT CCT GGG GAT TCA C (38, 40); and primer 9, AAG TAA GTG ACT GGG GTG AGC G (38, 40).

PCR. The reactions were performed in 1× PCR buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleotide triphosphate, 2.5 μg of primer, 2.5 μl (25 ng) of template DNA, and 1 U of *Taq* DNA polymerase (Boehringer Mannheim). Amplifications for RAPD analysis were carried out in a Perkin-Elmer Cetus DNA thermal cycler. The cycling parameters were 45 s at 94°C, 1 min at 36°C, and 2 min at 72°C for a total of 45 cycles. RAPD products were analyzed on a 1.3% agarose gel in TBE buffer containing 0.5 μg of ethidium bromide ml^{-1}. *Hae*III-digested ϕX174 DNA was the molecular mass marker, and a negative control contained all the reagents except the template DNA. The products were resolved by electrophoresis at 90 V for 3 h.

Staff sampling. The hands of 30 nurses and four doctors from the neonatal units and 24 nurses and five doctors from the hematology unit were sampled. This was done in August 1993. Fingerprint cultures were made on blood agar plates, which were incubated overnight at 35°C. Two colonies representing the predominant morphotypes of gram-positive clustering cocci were further identified phenotypically. If found to be *S. epidermidis*, they were analyzed by PFGE and RAPD analysis.

RESULTS

One hundred fifteen episodes of bacteremia caused by CoNS occurred in 44 adult hematology patients during the present study. The species distribution was 68.7% *S. epidermidis* and 14.8% *S. haemolyticus*. The other staphylococcal species detected were *S. hyicus*, *S. warneri*, *S. cohnii*, and *S. saprophyticus*. Among the neonates there were 89 episodes of CoNS bacteremia in 55 patients. The distribution was 75.3% *S. epidermidis* and 9% *S. haemolyticus*. The other staphylococcal species detected were *S. hyicus*, *S. caseolyticus*, *S. capitis*, *S. warneri*, *S. cohnii*, and *S. intermedius.*

Susceptibility testing demonstrated that a cohort of 38 isolates of *S. epidermidis* from 15 of the hematology patients was resistant to penicillin, ampicillin, methicillin, gentamicin, netilmicin, and ciprofloxacin and was sensitive to chloramphenicol, fusidic acid, rifampin, and vancomycin.

A second cohort of isolates of *S. epidermidis* was identified within the neonates. In this cohort, 35 isolates from 26 neonates were resistant to penicillin, ampicillin, methicillin, netilmicin, and gentamicin, while they were sensitive to ciprofloxacin, erythromycin, fusidic acid, rifampin, and vancomycin.

PFGE applied to the 146 isolates of *S. epidermidis* generated 64 types, based on a minimum of a single difference in fragments of 90, 100, 110, 120, 130, 140, 155, 185, 200, 224, 240, 280, 315, 400, 412, and 427 kb. There were constant bands at 60 and 75 kb. Isolates were interpreted according to the criteria of Tenover et al. (37) when isolates were either indistinguishable (no fragment differences), closely related (two to three fragment differences), possibly related (four to six fragment differences), or different (seven or more fragment differences). This identified two clusters of isolates (type A and type B) on the neonatal ward and one cluster of isolates (type C) on the hematology unit. The epidemiology is described in detail in Fig. 1. Figure 2 demonstrates isolates from individual patients of type A in lanes 1 and 2 and type B in lanes 5, 7, and 10. Type A was represented on the neonatal unit by 10 indistinguishable isolates from nine patients, 1 closely related isolate from another patient, and 6 possibly related isolates from an additional six patients. In addition, six isolates from four hematology unit patients produced a possibly related type. Type B was exclusive to the neonatal unit, where 20 indistinguishable isolates were obtained from 14 patients and 5 possibly related isolates were obtained from 5 additional patients. Type C was restricted to the hematological unit, where 26 indistinguishable isolates were obtained from 10 patients, 2 closely related isolates were obtained from another 2 patients, and 6 possibly related isolates came from 2 additional patients.

PFGE applied to the 25 isolates of *S. haemolyticus* from 15 patients produced 14 types, dependent on a minimum of a single band difference in fragments of 75, 90, 100, 120, 130, 140, 155, 185, 200, 240, 256, 280, 300, 315, and 412 kb. Bands at 110 and 60 kb were conserved. Nine of these types were different by the criteria of Tenover et al. (37). Figure 3 illustrates the heterogeneity of the results with identical isolates from the same patient on two separate occasions (lanes 4 and 5).

For 37 patients there were multiple positive blood cultures over a period ranging from 2 days to 2 months. A single species was isolated from 26 patients, with a constant PFGE pattern found for isolates from 11 patients. In seven patients there was a mixture of *S. epidermidis* and *S. haemolyticus*, and in four patients there was a mixture of *S. epidermidis* and *S. hyicus*. Fourteen patients were infected with multiple different types of *S. epidermidis*.

Reproducibility was established by the demonstration of a constant pattern when five isolates of *S. epidermidis* and two isolates of *S. haemolyticus* from each ward were tested at least three times.

For RAPD analysis nine primers were initially tested. Primers 1 and 2 produced between two and four bands for each isolate, while primer 3 yielded only a species-specific pattern. Primer 4 worked with only some of the isolates, whereas primers 5, 6, and 7 did not produce any bands. All isolates were typeable with primers 8 and 9. Primer 8 amplified nine bands of between 0.214 and 1.628 kb, with conserved bands at 0.364, 0.432, 0.540 and 0.805 kb. It discriminated on single band variation 58 types, of which 17 were represented by more than one isolate. Three patterns dominated and were found in 10, 24 (illustrated in Fig. 4), and 28 isolates which included the indistinguishable isolates from PFGE types A, B, and C, respectively. Primer 9 amplified bands of between 0.271 and 1.978 kb, discriminating on the basis of a single band difference 54 types, of which 19 were represented by more than one isolate. A selection of the results for isolates from the neonates is presented in Fig. 5. Three patterns dominated and were found in 12, 20, and 28 isolates, which included the indistinguishable isolates from PFGE types A, B, and C, respectively.

RAPD analysis of the 25 isolates of *S. haemolyticus* with primer 8 revealed 12 different banding patterns based on a

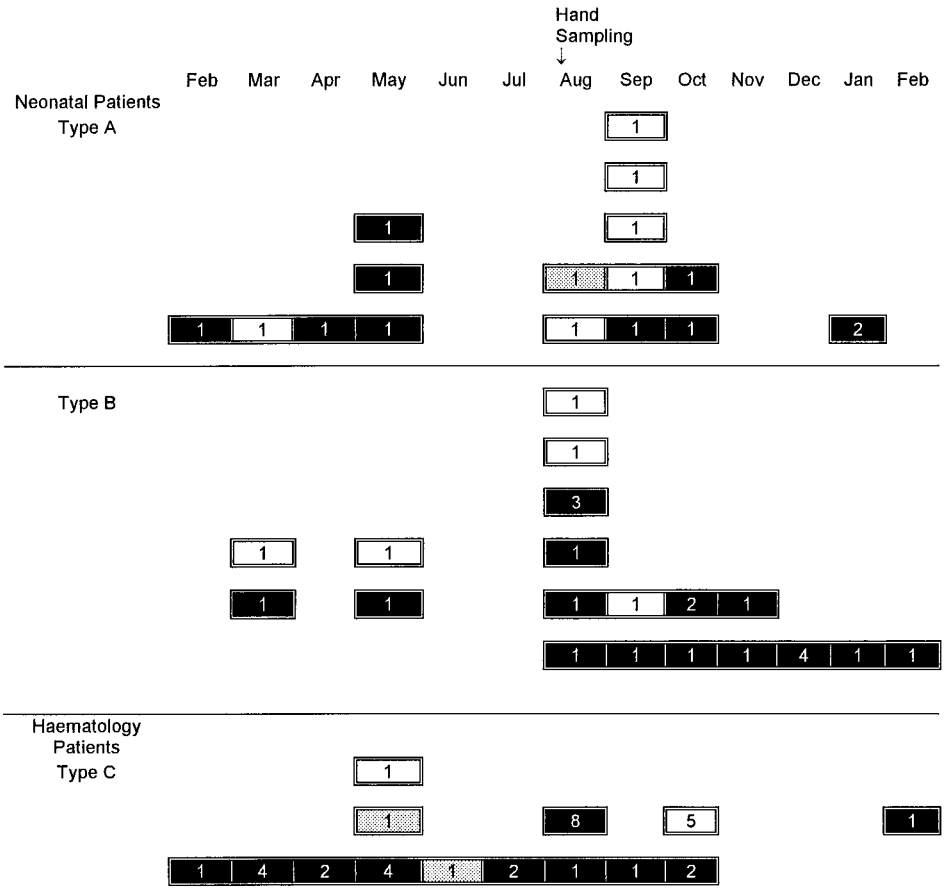

FIG. 1. Epidemiology of three strains defined by PFGE as outbreak related. ■, indistinguishable; ▨, closely related; □, possible related. The criteria of Tenover et al. (37) were used. Numbers refer to the number of separate positive blood cultures from each patient.

single variation in bands at 0.194, 0.234, 0.432, 0.488, 0.540, 0.603, 0.633, 0.841, 0.872, 0.955, 1.078, 1.133, and 1.463 kb. Primer 9 produced 10 types distinguished by at least a single difference in bands at 0.21, 0.271, 0.457, 0.603, 0.670, 0.872, 0.973, 1.078, and 1.353 kb.

Reproducibility was tested by using DNA prepared from different single colonies of the same strain on different days and amplifying the DNA from the strains on two different occasions. Sometimes the bands produced were less intense, but their overall position and their presence or absence were consistent.

Applying antibiograms to the isolates of *S. epidermidis* which were resistant to penicillin, ampicillin, methicillin, gentamicin, and netilmicin and sensitive to fusidic acid, rifampin, and vancomycin identified a ciprofloxacin-sensitive cohort from the neonatal unit and a ciprofloxacin-resistant cohort from the

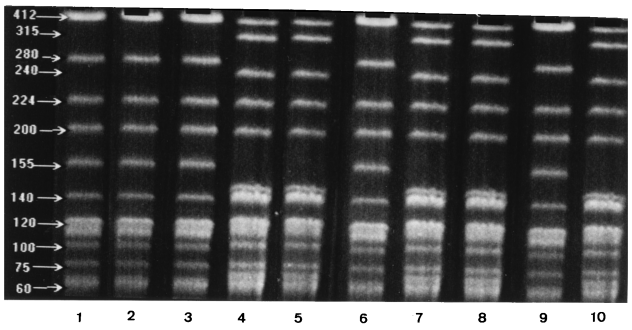

FIG. 2. PFGE of *S. epidermidis* isolates from patients (lanes 1, 2, 5, 7, and 10) and staff (lanes 3, 4, 6, 8, and 9) associated with the neonatal ward. Fragment sizes (in kilobases) are indicated on the left.

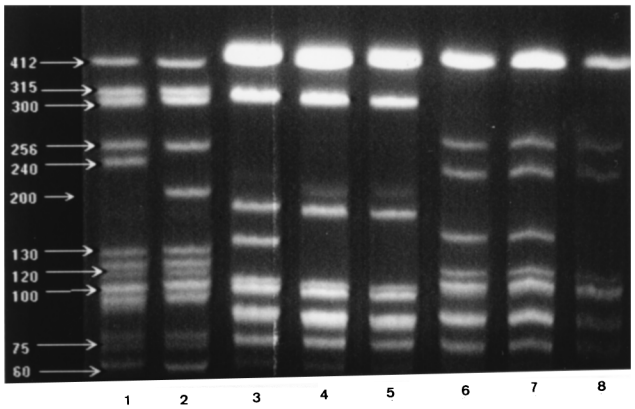

FIG. 3. PFGE of *S. haemolyticus* isolates from the hematology unit patients. Fragments sizes (in kilobases) are indicated on the left.

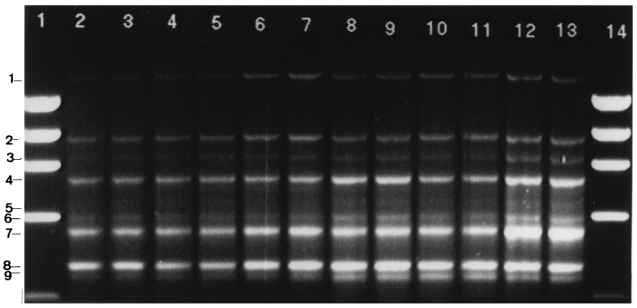

FIG. 4. RAPD fingerprinting of *S. epidermidis* isolates from the neonates (lanes 2 to 11) and from the staff of the neonatal unit (lanes 12 and 13) with primer 9. *Hae*III-digested ϕX174 DNA markers are in lanes 1 and 14. Bands 1 to 9 have molecular masses of 1.518, 1.078, 0.955, 0.805, 0.663, 0.603, 0.540, 0.432, and 0.364 kb, respectively.

hematology unit. The cohort from the neonatal unit was split by PFGE into types A and B. Type A was restricted by RAPD analysis with primer 8 to the 10 isolates from nine patients which were resistant to chloramphenicol and described by PFGE as being indistinguishable. Primer 9 found two further identical isolates described by PFGE as being closely related and possible related, respectively. There were two peaks of infection, in May and September 1993 (Fig. 1). Type B was restricted by RAPD analysis with primer 9 to the 20 isolates from 14 patients which were sensitive to chloramphenicol and described by PFGE as being indistinguishable. Primer 8 found that an additional four isolates from four further patients were identical. These were described by PFGE as being possibly related. There was a major cluster of infection between August 1993 and February 1994 (Fig. 1). The hematology unit cohort included the isolates defined by PFGE as type C. RAPD analysis with both primers 8 and 9 restricted this to the 28 isolates described by PFGE as being indistinguishable or closely related. Patients with a bacteremia due to this strain were present on the ward continuously between February 1993 and October 1994 (Fig. 1).

Staff screening identified PFGE type A isolates on the hands of four nurses and two doctors from the neonatal ward (Fig. 2, lanes 3, 6, and 9), PFGE type B on the hands of two nurses (Fig. 2, lanes 4 and 8), and PFGE type C on the hands of four nurses from the hematology ward. The similarity of the respective strains was confirmed by RAPD analysis with primers 8 and 9. In contrast, when the typing results for *S. haemolyticus* were combined, the only isolates which were identical came from the same patient.

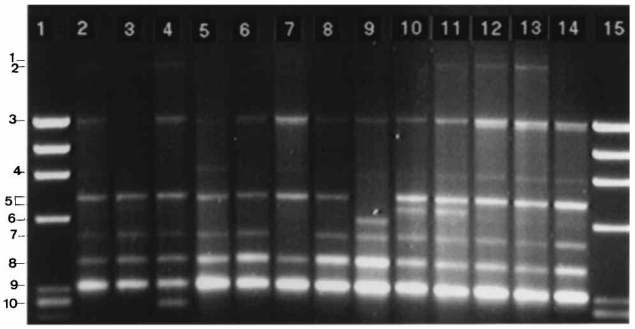

FIG. 5. RAPD fingerprinting of *S. epidermidis* isolates from the neonates with primer 9. *Hae*III-digested ϕX174 DNA markers are in lanes 1 and 15. Bands 1 to 10 have molecular masses of 1.978, 1.903, 1.353, 0.872, 0.738, 0.671, 0.530, 0.432, 0.310, and 0.271 kb, respectively.

DISCUSSION

This study confirmed the suitability of both PFGE and RAPD analysis for typing *S. epidermidis* and *S. haemolyticus* and the existence of three clusters of cross infection due to *S. epidermidis*. All strains could be typed, and reproducibility within the parameters described in the Results section was good. PFGE has previously been applied to multiple isolates from a single patient (19) and to multiple isolates from a single unit (11). Discrimination was highest with a single band difference, but this is scientifically unsound, so the criteria of Tenover et al. (37) were used for the definition of outbreak strains.

Among patients with multiple positive blood cultures, *S. epidermidis* was the sole isolate in 20 patients, while in the remaining 17 patients there were also bacteremias due to a different species. There was no evidence of a dominant type of *S. haemolyticus*, ruling out the possibility of occult cross infection, which has been reported on other units (8, 22, 26).

RAPD analysis demonstrated poor results with primers 1 to 7. Primers 1, 2, and 4 were 8-mers which had previously differentiated between species of bacteria (1) and between individual strains of pathogenic fungi (5, 21). Primers 5, 6, and 7 were 10-mers which typed methicillin-resistant isolates of *S. aureus*, in which the reported discrimination was lower than that achieved by PFGE (43). Primer 7 had also distinguished between isolates of CoNS from eight patients with a catheter-related bacteremia in two distinct hospital wards and six epidemiologically unrelated strains (4). Primer 3 was a 20-mer derived from a sequencing primer, the universal M13 primer, which had previously produced a mixture of species- and strain-specific products when applied to staphylococci (42).

Primers 8 and 9 represented enterobacterial repetitive intergenic consensus sequences which had previously successfully typed *S. aureus* (40). The present study extended this to *S. epidermidis* and *S. haemolyticus*. On the basis of a single band difference, primer 8 gave the higher level of discrimination for both species and was thus the primer of choice for distinguishing between isolates of CoNS. This analysis was based on a single band difference, which may be overdiscriminatory.

A rational scheme would involve the identification of a potential problem by the comparison of antibiograms followed by confirmation by PFGE by using the criteria of Tenover et al. (37) for strain differentiation. RAPD analysis would add refinement by confirming the degree of similarity of the closely and possibly related isolates.

The results demonstrated cross infection among the hematology patients due to a ciprofloxacin-resistant strain of *S. epidermidis* and two separate circulating strains in the neonatal unit. Both of these were sensitive to ciprofloxacin, while the type A isolates were resistant to chloramphenicol. This demonstrated that each unit had its own unique endogenous bacterial population in which the phenotype reflected, in part, ongoing antibiotic prescription. Ciprofloxacin was extensively used on the hematology unit but was never given to neonates, while chloramphenicol use was exclusive to neonates. Cross infection due to CoNS in neonatal units has previously been demonstrated by plasmid profiling. Clusters have included 11 *S. epidermidis* bloodstream infections over a 2-year period and three pairs of patients who harbored identical strains over a 6-month period (22). A combination of typing systems has shown three persistent strains of *S. epidermidis* over periods of 2 and 3 months and 4 years, respectively (23, 29). Others have

found evidence for endogenous infection due to CoNS in neonatal and adult intensive care units (2, 10, 41) and orthopedic patients due to ciprofloxacin-resistant strains (17, 27), but this has not been a universal finding (13).

The identification of the problem led to the instigation of measures to control it. In the hematology unit, ciprofloxacin was widely used to treat gram-negative sepsis. This antibiotic was withdrawn in November 1994 and was replaced by β-lactam-based antibiotics. Only one further isolate in the next 3 months was type C. In both units hands were implicated as a route of transmission. This had previously been reported in other outbreaks (6, 11, 17, 23) and was addressed by changing the hand-washing agent from chlorhexidine to either chlorhexidine gluconate in 70% isopropyl alcohol or povidone-iodine. This had an impact on the number of type A isolates on the neonatal ward, while the isolation of type B strains continued to the end of the study.

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